ATTACHMENT 15

Environmental Effects Literature

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技術報告

Effect of a Genetically Modified Strain of Commercial Baker's Yeast on Microbial Communities in Simulated Natural Environments

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Abstract

Disruption of the acid trehalase gene (*ATH1*) by genetic modification (GM) improves the freeze tolerance of baker's yeast, which is crucial for frozen-dough baking. We previously reported that *ATH1* disruption does not promote the survival of viable cells and DNA of baker's yeast in simulated natural environments. In this study, the effect of inoculation of a GM yeast strain on viable cell numbers and microbial communities of indigenous microorganisms in simulated natural environments was assessed by using the *ATH1* disruptant as a model GM yeast. The microbial community compositions were evaluated by using denaturing gradient gel electrophoresis (DGGE) of rDNAs. Changes in the number of viable cells and the DGGE band patterns of environmental samples inoculated with the GM strain were nearly the same as those inoculated with the wild-type (WT) strain, suggesting that the effect of the GM strain on microbial communities is not significantly different from that of the WT strain.

Key words: baker's yeast, genetically modified microorganism, DGGE, microbial community

Introduction

Genetic modification (GM) techniques for breeding baker's yeast *Saccharomyces cerevisiae* are well established. Characteristics such as fermentation ability and stress tolerance have been improved by using GM techniques[1-4]. Such improvements decrease the costs of baker's yeast production and of bakery processes. Therefore, GM techniques can be used in practical applications. However, the commercial use of GM strains is currently stalled due to a lack of scientific data on the survival of such strains in natural environments, as well as the effects of these organisms on the environment and on human health[5, 6]. A wide variety of yeast species have been detected in natural environments such as soil and water, and even strains of *S. cerevisiae* have been found in such environments[7, 8]. There is a need to assess the effects of GM yeasts on natural environments due to the potential for leakage of such yeasts into these environments. Such leakage might occur during the propagation process of yeast products in factories or during the leavening process in bakeries. It is important to provide the general public accurate information about the effects of GM yeast in order to promote its public acceptance in the commercial food industry.

We previously demonstrated that a GM yeast, which was an acid trehalase gene (*ATH1*) disruptant derived from commercial baker's yeast, exhibited a high accumulation of trehalose and improved freeze tolerance[1]. Based on that study, it is expected that commercial use of *ATH1*

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disruptants in frozen-dough baking would be effective[1]. We also previously assessed the survival of viable cells and DNA of *ATH1* disruptants constructed by GM and self-cloning techniques in simulated natural environments, and found that genetic modification of the *ATH1* locus apparently does not promote the survival of viable cells and DNA[9]. However, the effect of the *ATH1* disruptants on microbial communities was not studied at that time.

Despite the increased number of studies on GM plants and microorganisms[10-13], only a few studies on the behavior of GM yeasts in natural environments have been reported[9, 14, 15]. None of the studies have described the effect of GM yeasts on microbial communities in natural environments.

The aim of this study was to clarify the effects of GM yeasts on microbial communities in natural environments at the molecular level. For this purpose, the *ATH1* gene disruptant derived from commercial baker's yeast was used as a model of GM yeast, and was inoculated into two simulated natural environments (soil and water). To assess the effects of GM yeast on the microbial communities, changes in the number of indigenous microorganisms during 40 days were measured based on viable cell counts. The rDNA profiles of microbial communities in the inoculated environments were monitored by using DGGE (denaturing gradient gel electrophoresis) with PCR-amplified rDNAs as the samples. DGGE analysis is a cultivation-independent molecular analysis that has been used to profile complex microbial communities, including bacteria and fungi[16-18]. Here, we used these methods to conduct an environmental assessment of GM yeast at the microbiological level.

Materials and methods

Yeast strains

Prototroph diploid strains of *S. cerevisiae* T118CR-WT (*MAT***a**/α *ATH1/ATH1 cyh2/cyh2*) and T118CR-GM (*MAT***a**/α *ath1::kanMX4/ath1::kanMX4 cyh2/cyh2*) were used in this study. The strain T118CR-WT was a spontaneous cycloheximide-resistant mutant derived from commercial baker's yeast[9], and represented the wild-type (WT) strain in this study. The strain T118CR-GM was obtained from T118CR-WT by replacing the coding region of the *ATH1* gene with the kanamycin-resistant gene *kanMX4*[9], and represents the GM strain in this study.

Conditions of the simulated natural environments

The conditions of the two simulated natural environments (soil and water) were as follows. Horticultural non-sterile river sand (Matsuzaki™, Japan) was used as the model soil according to our previous study[9]. Sterile distilled water containing 10% (w/v) of the non-sterile sand was used as the model water. For the soil environment, each strain was inoculated into 70 g of soil in a 125-ml plastic bottle at a cell density of 10^6 cells/g of dry soil, and then was immediately mixed. For the water environment, each strain was inoculated into 500 ml of sand-containing water in a 1-liter flask at a cell density of 10^6 cells/ml of water, and then was immediately mixed. Both model environments were inoculated with either the WT or GM yeast cells grown at 30 ℃ for 48 h in YPD medium, and then was incubated at 25 ℃ for 40 d under dark conditions without shaking. During incubation, samples were taken every 5 d for viable cell counts and DNA extractions. At the time of sampling, the model environments were mixed to ensure homogenous samples.

Viable cell counts

The number of viable cells of inoculated yeast remaining in the soil and water environments was measured by using the plate count method as described previously[9]. In brief, the model environment samples were suspended in distilled water, and then plated onto CPS agar medium[9]. The CPS medium contained 5 μg/ml of cycloheximide and 0.75 mg/ml of sodium propionate to prevent the growth of indigenous fungi, including yeast. If the number of viable cells was expected to be less than 10^2 cells/g of soil or ml of water, the sample suspension was centrifuged to concentrate the viable cells. The number of colonies that appeared after incubation for 3 d at 30 ℃ on CPS medium was defined as the inoculated yeast viable cell number. To measure the number of indigenous bacteria, sample suspensions were plated onto tryptic soy agar (Difco Laboratory, USA) and then incubated at 37 ℃ for 3 d. The number of colonies that appeared on the agar was defined as the number of viable indigenous bacteria. To measure the number of indigenous fungi, the sample suspensions were plated onto rose bengal agar plates containing 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 5 g of pep-

tone (Difco), 10 g of glucose, 0.3 g of streptomycin, 33 mg of rose bengal, 20 g of agar (per liter), and then incubated at 25 ℃ for 5 d. The number of colonies that exhibited filamentous growth on the agar was defined as the number of indigenous fungi. The numbers of viable cells of bacteria, fungi, and yeast were expressed as the means of triplicate experiments.

DNA extraction

DNA extraction from the two simulated natural environments was conducted as follows. DNA contained in the soil environment samples (0.5 g) was extracted by using a FastDNA spin kit for soil (Q-Biogene, USA) as described by Takada-Hoshino and Matsumoto[19]. The extracted DNA was then dissolved in 50 μl of distilled water and used for PCR amplification as a template. DNA contained in the water environment samples (1 ml) was extracted as described by Davis et al.[20] with modifications. In brief, cells precipitated by centrifugation at 15,000 rpm for 3 min were incubated with 5 mg/ml of lysozyme (Siekagaku Kogyo, Japan) and 0.15 mg/ml of Zymolyase 100T (Seikagaku Kogyo), and then lysed with 0.4% SDS followed by the addition of 0.8 M potassium acetate. DNA in the supernatant of the lysate was ethanol-precipitated, dissolved in 50 μl of distilled water, and then was used for PCR amplification as a template.

DGGE analysis

DGGE analysis was performed according to Lopez et al.[21]. In brief, EX Taq-polymerase (Takara, Japan) and universal primers Ec338f-GC (5'-CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCCCCGCCCC ACTCCTACGG GAGGCAGCAG-3') and Ec518r (5'-ATTACCGCGG CTGCTGG-3') were used to amplify DNA fragments of bacterial 16S rDNA and fungal 18S rDNA by using PCR. The thermal cycling condition consisted of initial denaturing at 94 ℃ for 4 min, followed by 35 cycles of denaturing at 94 ℃ for 1 min, annealing at 54 ℃ for 1 min, and elongation at 72 ℃ for 1 min, and a final elongation at 72 ℃ for 5 min. DGGE was performed by using the DCode system (Bio-Rad, USA). The PCR product was loaded on 8% (w/v) polyacrylamide gels (1-mm thick) containing a linear gradient of 30 to 60% of denaturant, where 100% denaturant was 7 M urea and 40% (v/v) formamide. The gels were electrophoresed in 1 × TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA; pH 8.0) at 60 ℃ and 40 V for 15 h. The gels were stained by using ethidium bromide, washed twice with distilled water, and were examined by using UV transillumination.

Results and discussion

Changes in the number of viable cells in the soil environment

The changes in the number of viable inoculated yeast cells and indigenous microorganisms during 40 days in the soil environment were measured (Figs. 1A, B, and C). Figure 1A shows the viable cell count under control conditions, in which the model environment was not inoculated with yeast cells. Under the control condition, indigenous bacteria were detected at frequencies ranging from 1×10^7 to 5×10^6 CFU/g of dry soil throughout the entire 40-day incubation period. In contrast, indigenous fungi were detected at frequencies ranging from 8×10^2 to 1×10^2 CFU/g of dry soil throughout the entire 40-day incubation period. Neither the inoculation with the WT strain nor the GM strain significantly affected the numbers of bacteria or fungi (Figs. 1 B and C). These results strongly suggest that inoculation with WT or GM yeast strains did not affect the viability of indigenous microorganisms in the soil environment. Consistent with our previous observations[9], WT and GM yeast strains logarithmically decreased in a time-dependent manner.

Changes in the number of viable cells in the water environment

Similar to the results for the soil environment, the changes in numbers of viable indigenous bacteria and fungi in the simulated water environment were nearly identical for the WT, GM, and no-yeast control treatments (Figs. 1D, E, and F). These results suggest that inoculation with WT or GM yeast strains did not affect the viability of indigenous microorganisms in the water environment. In contrast to the comparable growth fitness of the WT and GM strains in the YPD medium, which were examined by using a growth competition assay as previously described[22] (data not shown), the number of viable cells of the GM strain decreased significantly more quickly than that of the WT strain in the water environment. This is consistent with our previous observations[9], which ex-

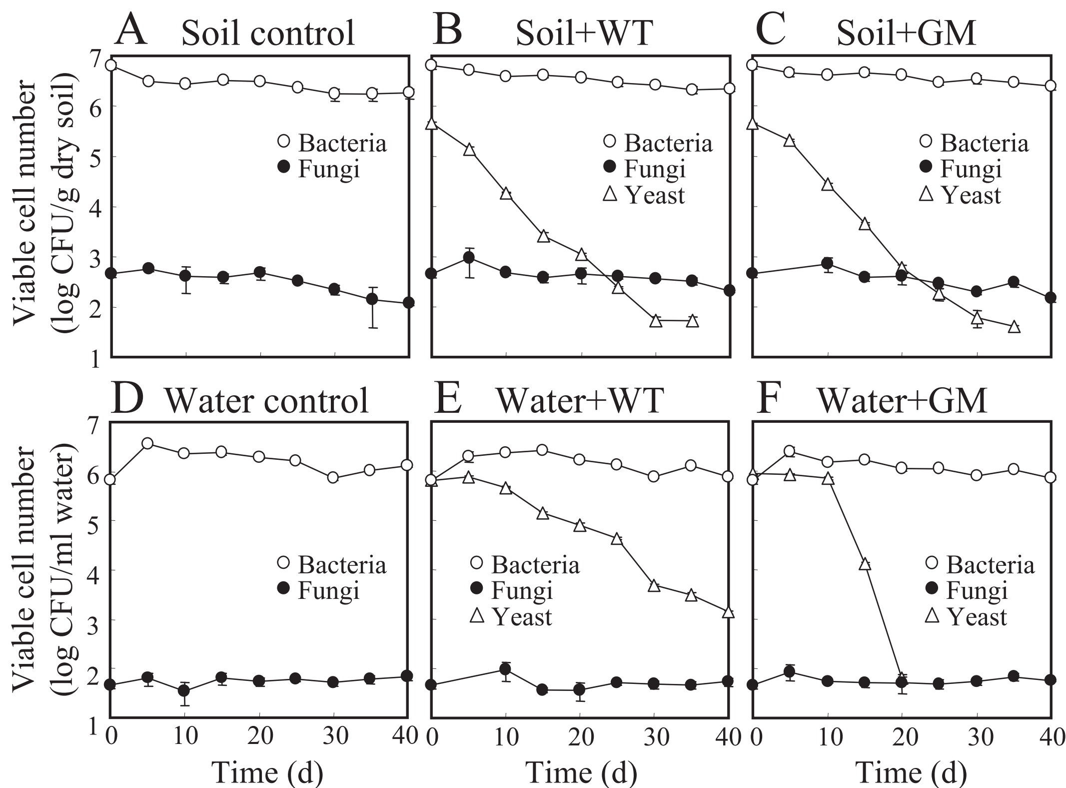

Figure 1.

Changes in the number of viable cells during 40-d cultivation in a simulated soil environment (A, B, and C) and water environment (D, E, and F) with and without inoculation of wild type (WT) and genetically modified (GM) yeast strains. Numbers of viable cells are expressed as means ± standard deviation from triplicate experiments.

amined the viability of co-inoculated WT and GM yeast strains in a water environment. The results suggest that the GM strain is less competitive than is the WT strain in a poor water environment.

DGGE analysis of the microbial communities in the soil environment

To gain further insight into the effects of GM yeast inoculation, we next examined the changes in rDNA profiles of the microbial communities in the soil and water environments by using DGGE analysis (Fig. 2). Figure 2A shows the changes in the DGGE pattern of PCR fragments amplified from DNA extracted from the soil environment. Almost all bands, such as bands A, B, and C, amplified from soil samples inoculated with either WT or GM strains, and from the no-yeast control, exhibited similar patterns throughout the entire 40-day incubation period. Although the intensity of band B increased transiently after 5 d of inoculation, the changes in patterns in GM strain-inoculated samples were similar to those in the WT strain-inoculated samples. Consistent with the viable cell count (Figs. 1B, C), bands representing the WT and GM yeast cells decreased in a similarly time-dependent manner (Fig. 2A). These DGGE data suggest that the effect of inoculation with the GM yeast strain was very similar to that observed with the WT strain, and that inoculation of neither strain affected the microbial community in the simulated soil environment.

DGGE analysis of the microbial communities in the water environment

Figure 2B shows the changes in DGGE patterns of PCR fragments amplified from DNA extracted from the water environment. In contrast to the case of the soil environment, the band pattern for no-yeast control samples was different from that for the yeast-inoculated samples. This result indicated that the microbial community in the simulated water environment was influenced by the inocu-

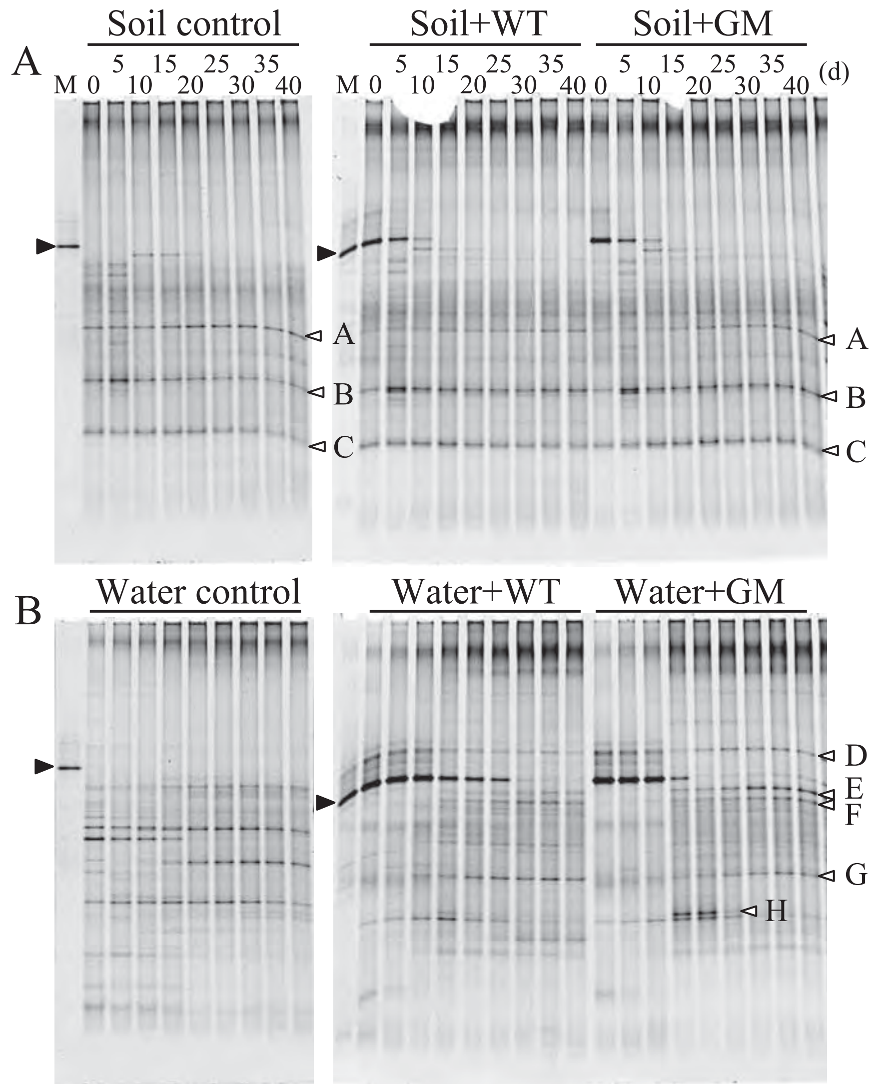

Figure 2.

Changes in microbial community compositions during 40-d cultivation in a soil environment (A) and water environment (B) analyzed by using rDNA-based DGGE profiling. The PCR product amplified by using genomic DNA from WT yeast cells was loaded on lane M as a control for rDNA fragment of baker's yeast (indicated by black arrowheads). White arrowheads indicate major bands.

lation with yeast. This could be simply explained by the assumption that the microbes in the simulated environment utilized nutrients derived from the dead cells of the inoculated yeast. As shown in Fig. 1, the simulated water environment exhibited a lower density of the viable bacteria and fungi compared with the simulated soil environment. Thus, the nutrient supply per microbial cell was relatively high in the simulated water environment, resulting in the changes in DGGE band patterns.

We next focused on the results from each yeast-inoculated sample. The intensities of bands D, E, F, and G were similar for the WT strain- and GM strain-inoculated samples throughout the entire 40-day incubation period. Although the intensity of band H for the GM strain-inoculated samples was higher than that for the WT strain -inoculated samples after about 20 d, this difference in intensity was transient. In fact, after 30 d, the H bands for both types of samples disappeared. These DGGE data suggest that the effect of inoculation with the GM yeast on the microbial community in the water environment was similar to that of the WT yeast. Consistent with our previous observations[9], the PCR fragments derived from the

GM yeast cells in the water environment decreased significantly more quickly than those from the WT yeast cells.

In the present study, the effects of a GM strain on both the total number of viable cells and on the composition of microbial communities in simulated soil and water environments were compared with those of the WT strain. Changes in the number of viable cells and in the microbial communities of GM strain-inoculated samples were nearly the same as those of WT strain-inoculated samples, suggesting that in these two simulated environments, there were no significant differences between the effects of inoculation with the WT and GM strains.

The present work suggests that inadvertent or intentional release of the GM yeast strain into natural environments will not affect the microbial communities. The survivability of the GM yeast strain was previously reported to be the same or lower than that of the WT strain[9]. Taken together, these results imply that the commercial utilization of GM yeast should have no negative effects on natural environments. However, the gene transfer from GM yeast to other organisms remains unknown. Further research is planned to study the horizontal gene transfer of genetically modified gene loci.

This is the first report of the effect of GM food microorganisms on natural environments. Other GM microorganisms used in the food industry, such as bacteria and fungi, will be developed in the near future. This study should be one of the advanced models for environmental risk assessment of GM food microorganisms.

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Survival of Genetically Modified and Self-Cloned Strains of Commercial Baker's Yeast in Simulated Natural Environments: Environmental Risk Assessment

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Although genetic engineering techniques for baker's yeast might improve the yeast's fermentation characteristics, the lack of scientific data on the survival of such strains in natural environments as well as the effects on human health prevent their commercial use. Disruption of acid trehalase gene (*ATH1*) improves freeze tolerance, which is a crucial characteristic in frozen-dough baking. In this study, *ATH1* disruptants constructed by genetic modification (GM) and self-cloning (SC) techniques were used as models to study such effects because these strains have higher freeze tolerance and are expected to be used commercially. Behavior of the strains in simulated natural environments, namely, in soil and water, was studied by measuring the change in the number of viable cells and in the concentration of DNA that contains *ATH1* loci. Measurements were made using a real-time PCR method during 40 days of cultivation. Results showed that the number of viable cells of GM and SC strains decreased in a time-dependent manner and that the decrease rate was nearly equal to or higher than that for wild-type (WT) yeast. For all three strains (SC, GM, and WT) in the two simulated natural environments (water and soil), the DNA remained longer than did viable cells but the decrease patterns of either the DNA or the viable cells of SC and GM strains had tendencies similar to those of the WT strain. In conclusion, disruption of *ATH1* by genetic engineering apparently does not promote the survival of viable cells and DNA in natural environments.

Molecular genetic engineering techniques for breeding of commercial baker's yeast are well established. Such techniques could improve the yeast's characteristics, such as fermentation ability and stress tolerance, and could decrease the cost for baker's yeast production and for bakery processes (16, 18, 22, 23, 25). Genetic engineering techniques produce two categories of yeasts: genetically modified (GM) yeast, which contains a heterologous DNA segment derived from organisms taxonomically different from their host cells, and self-cloning (SC) yeast, which does not contain any DNA derived from other organisms and does not produce any additional proteins except for proteins originally produced in the yeast (2, 10, 29). SC processes are considered the same as naturally occurring gene conversion, such as recombination, deletion, and transposition, and thus SC yeast is not considered a GM organism. For this reason, SC yeast might be more acceptable for consumers than GM yeast. However, genetically engineered baker's yeasts, not only GM yeasts but also SC yeasts, are currently not used commercially. One reason for the hesitation in commercial use of GM or SC strains of yeast is the lack of scientific data on the survival of such strains in natural environments as well as the effects on human health (5, 12, 14).

Assessment of the viability of yeasts constructed by GM and SC techniques in natural environments is important because such yeast might be inadvertently or intentionally released into natural environments, such as soil and water environments, during propagation processes of yeast products in factories or during baking processes in bakeries. It is important to provide the general public with accurate information about the behavior of genetically engineered yeast under natural conditions so that consumers can comfortably accept such techniques and the resultant products, resulting in a boost of the commercial use of GM or SC yeasts in the food industry. The aim of this study was to clarify the survival of viable cells and DNA of SC and GM yeast at the molecular level in natural environments.

In this study, gene disruptants of acid trehalase gene (*ATH1*) derived from commercial baker's yeast were constructed by using GM or SC techniques and then used as models of genetically engineered yeast. In *ATH1* disruptants, trehalose is highly accumulated and functional as a cryoprotectant under freezing conditions (22). Because disruption of *ATH1* improves the freeze tolerance of commercial baker's yeast, the commercial use of *ATH1* disruptants is expected in frozen-dough baking (22).

Despite the increased studies on the genetic engineering techniques of microorganisms, only a few studies on the survival of GM and SC yeasts under natural environments have been reported previously (3, 8). For example, Fujimura et al. (8) showed that under simulated environmental conditions, *Saccharomyces cerevisiae* that overproduces human coagulation factor XIIIa showed the same survival rate as the strain that harbors an empty vector. Specific methods for detecting genetically engineered yeast, however, have not

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TABLE 1. Strains and oligonucleotide primers used in this study

Strain or primer	Genotype or sequence	Source or reference
Strains		
T7	*MATa ATH1 CYH2 URA3*	22
T7CR	*MATa ATH1 cyh2 URA3*, derived from T7	This study
T7CRu	*MATa ATH1 cyh2 ura3*, derived from T7CR	This study
T7CR-SC	*MATa ath1::URA3 cyh2 ura3*, derived from T7CRu	This study
T7CR-GM	*MATa ath1::kanMX4 cyh2 URA3*, derived from T7CR	This study
T18	*MATα ATH1 CYH2 URA3*	22
T18CR	*MATα ATH1 cyh2 URA3*, derived from T18	This study
T18CRu	*MATα ATH1 cyh2 ura3*, derived from T18CR	This study
T18CR-SC	*MATα ath1::URA3 cyh2 ura3*, derived from T18CRu	This study
T18CR-GM	*MATα ath1::kanMX4 cyh2 URA3*, derived from T18CR	This study
T118	*MATa/α ATH1/ATH1 CYH2/CYH2 URA3/URA3*, obtained by mating T7 and T18	22
T118CR-WT	*MATa/α ATH1/ATH1 cyh2/cyh2 URA3/URA3*, obtained by mating T7CR and T18CR, defined as WT strain	This study
T118CR-SC	*MATa/α ath1::URA3/ath1::URA3 cyh2/cyh2 ura3/ura3*, obtained by mating T7CR-SC and T18CR-SC, defined as SC strain	This study
T118CR-GM	*MATa/α ath1::kanMX4/ath1::kanMX4 cyh2/cyh2 URA3/URA3*, obtained by mating T7CR-GM and T18CR-GM, defined as GM strain	This study
BY4741Δ*ath1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ath1::kanMX4*	EUROSCARF
Primers		
P1	5′-CAGTCCTTTATGAGAGCCTGC-3′	
P2	5′-AGAAGCCTCCGCATTGAACCA-3′	
P3	5′-CAATGAAGGCGTCGTTGT-3′	
P4	5′-ATGCTGGTCGCTATACTGC-3′	
P5	5′-GTAGATTCACGGACCAGGA-3′	
P6	5′-ACAATCTGAGTCAGCAAGGCA-3′	
P7	5′-CAGGAAGATCAAATTGGTAGG-3′	
P8	5′-TAGAGGTATGGCCGGTGGTA-3′[a]	
P9	5′-GCTTCCAGAAATGAGCTTGTTGC-3′	

[a] A 3′-terminal adenine base (underlined) corresponding to the *cyh2* mutation position.

yet been established. In contrast to only a few studies on genetically engineered yeasts, many studies on genetically engineered bacteria have been reported, such as *Pseudomonas* strains used for bioremediation and lactic acid bacteria used for probiotics (1, 7, 19, 21). Specific methods for the detection of genetically engineered bacteria have been reported previously (11, 26, 27, 30).

The goal of this current study was to clarify the survival of cells and specific DNA fragments of GM and SC yeasts in natural environments. Soil and water were chosen as models of natural environments because deliberate or accidental releases to such natural environments might occur. Diploid strains derived from commercial strains were used to simulate industrial baker's yeast in this study. First, a system to detect GM and SC yeasts in natural environments was constructed using quantitative real-time PCR (RTm-PCR) technology recently used to rapidly quantify genes and microorganisms in complex environments (6, 9, 24, 30). Then, the changes in the number of viable cells and in the concentration of DNA during 40 days in the two simulated natural environments (soil and water) were measured and compared for these three strains, namely, GM type of *ATH1* disruptants that harbor an antibiotic resistance marker gene derived from bacteria (28), SC type of *ATH1* disruptant constructed using an auxotrophic marker gene that was originally cloned from yeast (20), and wild-type (WT) strain.

MATERIALS AND METHODS

Construction of strains. Table 1 summarizes the strains and oligonucleotide primers used in this study. Strains T7 and T18 are haploid strains that are derivatives of commercial baker's yeast (22). T118 is a diploid strain obtained by mating haploid strains T7 and T18. The strain T118 has high fermentation ability (22). To discriminate these strains from indigenous yeast strains, diploid strains that harbor cycloheximide resistance were constructed. The spontaneous cycloheximide-resistant mutants (13) were isolated from strains T7 and T18, yielding T7CR and T18CR. T118CR-WT was constructed by mating T7CR and T18CR. T118CR-WT was resistant to 10 μg/ml of cycloheximide. Because T118CR-WT had an intact *ATH1* locus, T118CR-WT was defined as the WT strain in this study. The diploid strain of the SC type of *ATH1* disruptant was constructed as follows. To allow for utilization of the *URA3* gene as a selective marker, spontaneous *ura3* mutants from T7CR and T18CR were obtained by 5-fluoroorotic acid selection (4), yielding T7CRu and T18CRu. Gene disruption of *ATH1* with *URA3* was carried out as described previously (22), yielding T7CR-SC and T18CR-SC. T118CR-SC was constructed by mating T7CR-SC and T18CR-SC, and T118CR-SC was defined as the SC strain in this study. The diploid strain of GM type of the *ATH1* disruptant was constructed as follows. The *ath1::kanMX4* fragment was obtained by PCR using primers P6 and P7 (Table 1 and Fig. 1A) and genomic DNA of BY4741Δ*ath1* as a template. Gene disruption of the *ATH1* locus in strains T7CR and T18CR was achieved using the PCR fragment of *ath1::kanMX4*, yielding T7CR-GM and T18CR-GM. T118CR-GM was constructed by mating strains T7CR-GM and T18CR-GM, and T118CR-GM was defined as the GM strain in this study.

Cocultivation systems in soil and water environments. Model soil and water environments were inoculated with WT, SC, and GM yeast cells grown at 30°C for 48 h in YPD medium that contained 10 g of yeast extract (Difco, Detroit, Mich.), 20 g of peptone (Difco), and 20 g of glucose (per liter). Nonsterile river sand (Matsuzaki, Japan) for horticulture was used as the model soil, where the water content was 7.2% (wt/wt) and pH was 6.5. Sterile distilled water was used as the model water.

Two cocultivation systems were used: a series I cocultivation system, which harbored WT and GM strains, and series II, which harbored SC and GM strains. For the soil environment, two strains (either WT and GM or SC and GM) were coinoculated into 70 g of soil in a 125-ml plastic bottle at a cell density of 10^6 cells (each strain) per 1 g of dry soil and then immediately mixed. For the water environment, the strains were coinoculated into 500 ml of sterile distilled water

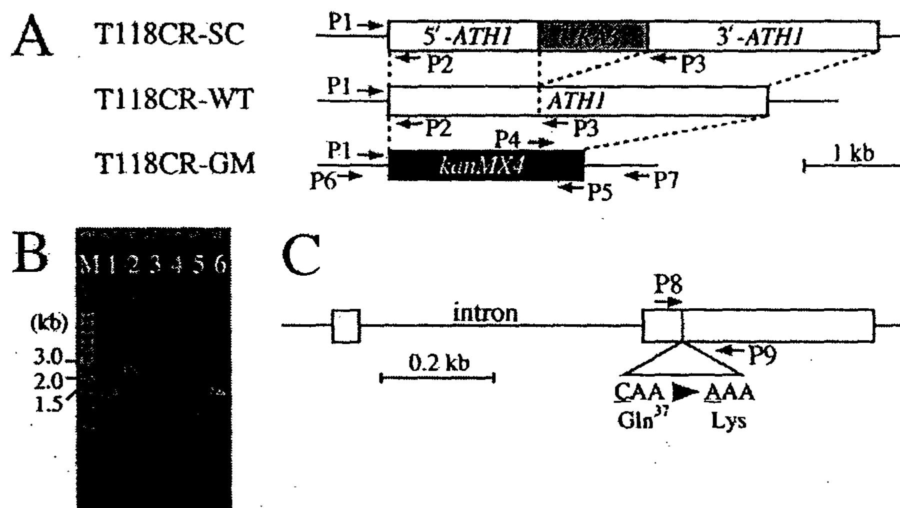

FIG. 1. (A) Schematic model of *ATH1* loci and PCR primers. Coding region of the *ATH1* gene is indicated by open boxes. Regions of *URA3* and *kanMX4* genes are indicated by shaded and filled boxes, respectively. Positions and directions of PCR primers are indicated by arrows. (B) *ATH1* loci of WT, SC, and GM strains were confirmed by PCR amplification. Lane M shows the molecular size marker. Lanes 1 to 6 show PCR product amplified using primers P1 and P3 from genome of T118CR-WT (lane 1), P1 and P3 from that of T118CR-SC (lane 2), P1 and P3 from that of T118CR-GM (lane 3), P1 and P5 from that of T118CR-WT (lane 4), P1 and P5 from that of T118CR-SC (lane 5), and P1 and P5 from that of T118CR-GM (lane 6). (C) Schematic model of *cyh2* locus and PCR primers. Coding region of the *CYH2* gene is indicated by open boxes. Position and direction of PCR primers are indicated by arrows. Mutation position of *cyh2* (CAA to AAA) is indicated by a large open triangle.

in a 1-liter flask at a cell density of 10^6 cells (each strain) per 1 ml of water and then immediately mixed. These soil and water cocultivation systems were then incubated at 25°C for 40 days and 42 days, respectively, under dark conditions without shaking. During incubation, samples were taken for measurement of the number of viable cells and the DNA concentration. At the time of sampling, the cocultivation systems were mixed to ensure homogenous samples.

Measurement of the number of viable cells remaining in soil and water environments. The number of viable cells of inoculated yeast cells remaining in the soil and water environments (series I and II cocultivation systems) were measured using the cultivation method as follows. The soil environment samples (0.5 g) were suspended in distilled water, and total volume was adjusted to 5 ml. Serial 10-fold dilutions of the soil suspension and the water environment samples were prepared using distilled water. The dilutions (200 μl) were then individually plated onto CPS agar medium, which is YPD medium containing 5 mg of cycloheximide (Sigma-Aldrich, St. Louis, Mo.), 0.75 g of sodium propionate, 0.3 g of streptomycin (Sigma-Aldrich), 5 g of lactic acid, and 20 g of agar (per liter). The colonies that appeared after incubation for 3 days at 30°C on CPS medium were considered total viable cells (VCt) of the inoculated yeast cells. These total survivors appearing in CPS agar medium (20 to 200 colonies) were then replica plated onto YPD-G418 agar medium (i.e., YPD agar medium supplemented with an aminoglycoside antibiotic, G418 [Sigma], at a concentration of 0.3 g/liter) and incubated at 30°C for 1 day. The colonies that appeared on YPD-G418 agar were considered cells that harbored *ath1::kanMX4* locus (VCg). The number of viable cells that harbored either wild-type *ATH1* (VCw) or self-cloned *ath1::URA3* locus (VCs) were calculated by subtraction of VCg from VCt. The number of viable cells was expressed as the mean from triplicate experiments.

To measure the number of indigenous bacteria, portions of each suspension of the soil and water environment samples were plated onto tryptic soy broth agar (Difco) and then incubated at 37°C for 3 days. The colonies that appeared on the agar were counted as the number of indigenous bacteria.

DNA extraction from soil and water environments and from yeast cells. DNA contained in 0.5-g soil environment samples was extracted using a FastDNA spin kit for soil (Q-Biogene, Carlsbad, Calif.) according to the manufacturer's instructions. The extracted DNA was then dissolved in 50 μl of distilled water and used for RTm-PCR analysis.

DNA contained in 1-ml water environment samples was extracted according to a protocol described by Philippsen et al. (17). The extracted DNA was then dissolved in 60 μl of distilled water and used for RTm-PCR analysis.

Yeast DNA used as standard DNA in RTm-PCR analysis was extracted from yeast cells, which were grown in 2 ml of YPD medium at 30°C for 48 h, using a FastDNA spin kit for soil according to the manufacturer's instructions. The extracted DNA was dissolved in 50 μl of distilled water. The DNA concentration was measured by using a spectrophotometer (Ultraspec UV2100 pro; Amersham Biosciences, Piscataway, N.J.).

Measurement of yeast DNA concentration in soil and water environments. The concentration of DNA from WT, SC, and GM strains in the soil or water environments was quantified using the RTm-PCR method (15, 24). RTm-PCR was done using a hot-start PCR kit (LightCycler FastStart DNA Master SYBR Green I; Roche, Mannheim, Germany) and LightCycler instrument (Roche), and the analysis was done using the LightCycler software version 3.5 (Roche). For RTm-PCR, 20 μl of the reaction mixture was used, consisting of 0.05% (wt/vol) of bovine serum albumin, 1 μM of each respective primer (Table 1) (also discussed in the next paragraph), 3 mM of $MgCl_2$, 0.29 μl of LightCycler FastStart enzyme (included in the kit), 1.71 μl of LightCycler FastStart reaction mix (included in the kit), and 2 μl of template DNA solution.

Primers for the detection of *ATH1* and *cyh2* loci were as follows. *ATH1* locus in the WT strain and *ath1::URA3* locus in the SC strain were detected as the 105 bp of PCR product using primers P1 and P2 (Table 1 and Fig. 1A). *ATH1* locus replaced by *kanMX4* in the GM strain was detected as the 101 bp of PCR product using primers P4 and P5 (Table 1 and Fig. 1A). *cyh2* locus of the WT, SC, and GM strains was detected as the 119 bp of PCR product using primers P8 and P9 (Table 1 and Fig. 1C). Primer P8 was designed based on the mutation position in the *CYH2* gene to enable the detection of the *cyh2* locus.

Thermal cycling conditions for detection of *ATH1* and *cyh2* loci were as follows. The thermal cycling conditions for *ATH1* and *ath1::URA3* locus in the WT and SC strains consisted of initially heating samples to 95°C and storage at 95°C for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 63°C and storage at 63°C for 4 s, and heating at 10°C/s to 72°C and storage at 72°C for 5 s. The thermal cycling conditions for detection of the *kanMX4* locus in the GM strain consisted of initially heating samples to 95°C and storage at 95°C for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 61°C and storage at 61°C for 5 s, and heating at 10°C/s to 72°C and storage at 72°C for 6 s. The thermal cycling conditions for detection of the *cyh2* locus of the WT, SC, and GM strains consisted of initially heating samples to 95°C and storage there for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 65°C and storage at 65°C for 4 s, and heating at 20°C/s to 72°C and storage at 72°C for 5 s. Fluorescence of double-stranded DNA-SYBR Green I complex was measured at the end of each 72°C cycle (extension process).

Melting curve analysis was done to confirm that the correct amplification of *ATH1* or *cyh2* loci occurred. The condition for this analysis consisted of heating

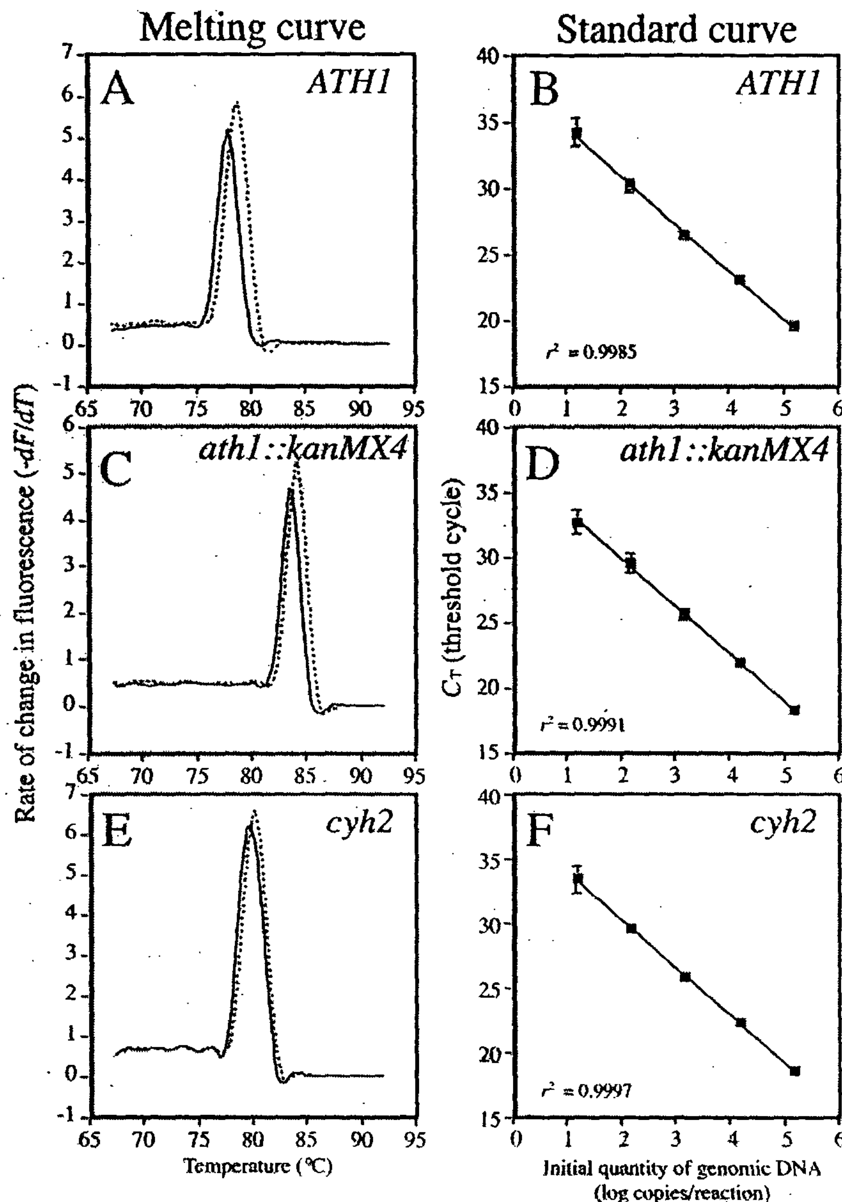

FIG. 2. Melting curves and standard curves of RTm-PCR products. (A, C, and E) Melting curves generated by plotting the negative first derivative of fluorescence against temperature ($-dF/dT$), which is a parameter for the rate of change in fluorescence based on DNA melting. (B, D, and F) Standard curves and correlation coefficients (r^2) by plotting C_T versus DNA quantity. C_T values are expressed as means ± standard deviations from triplicate experiments. Panel A is the melting curve of the *ATH1* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P1 and P2. Panel B is the standard curve for the *ATH1* locus. Panel C is the melting curve of the *ath1::kanMX4* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P4 and P5. Panel D is the standard curve for the *ath1::kanMX4* locus. Panel E is the melting curve of the *cyh2* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P8 and P9. Panel F is the standard curve for the *cyh2* locus.

at 20°C/s to 95°C, cooling at 20°C/s to 65°C and storage at 65°C for 15 s, and heating at 0.1°C/s to 95°C with continuous monitoring of the fluorescence. A melting curve was obtained by plotting the negative first derivative of fluorescence against temperature (i.e., $-dF/dT$). The melting temperature (T_m) of the double-stranded DNA products was represented by a peak in the melting curve.

Concentrations of the DNA contained in the soil and water environment samples were determined using the standard curves. The standard curves for measurement of *ATH1* and *cyh2* loci were generated by plotting the log of the number of copies in a 10-fold dilution series of the standard DNA extracted from he WT, SC, and GM strains against the C_T value, in which C_T was defined as the fractional cycle number (calculated using the LightCycler software) where the fluorescence increased above the detection threshold. The standard curve was then represented by a linear regression line of these C_T values. The quality of the standard curve was confirmed by the correlation coefficient (r^2) of C_T and DNA quantity. The DNA concentration was normalized based on the copy number per cell (two copies of *ATH1* loci and two copies of *cyh2* locus were contained in a diploid cell). DNA concentration was expressed as an equivalent of cell number and was the mean from triplicate experiments.

RESULTS

Construction of discriminative measurement systems of viable GM, SC, and WT strains. Homozygous SC and GM

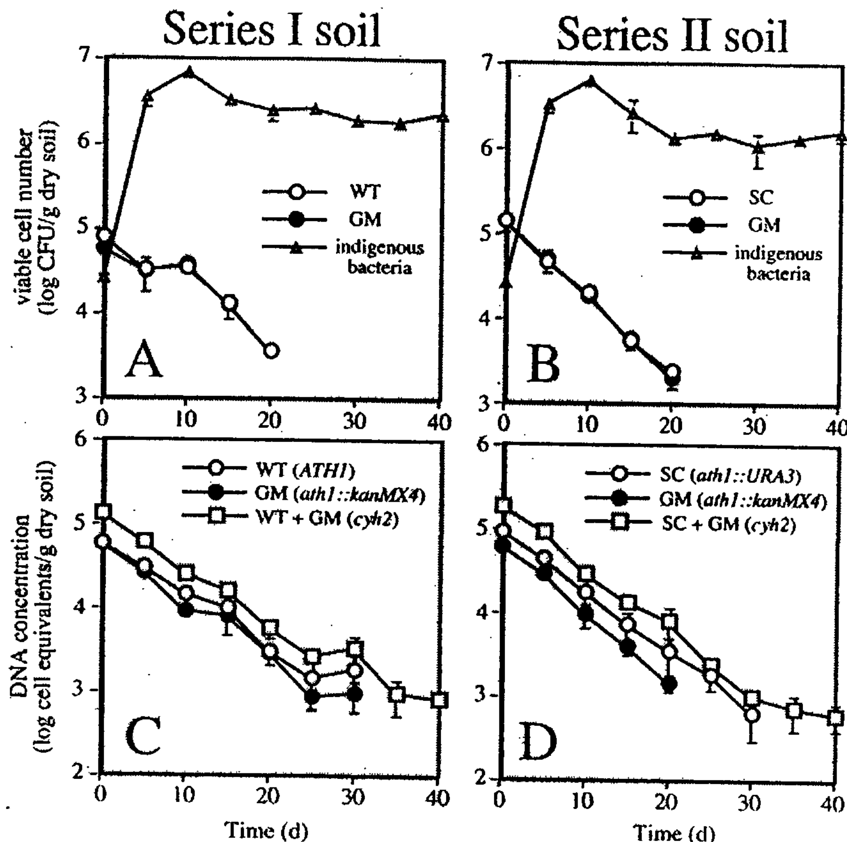

FIG. 3. Number of viable cells (A and B) and concentration of DNA containing the *AHT1* loci and *cyh2* locus (C and D) during 40 days (d) of cultivation in the soil environment. Panel A shows the number of viable cells in series I cocultivation (WT+GM) and panel B in series II cocultivation (SC+GM). Panel C shows DNA concentration of the *ATH1*, *ath1::kanMX4*, and *cyh2* loci in series I cocultivation (WT+GM) and panel D, that of the *ath1::URA3*, *ath1::kanMX4*, and *cyh2* loci in series II cocultivation (SC+GM). Number of viable cells and DNA concentration are expressed as means ± standard deviations from triplicate experiments.

strains of *ATH1* disruptants were constructed in the genetic background of T118. The expected *ATH1* loci of these strains are illustrated in Fig. 1A. Disruption of the *ATH1* loci of SC and GM was confirmed by PCR using primers P1 and P3 designed for the amplification of both intact *ATH1* and *ath1::URA3* and using primers P1 and P5 designed for the amplification of *ath1::kanMX4*, respectively (Fig. 1A). Amplifications of expected lengths of DNA from the WT, SC, and GM strains were observed (Fig. 1B), thus confirming that *ATH1* loci of the strains were correctly constructed.

Spontaneous mutations for cycloheximide resistance were introduced into the WT, SC, and GM strains to distinguish these strains from indigenous yeast cells under simulated soil environments. Sequence analysis of *CYH2* locus of the WT strain revealed that mutation occurred at position 623 (C to A) in the *CYH2* gene, suggesting the replacement of Gln^{37} by Lys (Fig. 1C). These strains grew on CPS agar plates, which contained 5 μg/ml of cycloheximide. Indigenous yeast included in the soil used in this study could not grow on CPS agar medium (data not shown). These results suggest that the three strains constructed here (WT, SC, and GM) are suitable for discriminative measurements from indigenous yeast using CPS agar nedium.

The G418 resistance of strains constructed here was examined to determine suitable conditions for discriminative measurement between the GM strain that harbors *kanMX4* and the WT and SC strains. The GM strain was resistant to 300 μg/ml of G418, whereas the WT and SC strains did not grow on agar medium containing G418 (data not shown). These data suggest that discriminative measurement between the GM strain and the SC and WT strains is possible by using agar medium containing 300 μg/ml of G418.

Molecular detection of *ATH1* and *cyh2* loci in soil and water environments. To construct a quantification system using RTm-PCR for the DNA fragments of the *ATH1* locus that survived in the soil and water samples, we designed specific PCR primers (P1 and P2) for amplification of the *ATH1* locus in both the WT and SC strains and specific primers (P4 and P5) for that in the GM strain (Table 1). The positions of the primers are indicated in Fig. 1A. The *cyh2* locus that was not modified by genetic engineering techniques was used for the neutral control to confirm the analysis results of the *ATH1* locus. Specific primers (P8 and P9) for amplification of the *cyh2* locus were designed based on the nucleotide sequence of *cyh2* in these strains (Table 1 and Fig. 1C).

Figure 2 shows the RTm-PCR profiles of DNA fragments amplified from the WT (*ATH1*) strain and those from the GM (*ath1::kanMX4*) strain. To confirm the correct amplification of each target locus, T_m of the PCR products was measured using melting curve analysis (Fig. 2A, C, and E). Figure 2A shows the melting curve analysis results for the *ATH1* locus amplified

from the WT strain using primers P1 and P2. The T_m of the PCR fragment amplified from DNA directly extracted from the soil samples was identical with that from standard DNA of the WT strain (Fig. 2A). In our assay system, the *ATH1* fragment amplified from the WT (*ATH1*) strain was expected to be the same as that from the SC (*ath1::URA3*) strain. Consistent with this expectation, both the T_m of the *ATH1* fragment amplified from DNA directly extracted from the soil samples and that from standard DNA of the SC strain were identical to that from the WT strain (data not shown). The T_m of the DNA fragments of *ath1::kanMX4* locus amplified from the soil samples was confirmed to be identical to the T_m from standard DNA of the GM strain using primers P4 and P5 (Fig. 2C). The T_m of the DNA fragments of *cyh2* locus amplified from the soil samples was confirmed to be identical to the T_m from standard DNA of the WT strain using primers P8 and P9 (Fig. 2E). The T_m of the amplified fragments from standard DNA of the SC and GM strains was identical to that of the WT strain (data not shown). Melting curve analysis using the genomic DNA of strain T118 (*CYH2*) using primers P8 and P9 as a template revealed that specific amplification did not occur (data not shown), suggesting that the *cyh2* locus is correctly detected by using primers P8 and P9. The molecular sizes of the PCR products from the *ATH1*, *ath1::kanMX4*, and *cyh2* loci measured by agarose gel electrophoresis were consistent with predicted DNA sizes (105, 101, and 119 bp, respectively) (data not shown). These results of T_m and molecular size analyses confirm the accuracy and high specificity of the PCR amplification of *ATH1* loci with *cyh2* locus as the neutral control.

To quantify the DNA fragments of *ATH1*, *ath1::kanMX4*, and *cyh2* in the water and soil environment samples, standard curves were constructed by using standard DNA (Fig. 2B, D, and F, respectively). The standard curves for each DNA fragment had a high r^2 (>0.99) and were not affected by other components, except for the concentration of DNA included in the samples directly extracted from the soil (data not shown). These curves shown in Fig. 2 confirm the reliability of the measurements of DNA containing the *ATH1*, *ath1::kanMX4*, and *cyh2* loci contained in a soil environment. The same experiments were done using DNA extracted from water samples. The RTm-PCR profiles for these water samples were identical to those for the standard DNA (data not shown), confirming the reliability of quantification of DNA in a water environment.

Survival rates of viable yeast cells and DNA concentration in the soil environment. To compare the survival rates of viable yeast cells and DNA concentrations of the three strains under strictly identical conditions, we constructed two series of cocultivation assay systems, namely, cocultivation of GM and WT strains (series I) and that of GM and SC strains (series II). In brief, two different strains (either GM and WT or GM and SC) were inoculated into soil and water samples and then cultured for 40 days and 42 days, respectively. During the culturing, the number of viable cells and the DNA concentration were measured using the discriminative methods described above (see Materials and Methods).

Figure 3 shows the measured number of viable cells and DNA concentration of the GM, SC, and WT strains in the soil environment. Figure 3A shows the results from the series I cocultivation system containing GM and WT strains. The number of viable cells of both strains logarithmically decreased in a similar time-dependent manner. The DNA fragment of the *ATH1* and *ath1::kanMX4* loci specified WT and GM strains, respectively, in the soil. The DNA concentration decreased more slowly than did the number of viable cells (Fig. 3C). The DNA concentration of *cyh2*, which was the neutral control for DNA quantification, decreased similarly to the concentrations of *ATH1* and *ath1::kanMX4* and was nearly equal to the sum of the concentrations of these two *ATH1* loci (Fig. 3C). Figure 3B and D show data from the series II cocultivation system containing GM and SC strains. Although the decrease rate of DNA concentration was lower than that of the number of viable cells, the DNA of both strains contained in the soil decreased logarithmically with the decrease in the number of viable cells (Fig. 3D). The data shown in Fig. 3 indicate that viable cells of both the GM and SC strains decreased in the same manner as those of the WT strain.

Based on these results, there was no significant difference in the survival of viable cells and DNA in the soil environment among the GM, SC, and WT strains.

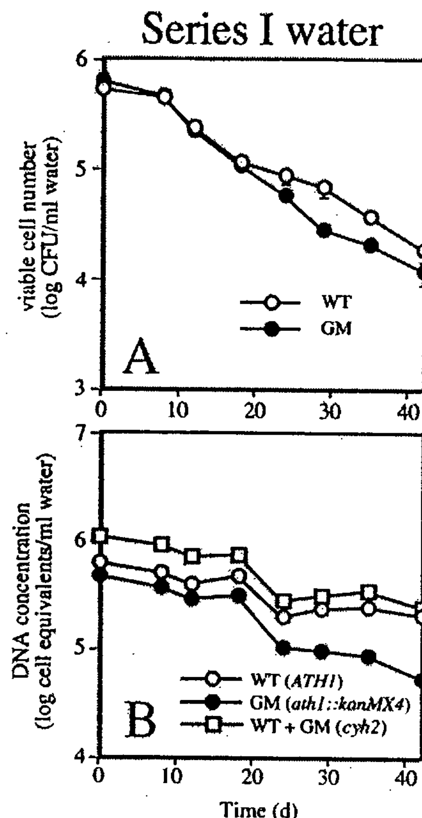

FIG. 4. Number of viable cells (A) and concentration of DNA containing *AHT1* loci and *cyh2* locus (B) of series I cocultivation (WT+GM) during 42 days (d) of cultivation in the water environment. Number of viable cells and DNA concentration are expressed as means ± standard deviations from triplicate experiments.

Survival rates of viable yeast cells and DNA concentrations in the water environment. Figure 4 shows the survival rates of viable yeast cells and the concentrations of DNA from the GM and WT strains (series I) in the water environment. Although the decrease rates of both strains in water were lower

than those in soil, the number of viable cells and the DNA concentration of the GM strain decreased in a time-dependent manner but the decrease rate of the GM strain was significantly higher than that of the WT strain.

DISCUSSION

In summary, the number of viable cells and DNA concentration of the GM and SC yeast strains in simulated natural environments, namely, in soil and water, were compared with that of the WT strain. The survival rate of yeast cells was not related to the presence or absence of genetically engineered *ATH1* loci in the soil environment because all three strains tested (GM, SC, and WT) showed similar kinetics of survivability. Decrease in the *ATH1* loci and the neutral gene *cyh2* were also investigated under the same conditions. The decrease in DNA that contained either *ATH1* or *cyh2* loci was correlated with the decrease in viable cells. For all three strains in both simulated environments, DNA remained longer than did viable cells, although the decrease rates of the SC and GM strains were the same as or higher than that of the WT strain.

The effectiveness of techniques using an RTm-PCR method to quantitatively measure a specific yeast DNA contained in soil was tested and demonstrated. Under the experimental soil conditions used here, the sensitivity of the RTm-PCR method was the same as that of the viable-cell-count method. After inoculation of yeast cells at a concentration of 10^6 cells/g of dry soil (Fig. 3C and D), the measured DNA concentration was approximately 10^5 cell equivalents/g of dry soil whereas the number of viable cells was approximately 10^5 cells/g of soil. The recovery rates of DNA and viable cells were approximately 10%. Although the behavior of the nonrecoverable cells and DNA remains unclear at present, the behavior of the recoverable cells and DNA is assumed to represent that of the nonrecoverable cells and DNA because we obtained similar data from three independent experiments with reproducibility. Under the experimental conditions for the water environment used in our study, the sensitivity of the RTm-PCR method was almost the same as that of the viable-cell-count method (Fig. 4). The RTm-PCR method therefore should be a useful tool for the detection of yeast cells in natural environments as well because the method can measure the number of specific yeast cells more rapidly and more easily than other detection methods, including the viable-cell-count method. We attempted to apply the RTm-PCR method to yeast detection in more complicated environments such as kitchen garbage, but we could not obtain specific amplification of the yeast DNA (data not shown). Although further research into the detection of DNA in such complicated environments is necessary, the RTm-PCR method should be a useful tool for detection in natural environments.

Bröker (3) and Fujimura et al. (8) reported that no differences could be detected in the survival rate of either recombinant or wild-type yeast cells under either sterile conditions (in water) or nonsterile conditions (in soil). Although in this study the presence of the genetically engineered loci of *ATH1* might not directly affect the survival of GM and SC types of commercial baker's yeast in nonsterile soil conditions, our results suggested that the GM type of baker's yeast was less stable than the WT in the sterile water condition.

The RTm-PCR assay showed that the DNA fragment derived from yeast strains decreased at a slower rate than did the viable cells under soil and water environments. The rates of decrease in the concentration of DNA of the GM and SC strains were not significantly different from that of the WT strain. These results suggest that the disruption of *ATH1* by genetic engineering does not promote the survival of viable cells and DNA in natural environments.

Although the survivability of baker's yeast constructed by GM and SC techniques was clarified here, the effects of any release of GM and SC yeasts on indigenous microflora remain unknown. Further research is planned to study these effects by determining the effect of yeast inoculation on the microflora of indigenous bacteria, fungi, and yeast.

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The assessment of the environmental impact of genetically modified wine yeast strains

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KEY WORDS: Yeasts, *Saccharomyces cerevisiae*, fermentation, genetically modified organisms, DNA.

ABSTRACT

In recent years, considerable efforts have been made to improve strains of the wine yeast *Saccharomyces cerevisiae* through the use of modern biotechnological tools. The main targets for these wine yeast strain development programmes have been, and still are, the improvement of fermentation performance, processing efficiency, and wine sensory quality, as well as the development of new strains with reduced risks and enhanced benefits for health. Currently, numerous stable genetically modified wine yeast strains already exist in laboratories, while many others are being constructed. Genetically modified wine yeast strains have not, as yet, been used in the wine industry. This situation is mainly due to concerns relating to GMOs in food. Nevertheless, the arrival on the market of genetically modified wine yeast strains appears imminent and an urgent need to assess the potential risks that may be associated with the use of this new technology throughout the wine production chain exists. This presentation will focus on the existing data generated by current research projects regarding the assessment of such risks. More specifically, we will present data and projects on (i) the available detection methods to monitor genetically modified wine yeast strains through the overall wine production chain, (ii) the survival and dynamics of industrial wild type and (iii) genetically modified wine yeast strains in natural habitats and winemaking environments, (iv) the potential for natural gene transfer between yeast and the natural wine micro-flora, and (v) the analysis of possible side effects of genetic modifications on yeast strain and product quality. *(Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 514-528).*

ZUSAMMENFASSUNG

Umweltverträglichkeitsprüfung gentechnisch veränderter Weinhefestämme

In den letzten Jahren wurden beträchtliche Anstrengungen unternommen, um die Weinhefestämme Saccharomyces cerevisiae durch den Einsatz moderner biotechnischer Hilfsmittel zu verbessern. Die hauptsächlichen Ziele dieser Entwicklungsprogramme für Weinhefestämme waren und bleiben bis heute die Verbesserung der Fermentationsleistung, die Verarbeitungseffizienz und die sensorische Weinqualität, sowie die Entwicklung neuer Hefestämme mit geringeren Risiken und gesundheitsfördernden Eigenschaften. Zahlreiche stabile, gentechnisch veränderte Weinhefestämme existieren heute bereits in den Labors und viele andere befinden sich in der Entwicklung. Bisher wurden genetisch veränderte Weinhefestämme noch nicht in der industriellen Weinbereitung eingesetzt. Der Grund dafür liegt in Bedenken hinsichtlich des Einsatzes von GVOs in Nahrungsmitteln. Trotzdem steht die Markteinführung gentechnisch veränderter Weinhefestämme offensichtlich unmittelbar bevor und es besteht die dringende Notwendigkeit, die potentiellen Risiken, die mit dieser neuen Technologie innerhalb der gesamten Kette der Weinproduktion bestehen könnten, abzuschätzen. Dieser Beitrag konzentriert sich auf existierende Daten, die aus verschiedenen aktuellen Forschungsprojekten hinsichtlich diesbezüglicher Risikoabschätzung stammen. Insbesondere stellen wir Daten und Projekte bezüglich (i) der verfügbaren Erkennungsmethoden zur Kontrolle gentechnisch veränderter Weinhefestämme entlang der Kette der Weinproduktion, (ii) das Überleben und die Dynamik industrieller Wildtypen und (iii) gentechnisch veränderte Weinhefestämme im natürlichen Lebensraum und im Umfeld der Weinbereitung, (iv) das Potential natürlichen Gentransfers zwischen der Hefe und der natürlichen Weinmikroflora und (v) die Analyse möglicher Nebenwirkungen gentechnischer Veränderungen auf Hefestämme und die Qualität des Endprodukts vor. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

RÉSUMÉ

Evaluation des effets sur l'environnement des souches de levures du vin génétiquement modifiées

Des efforts considérables ont été réalisés pour améliorer des souches de levures du vin Saccharomyces cerevisiae à l'aide des outils modernes de la biotechnologie. L'objectif principal de ces programmes de développement des souches de levures a toujours été l'amélioration de la qualité de la fermentation, l'efficacité dans l'élaboration, l'amélioration de la qualité sensorielle du vin, ainsi que le développement de nouvelles souches avec des risques minimes et des bénéfices accrus pour la santé. Actuellement, de nombreuses souches de levures génétiquement modifiées existent en laboratoire, tandis que beaucoup d'autres sont en cours de développement. A ce jour, les souches de levures génétiquement modifiées n'ont pas été utilisées dans le secteur du vin. Ceci est dû, en grande partie, à des préoccupations vis-à-vis de l'utilisation des OGM dans les produits alimentaires. Cependant, l'arrivée sur le marché des souches de levures génétiquement modifiées semblerait être imminente et il existe un besoin urgent d'évaluer les risques potentiels associés à l'utilisation de cette nouvelle technologie dans l'élaboration du vin. Cette présentation montrera les données existantes issues des

projets de recherches actuels concernant l'évaluation de ces risques. Plus précisément, nous exposons des données et des projets concernant: (i) les méthodes de détection disponibles pour contrôler les souches de levures génétiquement modifiées pendant les différentes étapes de l'élaboration du vin, (ii) la survie et la dynamique du type sauvage industriel, (iii) les souches de levures génétiquement modifiées en milieu naturel et dans les milieux de l'élaboration du vin, (iv) le potentiel de transfert naturel de gènes entre les levures et la microflore naturelle du vin et (v) l'analyse des effets secondaires potentiels des modifications génétiques sur les souches de levures et la qualité du produit. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

Resumen

Evaluación del impacto medioambiental de las cepas de levaduras geneticamente modificadas

Se han realizado esfuerzos considerables para mejorar las cepas de levaduras del vino Saccharomyces cerevisiae utilizando las herramientas modernas de la biotecnología. El objetivo principal de estos programas de desarrollo de las cepas de levaduras ha sido siempre el de mejorar la calidad de la fermentación, aumentar la eficacia del proceso de elaboración, mejorar la calidad sensorial del vino, así como desarrollar nuevas cepas con una reducción de los riesgos y un mayor beneficio para la salud. Actualmente, numerosas cepas de levaduras genéticamente modificadas existen en laboratorio, mientras que muchas otras se están desarrollando. Hasta el día de hoy, las cepas de levaduras genéticamente modificadas no han sido utilizadas en el sector del vino. Esto se debe en gran parte a la preocupación respecto a la utilización de los OGM en los productos alimentarios. Sin embargo, la llegada al mercado de las cepas de levaduras genéticamente modificadas parece inminente y existe una necesidad urgente de evaluar los riesgos potenciales que podrían esta ligados a la utilización de esta nueva tecnología en la elaboración del vino. Esta presentación mostrará los datos existentes provenientes de los actuales proyectos de investigación relativos a la evaluación de estos riesgos. Más precisamente, exponemos los datos y los proyectos relativos: (i) los métodos de detección disponibles para controlar las cepas de levaduras genéticamente modificadas durante toda la cadena de producción del vino, (ii) la supervivencia y la dinámica de los tipos industriales, (iii) las cepas de levaduras genéticamente modificadas en medio natural y en los medios de la elaboración del vino, (iv) el potencial de transferencia natural de genes entre las levaduras y la microflora natural del vino, (v) el análisis de los posibles efectos secundarios de las modificaciones genéticas en la cepa de levadura y en la calidad del producto. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

Riassunto

La valutazione d'impatto ambientale di lieviti enologici geneticamente modificati

Negli ultimi anni, sono stati compiuti innumerevoli sforzi per perfezionare le varietà del lievito enologico Saccharomyces cerevisiae mediante l'uso di moderni strumenti biotecnologici. I principali obiettivi dei programmi per lo sviluppo dei lieviti sono stati, e sono tuttora, il miglioramento della qualità della fermentazione, l'efficacia del processo stesso, la qualità sensoriale del vino, nonché lo sviluppo di nuove varietà che presentino minori rischi e

maggiori benefici per la salute. Attualmente esistono già in laboratorio numerose varietà di lieviti stabili geneticamente modificati, mentre molti altri sono in via di realizzazione. I lieviti geneticamente modificati non sono stati usati, finora, nell'industria enologica. Tale situazione è dovuta principalmente alle preoccupazioni riguardanti gli OGM nei prodotti alimentari. Tuttavia, l'arrivo sul mercato di lieviti geneticamente modificati appare imminente, mentre c'è l'urgente necessità di valutare i rischi potenziali che possono essere legati all'uso di questa nuova tecnologia in tutta la catena produttiva del vino. Questa presentazione sarà incentrata sui dati esistenti ottenuti dagli attuali progetti di ricerca che riguardano la valutazione di tali rischi. Più specificatamente, verranno presentati dati e progetti su (i) i metodi di rilevamento disponibili per monitorare i lieviti geneticamente modificati in tutta la catena di produzione del vino, (ii) la sopravvivenza e la dinamica dei lieviti usati a livello industriale, sia naturali che (iii) geneticamente modificati, negli habitat naturali e negli ambienti di vinificazione, (iv) il potenziale del trasferimento del gene naturale dal lievito alla microflora naturale del vino, e (v) l'analisi dei possibili effetti collaterali di modificazioni genetiche sul lievito e sulla qualità del prodotto. (Bulletin O.I.V., 2004, vol. 77, n° 881-882, pp. 515-528).

INTRODUCTION

Wine fermentation used to be conducted by the microorganisms present on the grapes or the winery equipment, including yeast, fungi and bacteria. During these spontaneous fermentations, a succession of microorganisms dominates the early stages of the alcoholic fermentation, while yeasts, mainly of the species *Saccharomyces cerevisiae*, invariably dominate the latter stages of the process, when alcohol concentration is high. Today, spontaneous fermentations are still employed in some wineries, mainly by smaller producers of quality wines. However, most wine makers use commercially produced active dried wine yeast strains (ADWY) to inoculate grape must and kick-start the fermentation process. Inoculation presents several advantages that off-set the additional costs incurred when purchasing the yeast. The advantages include (i) the improved control of the fermentation process, (ii) the reduction of the risk of sluggish or stuck fermentations and of microbial contamination, and (iii) the choice of yeast strains that impart specific characteristics to the wine and achieve a desired outcome. No disadvantages have been associated with the use of ADWY, but for the observation that wines fermented by a single yeast strain may sometimes display reduced complexity when compared to those having undergone spontaneous fermentation.

Most commercial wine yeast strains available today have been bred and/or selected to display particular oenological characteristics, and yeast producers market their strains for specific applications and for specific styles of wines. These and other technological developments have without doubt contributed to an improvement in the average quality of wine, and have enhanced the ability of wine makers to control the fermentation process and to achieve specific outcomes. In this context, it is not surprising that continuous innovation is widely considered essential to maintain a competitive edge in the wine market. Innovation will be driven by several key technologies. Progress may still be made on improving the existing traditional viti- and vini-cultural practices. However, the probably biggest potential for

developing innovative solutions and for generating new products resides in the application of modern biotechnological tools, in particular genetic engineering.

The last two decades have seen a rapid increase in biotechnological know-how and in the development of modern molecular biology. The yeast *S. cerevisiae* has played an essential part in the development of these technologies, and a tremendous amount of knowledge has been accumulated about this organism. The potential for significant scientific and technological breakthroughs, based on the application of our accumulated know-how of yeast molecular biology to wine-related problems, promises therefore benefits for wine producers and consumers alike.

However, the application of modern biotechnology, particularly in agriculture and food production, remains controversial. Countless reports on the topic have been published, and the arguments for and against the use of genetic modification, be they of an ethical, economic, environmental or otherwise scientific nature, have been made extensively in public debates. Most of these discussions have not been conducted in a rational environment, and have frequently been hijacked by particular interests. This situation, combined with the sometimes irresponsible political handling of sensitive, public health-related issues and a silent scientific community, has created a negative public perception of GM-technology, particularly in European countries.

The future application of this technology in the wine industry therefore hinges on a scientifically sound evaluation of the safety and of the potential environmental and economic impact of genetically modified organisms. This evaluation requires the study of complex interactions and ecosystems, and needs to assess a large number of interrelated parameters. It therefore has to integrate multidisciplinary approaches, and include ecologists, microbiologists, geneticists, biochemists and other scientists. It is probably due to the intrinsic complexity of the topic that few studies have been conducted to holistically assess the environmental impacts of microbial GMOs (*Table I*).

Table I

Problems associated with the investigation of the ecological impact of introduced microorganisms

Conceptual	Inability to predict how potential impacts will be expressed within a complex ecosystem. Inability to define the level at which ecological impacts should be sought (micro- vs. macro-ecology) Absence of a framework of references for scientific interpretation of results (e.g. statistical vs. ecological significance) Impossibility to extrapolate data obtained in closed model systems to open natural systems
Technological	Monitoring techniques not adapted for specific purpose (I.e. sensitivity too high-too low) Techniques have only been assessed in model systems Quantitative data difficult to generate reliably

The research presented here describes several approaches that, when taken together, should provide us with a holistic view of the potential impact of GM yeast in a winery and associated environments. It is specifically focused on addressing several unresolved issues, including:

(i) Evaluating available detection methods for GM yeast.
(ii) Assessing the spreading of commercial wine yeast strains in the environment.
(iii) Comparing the behaviour of parental and genetically modified strains in model systems to assess whether GM strains may possess a selective advantage which could lead to their spreading.
(iv) Evaluating the probability of trans-genes spreading vertically to other yeast strains or horizontally to other species.
(v) Assessing the consequences of genetic modification on the modified yeast itself.

In the following sections, the current status of some research projects within each of these major topics will be described.

AVAILABLE METHODS FOR THE DETECTION OF GM YEAST

The available methods for the detection of genetically modified organisms in food were recently reviewed by Ahmed (2002). The most commonly used techniques are listed in *Table II*, and include Southern blotting and PCR analysis for the detection of transgenic DNA, and immunological tests for the detection of GM proteins. All of these methods require detailed *a priori* knowledge of the specific GMO to be monitored, in particular regarding the specific sequences used for the modification.

If no such information is available, it may be possible to investigate strains for the presence of those sequences that are most commonly used in genetic engineering, including selection markers (e.g. antibiotic resistance genes), particular promoters (sequences that regulate the expression of an inserted gene) or secretion signal sequences (if the GMO is suspected to produce a secreted protein), but modern strategies provide a wide, indeed virtually limitless variety of choices to biotechnologists, making such a search a rather hazardous undertaking.

PCR-based methods are highly sensitive, but high sensitivity also leads to an increased risk of generating false positive results. This problem is amplified when investigating the presence of a GMO or of GM-products in a complex food-product like wine, where the donor species of the investigated heterologous gene may also have been present during the production process. Indeed, many currently ongoing yeast improvement projects are using DNA isolated from organisms that are naturally present in wine.

Our data show that all techniques described in *Table II* can be used to trace GM yeast in a wine or vineyard environment. However, when complex populations have to be assessed, none of the techniques offered easily reproducible results. In addition, quantification of GM yeast within a mixed culture using quantitative DNA methods has proved extremely difficult. Furthermore, none of the currently available methods is suitable for standard analysis in a wine cellar environment.

Table II

Summary of methods that specifically detect recombinant DNA or its products in food-stuff (Adapted from Ahmed, 2002)

Parameter	Protein-based			DNA-based			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR and limiting dilution	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High	High
Duration	2 d	30-90 min	10 min	6 h’	1.5 d	2 d	1 d
Gives quantitative results	No	Yes	No	No	No	Yes	Yes
Suitable for field test	No	Yes	Yes	No	No	No	No
Employed mainly in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

THE SPREADING OF COMMERCIAL WINE YEAST STRAINS IN VINEYARDS

Commercially used yeast strains are released in large numbers and on an annual basis by most wine producers. These strains will in most cases not have originated from the area of release, and may therefore be expected to have an impact on the naturally occurring yeast micro-flora. This impact has to be carefully assessed, since the existing commercial wine yeast strains will be the target for genetic manipulations. It is absolutely necessary to know to what extend these existing commercial wine yeast strains survive and spread in nature and to what extend they influence the fermentations of the following year.

To study the effects of introduced new yeast varieties, be they genetically modified or not, we assessed the spread of commercially used wine yeast strains. We specifically investigated the potential spreading of the wine yeast strains most commonly used in South Africa and in the wine estates investigated, including VIN13, VIN7, WE228, N96, WE14, WE372, D254, CY3079, Bordeaux Red, EC1118, D47, DV10, L2056 and QA23. The yeast strains were isolated during the 1998 and 1999 harvesting seasons from vineyards on six wine farms in different climatological regions in the Western Cape. Six sampling sites, identified according to their relative position to the cellar, the dumping sites of grape skins, the topology of the area (including drainage lines etc) and the prevailing wind direction were selected on each farm. Due to the large number of yeasts isolated, a near-infrared spectroscopy (NIR) technique to identify the yeast isolates was developed. The results of the NIR were further verified by pulsed-field gel electrophoresis (CHEF).

The data confirmed previous reports (Van der Westhuizen *et al.*, 2000) indicating that *Saharomyces cerevisiae* is not present in large numbers in vineyards, since the vast majority of isolated yeast belonged to the genera *Kloeckera* and its anamorph *Hanseniaspora*, as well as *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Rhodotorula*. A total of 34 and 55 different "wild" *Saccharomyces* strains were identified after spontaneous fermentation of the grape must during the 1998 and 1999 seasons, respectively. Out of 1500 individual colonies screened, only eight (or less than 1%) were identified as commercial wine yeast strains, seven corresponding to VIN13, while one isolate was identified as N96. Both of these strains are widely used in the South-African wine industry. None of the other commercial strains could be detected at any of the sampling sites. All industrial *Sacchromyces cerevisiae* strains identified were found at sites closely associated with the winery dumping sites. Considering the position of the sampling sites, which were selected for the high probability of accidental dispersal of yeast from the dumping sites, and taking into account the large number of yeast isolated, the data suggest that commercial wine strains are not easily dispersed from the cellar to the vineyard. The data are in accordance with findings by other groups in France and Portugal that will also be presented at this meeting.

THE DETECTION AND MONITORING OF GENETICALLY MODIFIED YEAST STRAINS WITHIN MICROBIAL VINEYARD POPULATIONS

To monitor the behaviour of genetically modified yeast strains during wine fermentation and in the vineyard, and to compare the relative fitness of

these strains and of the corresponding parental strains, several trials have been conducted at the Wine Research Institute in Geisenheim, Germany, and at the Department of Microbiology and the Institute for Wine Biotechnology in Stellenbosch, South Africa. Since GM yeast may not be released into the environment, the studies were conducted in confined wine cellars and vineyards established in greenhouses.

The greenhouse trial was started in 1999, using a newly established vineyard of one year old vines. In the first year of investigation, only the naturally occurring yeast population was assessed. The most prevalent yeast genera and species that were isolated from the berries, leaves, stems and the soil included *Rhodotorula*, *Saccharomycodes*, *Yarrowia lipolytica*, *Pichia guilliermondii*, *Metchnikowia pulcherima* and *Hanseniaspora*. To a lesser extent *Candida parapsilosis* and *Debaromyces hansenii* were also detected.

A well-known industrial wine yeast strain, *Saccharomyces cerevisiae* VIN13, was used as the parental strain. Several genes were transformed into this strain, either as single genes or in combination. These genes originated either from bacteria or non-*Saccharomyces* yeast, and included the *LKA1* gene, which encodes a raw starch degrading α-amylase, the end1 gene (encoding endo-β1,4-glucanase) and the *XYN4* gene (encoding xylanase), as well as the pelE (encoding pectate lyase) and peh1 (encoding polygalacturonase) genes. All genes are under the control of strong yeast promoters and terminators and have been integrated into the genome of VIN13, together with the yeast-derived resistance marker *SMR*-410 or the kanMX gene which confers resistance to geneticin.

Before the spraying of the yeasts, the greenhouse was divided into four individually contained blocks, each block consisting of 20 vines. In the first year of the trial, block 1 was left untouched, and no yeast strain was released, whereas the other three blocks were each treated with 1.5 l of a solution containing 2.5×10^6/ml of colony forming units (CFU) of pure yeast culture. Treatment was applied by directly spraying the yeast solution onto the vines. Block 2 was sprayed with the GM yeast alone, block 3 with the parental strain VIN13, whereas block 4 was sprayed with a 1:1 mixture of both strains (Figure 1).On a weekly basis, the yeast populations on the grapes, leaves, stem and soil in the different blocks were monitored. Although a high concentration of yeast was sprayed, few *S. cerevisiae* strains could be isolated

FIGURE 1

Arrangements of blocks in greenhouse trials. Control: No yeast sprayed. VIN13: Only parental strain sprayed. LKA: GM yeast with *LKA1* gene. Gluc: GM Yeast with glucanase-encoding genes. Pect: GM yeast with pectinase-encoding genes.

YEAR 1

1 Control	4 LKA + VIN13
LKA 2	VIN13 3

YEAR 2

1 Control	4 LKA + Gluc + Pect
LKA 2	LKA + Gluc 3

at any given time. The yeast population in the sprayed blocks was otherwise very similar to the one found on the control vines, indicating that the commercial or GM yeast did not affect the overall ecological balance of the micro-flora. Furthermore, no significant differences between the behaviour of the genetically modified and the parental strains could be detected.

Micro-vinifications were conducted from the grapes harvested from each block, and the numbers of *S. cerevisiae* cells present during the fermentations was monitored. In all cases, the must obtained from grapes harvested from the blocks that had been sprayed with commercial or GM yeast strains fermented significantly more efficiently than the must obtained from the control block, indicating the presence of increased numbers of *S. cerevisiae* strains on the grapes. However, no significant differences were observed between the blocks that had been sprayed with either the GM yeast or with the parental strain.

In the second year, the four blocks were again sprayed, but GM yeasts with activities that may provide a selective advantage over the parental strains were used (*Figure 1*). Indeed, the GM yeasts were designed to secrete significant amounts of polysaccharide-degrading enzymes, in particular glucanases and pectinases, which may provide some advantages to the strains when released on the vines. However, the same patterns as in the previous year were observed, and no significant differences were detected between the transformed and the parental strains, both with regard to their presence in the vineyard and to the cell numbers and fermentation efficiencies during spontaneous fermentations.

The data again suggest that the GM yeasts did not benefit from any specific advantage in terms of overall fitness when released in the vineyard, since the weekly sampling did not reveal any statistically significant differences between the three blocks that had been sprayed. During spontaneous fermentations of the grape musts derived from the different blocks, no significant difference in fermentation speed, efficiency or the number of colony forming units could be observed, and none of the different yeast was able to dominate other yeast strains in both the vineyard and during spontaneous fermentation (*Figure 2*).

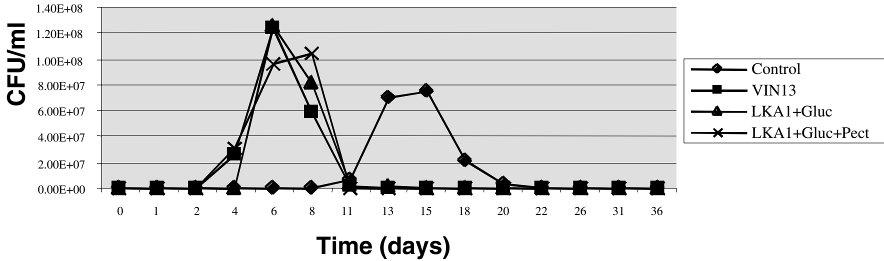
We are currently assessing the fermentation behaviour of the same strains when directly inoculated into grape must, either as single strains or as a mixture of GM and parental strains. These experiments will allow assessing the relative fitness of these strains when subjected to direct competition during wine fermentation.

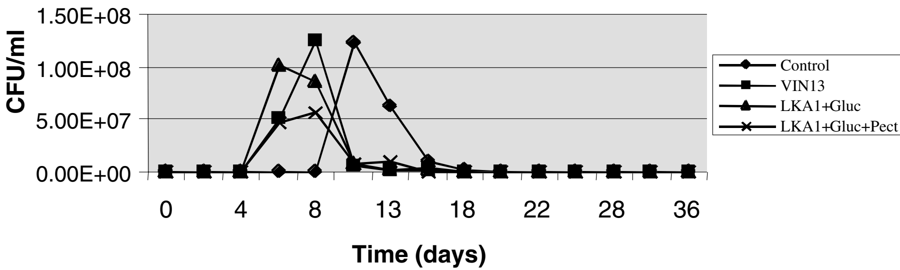
The assessment of vertical and horizontal gene transfer from wine yeast strains to other strains or microorganisms

The assessment of potential risks associated with GMOs has to take into account the possibility that a specific character conferred through GM technology may be able to spread beyond the confines of the initially modified strain. Vertical gene transfer refers to the transmission of DNA through sexual reproduction. In the case of wine yeast strains, it would require sporulation of the strain, followed by mating of the spores with either each other or with spores of another *S. cerevisiae* strain that may have been present during the fermentation process. To our knowledge, no direct observations

FIGURE 2

Spontaneous fermentations of grapes harvested from the different blocks. The graphs represent two independently conducted experiments (A and B), and indicate the number of colony forming units observed during the fermentations. The data correlate well with fermentative activity (data not shown).





of sporulation in commercial wine yeast strains during wine fermentation have been published, and its occurrence will therefore have to be assessed during this study. Horizontal gene transfer, on the other hand, refers to the asexual transfer of DNA from one species to another, and has recently attracted wide-spread interest and media coverage. The analysis of entire genome sequences has revealed that most, if not all species, have been subjected to horizontal transfers in their evolutionary history, and that the process has probably played an essential role in evolution. Horizontal transfer of DNA has been observed between a number of microorganisms, both between two strains of the same species and between unrelated species. However, few if any data exist regarding its occurrence and possible mechanisms in eukaryotic organisms, but for the transfer of genes by viruses. Furthermore, no data are available regarding the possible occurrence of such transfers between microorganisms present during wine fermentation, and in particular between a wine yeast strain and either other yeast strains and species or bacteria.

Two projects are currently underway at the Institute for Wine Biotechnology (South Africa) to assess the probability of DNA transfer between two

wine yeast strains and between wine yeast strains and other microorganisms during wine fermentation. The first project more specifically assesses the probability of sexual DNA transfers, whereas a second project investigates the general probability of DNA transfer, particularly through non-sexual mechanisms.

Most industrial wine yeast strains are either diploid or polyploid. Sexual reproduction of these strains requires that cells sporulate, which results in the formation of four haploid or aneuploid ascospores. A single spore can multiply vegetatively until encountering a cell of the opposite mating type, which will lead to the fusion of the two cells. Sporulation and mating would be the most obvious and easiest way through which specific characters or genes, whether established through genetic modification or traditional methodologies, can be transferred from one strain to another. The presence or absence of sporulating cells in a culture is difficult or impossible to assess visually if the percentage of these cells is low, which will be the case during wine fermentation. To overcome this problem, we will fuse an Open Reading Frame (ORF) encoding a Green Fluorescent Protein (GFP) to the promoter of a strong sporulation-specific gene. Several such genes have been identified. A plasmid carrying a dominant selection marker combined with the above mentioned construct will be transformed into industrial wine yeast strains, and the transformed strains will be inoculated individually into experimental fermentors containing grape must. Each wine yeast strain of interest can be monitored, and the frequency of sporulation can be established. The influence of environmental factors on sporulation frequency can also be assessed.

To assess the occurrence of horizontal gene transfer, several industrial wine yeast strains with a well established genetic background and karyotype will be transformed with plasmid DNA carrying a single dominant selection marker, which confers resistance to either sulfomethurone (*SMR1*) or geneticin (kanMX). Plasmids will be both centromer (single copy) and 2-micron (multiple copy) based. Two transformed strains, each carrying a different marker, will be co-inoculated at variable initial ratios into grape must or specific laboratory media. After having completed micro-vinifications under various conditions, possible transfers of DNA will be assessed by isolating strains resistant to both compounds. Should such strains be found, they will be analysed genetically and karyotyped in order to assess whether the transfer was limited to plasmid DNA or involved the transfer of chromosomal fragments as well.

The experimental set up provides a framework to assess a "worst case scenario", since it uses conditions which might be optimal for horizontal DNA transfer to occur:

- Plasmid based genes are probably more likely to be transferred. Plasmids are small, autoreplicating elements and therefore are more likely to be both released from cells and to be taken up by another cell. The possibility of transfer will be further increased through the use of high copy number plasmids;
- Cells will be grown to high density, increasing contact between cells and therefore increasing the likelihood of transfer;
- Grape must fermentation results in high levels of ethanol which is known to increase membrane permeability, and should therefore increase the

possibility of DNA transfer. Laboratory media with high ethanol content will also be used during the experimental phase;
- Each assay will be monitored for a prolonged period of time, even after the end of fermentation. Numerous cells undergo autolysis during the late stages of fermentation which should lead to increased release of DNA and increased transfer probability.

The data will provide essential information regarding the assessment of risks associated with genetically modified yeast. Since the experiments are designed to improve the chances of transfer, a negative result would provide a strong indication that such a transfer is very unlikely to occur and that the spreading of characters that have been modified in GMO yeast is an unlikely event.

ASSESSING UNFORESEEN CONSEQUENCES OF GENETIC MODIFICATION ON THE MODIFIED ORGANISM

Genetic engineering has a number of advantages over classical breeding and selection. In particular, it allows the specific modification of a single trait of a target organism, without changing the genetic background. In most cases, genetic engineering aims either to introduce new enzymatic activities or to change the metabolic flux through specific pathways by changing existing enzymatic activities. In both cases, the change will have some metabolic consequences, be they due to the demand for additional protein synthesis in the case of expressing new enzymes destined for secretion, the modification of metabolite concentrations or the presence of new, foreign metabolites.

It is likely that the changed parameters will be sensed by the complex regulatory mechanisms that exist within any living cell, and will lead to a specific molecular response. This response may result in some unforeseen, indirect consequences regarding the metabolic activity of the cell. In several cases of attempted metabolic engineering of wine yeast strains, such unforeseen consequences have been described, an example being the increased production of acetate in strains with increased levels of glycerol-synthesising enzymes. Modern biotechnological tools allow to systematically assess any biological process on a 'global' level, by analysing the entire transcriptome (all mRNAs present in a cell), and – in the near future – the proteome (all proteins) and metabolome (all metabolites). The transcriptome can be analysed by microarrays, which monitor the transcription of all protein-encoding genes present in the genome of yeast.

Several studies have already been conducted to compare the transcriptional regulation in parental and genetically modified yeast strains. By monitoring the transcriptome, possible unexpected side-effects of the genetic modification can be revealed and analysed. In Montpellier, the effects of the introduction of a gene that lead to the production of high levels of lactate by *S. cerevisiae* (Dequin *et al*., 1999) has been studied in some detail. This yeast has been transformed with a bacterial gene encoding lactate dehydrogenase, and produces up to 35 g/l of lactate. The high level of lactate suggests that the metabolism of this specific yeast strain has been profoundly modified, and the strain indeed displays several additional phenotypes, including reduced fermentative activity and numerous knock-on effects

are therefore expected. The data show that the modified strain responds to the increased levels of acid by modifying the expression of more than 100 genes. For the most, these genes belong to groups which are required to adjust or to maintain the internal pH of the cell or to protect the cell against environmental stress, including proton transporters and heat-shock proteins. Other genes whose expression has been modified are involved in general metabolism (glycolytic genes). Similar studies are underway in several laboratories, and will lead to the establishment of databases where all metabolic side effects of genetic modifications can be analysed. In the near future, it will be possible to fully assess the consequences of any genetic modification at all levels of cellular metabolism.

Conclusion

The projects described in this report are designed to generate a scientifically sound, holistic view of the potential effects of genetically modified yeasts in the wine industry. Most of these projects will have been finalised within the next two years, and the data will provide a framework for the assessment of individual strains by regulatory bodies, including the OIV and national governments.

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Interaction between *Glomus mosseae* and soil yeasts on growth and nutrition of cowpea

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KEYWORDS
Mycorrhiza;
Yeasts;
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Summary

A glass house experiment was conducted to study the interaction between the mycorrhizal fungus, *Glomus mosseae* and six soil yeasts (*Rhodotorula mucilaginosa*, *Metschnikowia pulcherrima*, *Trichosporon cutaneum* var. *cutaneum*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii*, *Debaryomyces occidentalis* var. *occidentalis*), and their effect on growth and nutrition of cowpea. All the yeasts had a synergistic interaction with the mycorrhizal fungus and dual inoculation improved plant growth compared to single inoculation with *G. mosseae* alone. Nitrogen and phosphorus uptake of plants was also enhanced significantly in *G. mosseae* and soil yeasts combinations. Growth, N, P, chlorophyll and phenol content and yield of cowpea were highest in plants treated with *G. mosseae*+*R. mucilaginosa*. Mycorrhizal root colonization, spore numbers and population of yeasts in the root zone soil were also highest in the treatment *G. mosseae*+*R. mucilaginosa* and least in the uninoculated plants.
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Introduction

Arbuscular mycorrhizal fungi (AMF) are probably most abundant in agricultural soils, accounting for about 5–50% of the biomass of soil microbes (Olsson et al., 1999). They are recipients of wide attention as part of popular paradigm that considers an active and diverse soil biological community, essential for increased sustainability of agricultural systems (Gianinazzi and Schuepp, 1994). Inoculation with AMF is known to increase the growth of many plant species. This is attributed to increased uptake of nutrients (especially diffusion limited nutrients like P, Zn, Cu, etc.) production of growth promoting substances, tolerance to drought, salinity and transplant shock, resistance to plant pathogens and synergistic interaction with other beneficial soil microorganisms such as N_2-fixers, P solubilisers (Bagyaraj and Varma, 1995). It has been established that mycorrhizal plants grow better than nonmycorrhizal plants in infertile soils because of improved mineral nutrition through hyphae, which help in exploring a greater volume of soil beyond the root hairs (Rajan et al., 2000). Enhanced mineral nutrition helps in increased

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chlorophyll content thus helping in higher photosynthetic rate (Bian et al., 2001; Feng et al., 2002). Further, reduction in the severity of disease caused by root pathogens is attributed to morphological alterations caused by AMF on the host like thickening of the cell wall, stronger vascular bundles, etc or because of physiological changes in host like increased concentration of P, phenoloics, sulfur containing aminoacids (Raman et al., 2001; Boby and Bagyaraj, 2003).

Mycorrhizal fungi interact with a wide range of other soil organisms in the root, rhizosphere, and in the bulk soil. These interactions may be inhibitory or stimulatory; some are clearly competitive and others may be mutualistic. Though, AMF are not capable of fixing atmospheric N_2, they are known to increase N_2-fixation and positively interact with N_2-fixers (Bagyaraj and Menge, 1978; Bethlenfalvay, 1992; Barea et al., 1992). Combined inoculation of phosphate solubilizing microorganisms and AMF have shown better N and P uptake and improved crop yields in nutrient deficient soils (Singh and Kapoor, 1999). Fracchia et al. (2000) reported existence of synergistic interaction between saprophytic fungus *Fusarium oxysporum* and AMF resulting in increased root colonization and enhanced growth. Singh et al. (1991) observed enhancement of root colonization of legumes by native AMF through inoculation of legume seeds with a commercial yeast (*Saccharomyces cerevisiae*). Yeasts are one of the important sources of Vitamin B_{12}. Though Vitamin B_{12} produced by yeasts may not be required directly for plant growth, yeasts in the root zone may influence plant growth indirectly by encouraging the growth of other plant growth promoting rhizomicroorganisms (PGPRs). Enhancement of root colonization of legumes by native AM fungi through inoculation of seeds or soil with the yeasts *S. cerevisiae* and *Rhodotorula mucilaginosa* has been reported by earlier workers (Singh et al., 1991; Fracchia et al., 2003).

So far, no information is available on the influence of soil yeasts on multiplication and establishment of introduced AMF in soils. So, a glass house experiment was conducted to study the interaction of soil yeasts and AMF by taking cowpea as the host.

Materials and methods

AM inoculum

Glomus mosseae, a local isolate obtained from the rhizosphere of *Leucaena leucocephala*, maintained as pot culture in sterilized sand: soil (1:1 v/v) mixture on Rhodes grass (*Chloris gayana*) was used as the AMF in the present study. The air-dried inoculum contained AM hyphae, spores and root pieces. About 12,500 infective propagules based on the most probable number estimation (Porter, 1979) was added as a layer to the soil 3 cm below the surface before sowing cowpea.

Yeast cultures

Six yeast cultures namely *Debaryomyces occidentalis* var. occidentalis (MTCC 29), Metschnikowia pulcherrima (MTCC 632), *S. cerevisiae* (MTCC 170), *Cryptococcus laurentii* (MTCC 3953), *Rhodotorula mucilaginosa* (MTCC 850) and *Trichosporon cutaneum* var. cutaneum (MTCC 255) were obtained from Microbial Type Culture collection, Institute of Microbial Technology, Chandigarh, India. The first three yeast cultures are fermentation positive with sexual reproduction and the last three yeast cultures are fermentation negative with no sexual reproduction (Barnett et al., 2000). The yeast cultures were grown in malt yeast extract broth for 6 days at 30 °C in a shaker at 120 rpm. The cultures were centrifuged at 5500 rpm for 20 min and the supernatant discarded and the pellets containing yeast cells were suspended in 0.01 M $MgSO_4$ solution. The colony-forming units (CFU) of soil yeasts were $8–10 \times 10^5$/ml inoculum. The yeast cultures were applied to the seed hole at the rate of 25 ml per hole. The control plants were provided with 25 ml of 10 mM $MgSO_4$ solution.

Seed treatment and planting

Cow pea seeds (Variety TC-201) were surface sterilized with 70% ethyl alcohol for 1 min followed by washing three times with sterilized distilled water. Seeds were later treated with 0.01% $HgCl_2$ for 3 min and washed with sterilized distilled water six times. Cowpea seeds were sown in pots containing soil and were thinned to 2 plants per pot after one week of germination. The soil used in the experiment was an Alfisol of a fine kaolinitic, isohyperthermic Typic Kanhaplustalfs type having pH 5.8, available P of 5.6 $\mu g\, g^{-1}$ (NH_4F+HCl extractable) and an indigenous mycorrhizal spore count of 37 per 50 g of soil. The plants were watered once in 2 days and were grown for 90 days under glass house conditions.

The experiment consisted of the following 8 treatments with 8 replications:

(1) Uninoculated control and inoculated treatments with (2) *G. mosseae* (Gm) (3) Gm+*R. mucilaginosa* (4) Gm+*Metschnikowia pulcherrima*

(5) Gm+*T. cutaneum* var. cutaneum (6) Gm+*S. cerevisiae* (7) Gm+*C. laurentii* (8) Gm+*D. occidentalis* var. *occidentalis*.

Plant parameters studied

Plant height and number of leaves were recorded once in 20 days interval up to the time of harvest, i.e., 90 days after sowing (DAS). Only values at the time of harvest are presented in this paper. Dry weights of shoot and root were determined after drying to a constant weight. Yield of pods/plant was also recorded. The chlorophyll content of leaves was estimated by dimethyl sulfoxide (DMSO) method (Hiscox and Israedstam, 1979). The phenolic content of root and leaf samples were estimated by the method given by Sadasivam and Manickam (1996). Plant phosphorus concentration was estimated colorimetrically following the vanadomolybdate yellow color method (Jackson, 1973). The nitrogen content of plant samples was determined by the method given by Catalado et al. (1975).

Microbiological analysis

Mycorrhizal root colonization was determined by the grid line intersect method (Giovannetti and Mosse, 1980) after staining the roots with trypan blue (Philips and Hayman, 1970). The number of extramatrial chlamydospores produced by AMF in the root zone soil was estimated by wet sieving and decantion method (Gerdemann and Nicolson, 1963). The population of soil yeasts in the root zone soil was determined at 20 days interval by serial dilution plate method using malt extract agar (Galloway and Burgess, 1952). But only the values at the time of harvest are given in this paper. The vitamin B_{12} production of yeasts was determined by the microbiological assay using *Lactobacillus delbrueckii* sub sp. *lactis* (MTCC 0911) as the test organism. The culture filtrate of yeasts grown on malt yeast extract broth was used in the assay solution. Vitamin B_{12} agar was seeded with the test bacterium. One cm filter paper discs dipped in assay solution were placed on seeded agar plates and inoculated. The zone of exhibition by the test bacterium indicated the presence of vitamin B_{12} in the assay solution (Foster et al., 1949).

Statistical analysis

The results of various parameters obtained from the experiments were analyzed by statistical analysis suitable to RCBD and were separated using Duncan's Multiple Range Test (DMRT) (Duncan, 1965).

Table 1. Interaction effects of *Glomus mosseae* and soil yeasts on growth and yield of cowpea

Treatment	Plant height (cm)	No. of leaves	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Total biomass (g/plant)	Yield (g/plant)
Uninoculated control	28.23[d]	7.0[c]	3.91[f]	1.42[d]	5.33[f]	9.21[e]
Inoculated with *G. mosseae* (Gm)	29.08[cd]	7.3[bc]	4.3[de]	1.62[cd]	5.92[e]	10.1[de]
Inoculated with Gm+*R. mucilaginosa*	33.18[a]	10.3[a]	6.26[a]	2.12[a]	8.38[a]	13.8[a]
Inoculated with Gm+*M. pulcherrima*	33.08[a]	8.6[abc]	5.93[b]	1.91[ab]	7.84[b]	11.03[cd]
Inoculated with Gm+*T. cutaneum* var. *cutaneum*	31.55[ab]	9.2[a]	5.21[c]	1.96[ab]	7.17[c]	10.34[de]
Inoculated with Gm+*S. cerevisiae*	31.99[ab]	9.0[ab]	4.84[d]	1.74[bc]	6.58[d]	13.71[a]
Inoculated with Gm+*C. laurentii*	30.19[bc]	9.4[a]	6.12[a]	1.70[bc]	7.82[b]	12.9[ab]
Inoculated with Gm+*D. occidentalis* var. *occidentalis*	31.13[b]	9.5[a]	5.83[b]	2.02[a]	7.85[b]	12.0[bc]

Means having same superscript do not differ significantly at $P = 0.05$ level by DMRT.

Results

Response of cowpea to inoculation with *G. mosseae* and soil yeasts

Among the various treatments, plants inoculated with *G. mosseae*+*R. mucilaginosa* showed highest plant height, number of leaves, total biomass, number of pods and yield of cowpea (Table 1). At the time of harvest, highest height was observed in plants inoculated with *G. mosseae*+*R. mucilaginosa*, which was found to be significantly different from that of *G. mosseae* alone and uninoculated control. Highest number of leaves were observed in *G. mosseae*+*R. mucilaginosa* treatment, which was on par with all treatment combinations of *G. mosseae* plus soil yeasts but significantly different from *G. mosseae* alone and uninoculated control. Single inoculation with *G. mosseae* did not result in significantly higher number of leaves compared to uninoculated control.

Shoot dry weight was highest in plants inoculated with *G. mosseae*+*R. mucilaginosa* which was on par with the treatment *G. mosseae*+*S. cerevisiae*. This was followed by *G. mosseae*+*C. laurentii*, *G. mosseae*+*M. pulcherrima* and *G. mosseae*+*D. occidentalis*. Inoculation with *G. mosseae* alone resulted in significantly higher dry weight compared to uninoculated plants but on par with *G. mosseae*+*S. cerevisiae* treatment. Root dry weight was highest in *G. mosseae*+*R. mucilaginosa* treated plants which was on par with *G. mosseae*+*D. occidentalis*, *G. mosseae*+*T. cutaneum* and *G. mosseae*+*M. pulcherrima* treatments. Single inoculation of *G. mosseae* did not result in significantly higher root dry weight compared to uninoculated plants but the difference in total plant biomass (shoot and root) was significantly more. Total biomass of plants in all the dual inoculated plants was significantly more compared to plants inoculated with *G. mosseae* alone. In *G. mosseae*+*R. mucilaginosa* treated plants, the total plant biomass was significantly more compared to all other treatments. This was followed by *G. mosseae*+*D. occidentalis*, *G. mosseae*+*M. pulcherrima* and *G. mosseae*+*C. laurentii* inoculated plants, all the three not differing significantly. Uninoculated plants had the least plant biomass compared to all inoculation treatments.

The yield of pods was also highest in plants inoculated with *G. mosseae*+*R. mucilaginosa* which was on par with the treatments *G. mosseae*+*S. cerevisiae* and *G. mosseae*+*C. laurentii*. This was followed by those treated with *G. mosseae*+*D. occidentalis*, *G. mosseae*+*M. pulcherrima*, but not differing significantly from the treatments *G. mosseae*+*T. cutaneum* and *G. mosseae* alone.

Nitrogen and phosphorus uptake

The shoot P content was highest in plants inoculated with *G. mosseae*+*R. mucilaginosa* which was on par with *G. mosseae*+*C. laurentii* followed by *G. mosseae*+*D. occidentalis* and *G. mosseae*+*M. pulcherrima* both not differing significantly (Table 2). Single inoculation with *G. mosseae* resulted in significantly higher shoot P content compared to uninoculated plants. Root P content also showed more or less a similar trend, except that the roots of plants treated with *G. mosseae*+*C. laurentii* had lower P content, which was on par with *G. mosseae* alone treatment.

Highest shoot N content was recorded in plants inoculated with *G. mosseae*+*R. mucilaginosa* which was significantly different from all other inoculation, the next best treatments being *G. mosseae*+*M. pulcherrima* and *G. mosseae*+

Table 2. Interaction effects of *Glomus mosseae* and soil yeasts on P and N content of cowpea

Treatment	Shoot P content (mg/plant)	Root P content (mg/plant)	Shoot N (mg/plant)	Root N (mg/plant)
Uninoculated control	0.59^{e}	0.23^{e}	15.29^{g}	4.43^{g}
Inoculated with *G. mosseae* (Gm)	0.73^{d}	0.35^{d}	16.81^{f}	5.38^{f}
Inoculated with Gm+*R. mucilaginosa*	1.18^{a}	0.53^{a}	26.23^{a}	7.39^{a}
Inoculated with Gm+*M. pulcherrima*	1.01^{b}	0.45^{abc}	24.37^{b}	6.13^{d}
Inoculated with Gm+*T. cutaneum* var. *cutaneum*	0.93^{c}	0.48^{ab}	20.84^{d}	6.47^{b}
Inoculated with Gm+*S. cerevisiae*	0.89^{c}	0.43^{bc}	19.94^{e}	5.60^{e}
Inoculated with Gm+*C. laurentii*	1.11^{a}	0.40^{cd}	23.93^{b}	5.41^{f}
Inoculated with G+*D. occidentalis* var. *occidentalis*	1.03^{b}	0.46^{abc}	23.32^{c}	6.34^{c}

Means having same superscript do not differ significantly at $P = 0.05$ level by DMRT.

C. laurentii. Plants inoculated with *G. mosseae* alone had significantly higher N than the uninoculated plants. The root N content was also maximum in plants treated with *G. mosseae*+*R. mucilaginosa* significantly differing from all other treatments. Plants treated with *G. mosseae* and the other soil yeast combinations showed better root N content, except *G. mosseae*+*C. laurentii*, than plants inoculated with *G. mosseae* alone and uninoculated control.

Chlorophyll and phenolic content

The plants inoculated with *G. mosseae*+*R. mucilaginosa* had significantly higher chlorophyll content compared to all other treatments (Table 3). Chlorophyll content in the dual inoculated treatments did not differ significantly from the *G. mosseae* alone treated plants. Uninoculated plants had the least chlorophyll content.

Plants inoculated with *G. mosseae*+*R. mucilaginosa* had highest phenolic content in leaves significantly differing from all other treatments. All other *G. mosseae*+soil yeast combinations had significantly higher phenolic content than *G. mosseae* alone treated plants; uninoculated control plants had the least phenolic content. Root phenolic content in *G. mosseae*+*R. mucilaginosa* treated plants was almost thrice as that of *G. mosseae*+other soil yeast combinations. All the *G. mosseae*+soil yeast treatments were significantly different from *G. mosseae* alone treatment and uninoculated plants had the least root phenol content.

Mycorrhizal root colonization and spore count

Inoculation of plants with *G. mosseae* significantly increased root colonization compared to the uninoculated plants (Table 4). When *G. mosseae* was co-inoculated with soil yeasts, root colonization was further increased. Highest percent root colonization was observed in the treatment *G. mosseae*+*R. mucilaginosa*, which was significantly different from all other *G. mosseae*+soil yeast treatment combinations. The next best treatments were *G. mosseae*+*S. cerevisiae* and *G. mosseae*+*T. cutaneum*. Mycorrhizal spore count in the root zone soil also showed more or less a similar trend.

Population of yeasts in the root zone soil of cowpea

The population of yeasts in the root zone soil of cowpea showed fluctuations from 20 to 90 DAS. The population of *R. mucilaginosa* was highest throughout the period followed by *S. cerevisiae*. At the time of harvest, highest population of soil yeasts was observed in the root zone soil of plants treated with *G. mosseae*+*R. mucilaginosa* followed by *G. mosseae*+*S. cerevisiae* both differing significantly. This was followed *G. mosseae*+*T. cutaneum* and G. *mosseae*+*C. laurentii* both being on par with each other. The root zone soil of uninoculated plants and those treated with *G. mosseae* alone had significantly lower yeast population.

Vitamin B_{12} production by soil yeasts

An exhibition zone of approximately 5 mm was produced by all the 6 yeast cultures used in the present study suggesting that they are positive for vitamin B_{12} production in *Lactobacillus* seeded Petri plates.

Discussion

Reports available till now reveal that in spite of the presence of natural endophytes, introduced

Table 3. Interaction effects of *Glomus mosseae* and soil yeasts on the chlorophyll and phenolic content of cowpea

Treatment	Chlorophyll content (mg/g)	Phenolic content (mg/g)	
		Leaves	Root
Uninoculated control	0.401^{c}	8.61^{g}	0.10^{g}
Inoculated with *G. mosseae* (Gm)	0.455^{b}	9.12^{f}	0.47^{f}
Inoculated with Gm+*R. mucilaginosa*	0.510^{a}	15.5^{a}	3.06^{a}
Inoculated with Gm+*M. pulcherrima*	0.500^{ab}	13.7^{c}	0.93^{d}
Inoculated with Gm+*T. cutaneum* var. *cutaneum*	0.470^{ab}	10.61^{e}	0.76^{e}
Inoculated with Gm+*S. cerevisiae*	0.483^{ab}	14.25^{b}	1.06^{c}
Inoculated with Gm+ *C. laurentii*	0.50^{ab}	10.5^{e}	1.07^{c}
Inoculated with Gm+*D. occidentalis* var. *occidentalis*	0.477^{ab}	11.86^{d}	1.25^{b}

Means having same superscript do not differ significantly at $P = 0.05$ level by DMRT.

Table 4. Interaction effects of *Glomus mosseae* and soil yeasts on mycorrhizal root colonization, spore count and yeast population in the root zone soil of cowpea

Treatment	Root colonisation (%)	Spore numbers/50 g soil	Yeast population (CFUx10^4)
Uninoculated control	40.0^{e}	37.0^{e}	18.0^{f}
Inoculated with *G. mosseae* (Gm)	52.0^{d}	44.0^{d}	22.0^{f}
Inoculated with Gm+*R. mucilaginosa*	77.7^{a}	72.0^{a}	80.4^{a}
Inoculated with Gm+*M. pulcherrima*	68.0^{c}	58.0^{c}	27.4^{e}
Inoculated with Gm+*T. cutaneum* var. *cutaneum*	70.3^{bc}	66.0^{ab}	35.5^{c}
Inoculated with Gm+*S. cerevisiae*	72.7^{b}	58.7^{c}	45.2^{b}
Inoculated with Gm+*C. laurentii*	69.0^{c}	60.0^{bc}	32.8^{cd}
Inoculated with Gm+*D. occidentalis* var. *occidentalis*	69.7^{c}	55.0^{c}	28.1^{de}

Means having same superscript do not differ significantly at $P = 0.05$ level by DMRT.

mycorrhizal fungi could improve growth under unsterile soil conditions. It has also been reported that dual inoculation of AM fungi with other beneficial microorganisms enhances P uptake and increased dry matter yields (Antunes and Cardoso, 1991; Ortas et al., 2002). In the present study, cowpea plants inoculated with the AMF *G. mosseae* alone grew taller than the uninoculated plants but better growth was observed when AMF was co-inoculated with soil yeasts. Plants inoculated with *G. mosseae*+*R. mucilaginosa* exhibited maximum height, number of leaves and biomass. In *G. mosseae*+*R. mucilaginosa* treated plants the increase in total biomass was 60 % more compared to uninoculated plants. Dual inoculation with other yeasts like *M. pulcherrima, D. occidentalis, C. laurentii* resulted in increase in biomass of 47% each while treatment with *T. cutaneum* and *S. cerevisiae* resulted in 36% and 23% increase, respectively. An increase in plant biomass due to inoculation with the native AM fungi+*S. cerevisiae* has been reported by Singh et al. (1991). This was attributed to the stimulatory effect of yeasts on multiplication, spore germination and establishment of native AMF. The present study brings out that *R. mucilaginosa* is a better mycorrhizal helper organism stimulating colonization of mycorrhizal fungus and thus in turn better plant growth response compared to *S. cerevisiae*. There is evidence that B vitamins can be absorbed by roots, producing favorable effects on root development (Dobbelaere et al., 2003). Perhaps this valorized root development is responsible for the enhanced activity of AMF.

The AMF *G. mosseae*, which improved plant biomass, also enhanced P uptake by the plants. Plants inoculated with *G. mosseae* in combination with soil yeasts further enhanced plant P uptake. Plants inoculated with *G. mosseae*+*R. mucilaginosa* had highest P content. The shoot P uptake was two times higher in *G. mosseae*+*R. mucilaginosa* treated plants compared to uninoculated plants. The root P uptake was 30 % higher in *G. mosseae*+*R. mucilaginosa* treatment compared to the uninoculated plants. AM fungi are known to improve P nutrition of plants especially in P deficient soil and can translocate phosphate by scavenging a larger volume of soil with extensive hyphae (Kothari et al., 1990; Ortas et al., 2002).

Shoot and root N content of plants inoculated with *G. mosseae*+*R. mucilaginosa* was 70% and 65% more, respectively, compared to uninoculated plants. Singh et al. (1991) reported increased nodule number and dry weight of legumes due to inoculation with yeasts because of stimulation of indigenous microflora. The increase in nodulation and other symbiotic parameters of forage legumes (*Trifolium alexandrium* and *Medicago sativa*) due to combined inoculation of yeasts (*S. cerevisiae* and *Candida torpicalis*) and specific *Rhizobium* sp. have been reported earlier (Tuladhar and Subba Rao, 1985) and it was attributed to the stimulatory action of yeasts on the multiplication of native rhizobia (Tuladhar, 1983). Therefore, it is possible that the increased uptake of N observed in the present study could be due to stimulation of native rhizobia by AMF+soil yeast combinations. Synergistic interaction between rhizobia and AMF is well documented (Dela Cruz et al., 1988; Mandal et al., 1995).

Inoculation with *G. mosseae* alone resulted in significantly higher chlorophyll content compared to control plants. Increase in chlorophyll content due to inoculation with AMF in plants has been reported by earlier workers (Bian et al., 2001; Feng et al., 2002). Plants treated with *G. mosseae*+*R. mucilaginosa* exhibited 80% increase in phenolic content in leaves compared to uninoculated con-

trol. Leaves of plants inoculated with *G. mosseae* alone, also had 20% higher phenolic content compared to uninoculated plants. Increased polyphenol oxidase, phenyl alanine ammonia lyase and peroxidase activity in mycorrhizal plants, which are responsible for the oxidation of phenolic compounds to quinones to keep off pathogens has been reported earlier (Mathur and Vyas, 1996; Raman et al., 2001). The present study brings out that coinoculation of soil yeasts with AMF can enhance the chlorophyll and phenolic content of plants significantly compared to inoculation with AMF alone. The reason for such an increase needs further investigation.

In general, mycorrhizal inoculation increased the percent mycorrhizal root colonization and spore numbers in the root zone soil compared to uninoculated plants. The extent of mycorrhizal root colonization and spore numbers in the root zone soil varied with *G. mosseae* and different soil yeast combinations. The maximum percent mycorrhizal root colonization and spore numbers in the root zone soil was observed in plants inoculated with *G. mosseae*+*R. mucilaginosa*. Singh et al. (1991) observed increased production of vesicles, arbuscules and spores of native AMF because of inoculation with *S. cerevisiae* in legumes while Fracchia et al. (2003) reported enhanced AM colonization of soyabean and red clover when the yeast *R. mucilaginosa* was applied to the soil.

The population of soil yeasts in the root zone soil was stimulated in plants inoculated with *G. mosseae*+soil yeasts. The plants inoculated with *G. mosseae* alone also had higher yeast population in the root zone soil compared to uninoculated plants. This upholds the observation made by earlier workers that AM fungi stimulated the activity of beneficial soil microorganisms (Meyer and Linderman, 1986; Boby and Bagyaraj, 2003).

All the characters studied such as plant height, number of leaves, dry weight, P and N uptake were highest in plants inoculated with *G. mosseae*+*R. mucilaginosa*. Mycorrhizal root colonization and spore numbers in the root zone soil also increased in the presence of yeast. It appears that increase in N content with yeast inoculation may not merely be due to its effect on the proliferation of native rhizobia but could also be due to stimulatory effect of yeast spore germination and multiplication of AMF (Singh et al., 1991) which are known to increase the uptake of P and other micronutrients involved in N fixation (Bethlenfalvay, 1992; Barea et al., 1992). All the soil yeasts used in the experiment whether fermentation and sexual reproduction positive or negative, were positive for Vitamin B_{12} production. The yeasts may enhance AMF development by supplying vitamin B_{12} to the rhizosphere, because AMF have been shown to be stimulated by this vitamin (Singh et al., 1991). Thus vitamin B_{12} produced by the soil yeasts might have resulted in better plant growth and yield in plants treated with *G. mosseae* plus soil yeasts. Singh (Pers. Commun.) observed that inoculation with *S. cerevisiae* had negligible effect on non-mycorrhizal plants while it increased the root colonization and spore count of mycorrhizal plants. This suggests that the yeasts specifically stimulate AM development rather than the host plant, which upholds the observation made by Larsen and Jacobsen (1996). It is quite possible that Vitamin B_{12} production by soil yeasts could be the main reason for the stimulation of mycorrhizal development observed in the present study, which needs further investigations.

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The influence of mannan oligosaccharides, acidifiers and their combination on caecal microflora of Japanese quail (*Coturnix japonica*)

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ABSTRACT

The aim of this study was to investigate the effects of the dietary supplementation of mannan oligosaccharides (MOS) extracted from yeast *Saccharomyces cerevisiae*, acidifiers -calcium formate (CF), calcium propionate (CP)- and their combination on the caecal microflora of Japanese quail (*Coturnix japonica*). Four hundred and fifty 1-day old quail where divided in six groups with three replicates each. One group that served as control received the basal diet. The five experimental diets consisted of the basal diet to which either 1 g MOS/kg, or 6 g CF/kg, or 6 g CP/kg, or 1 g MOS plus 6 g CF/kg or 1 g MOS plus 6 g CP/kg were added. The body weight was examined at weekly intervals and mortality was recorded daily. At days 21 and 42 of age, the total count of aerobic bacteria, lactic acid bacteria, *enterobacteriaceae* and coliforms in the caecal content of one bird of each replicate was determined. Also, at day 42 of age, two birds of each replicate were slaughtered and their carcass weight was determined. The results showed that MOS significantly ($P \leq 0.050$) increased the total aerobic plate and lactic acid bacteria counts on day 21. Furthermore, CP significantly ($P \leq 0.050$) decreased the total aerobic plate and lactic acid bacteria counts compared to controls on day 21. Significant interaction between MOS and acidifiers was noticed on total aerobic plate count on day 21. No significant ($P > 0.050$) difference was found in the caecal microflora on day 42. Finally, no significant ($P > 0.050$) difference was noticed on mortality, body and carcass weight.

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1. Introduction

The use of antibiotic growth promoters in animal feed diets has been highly regulated in recent years, due to their residual effects and the risk of development of drug resistance in animal and human pathogenic bacteria. As a result, today there is a growing interest in the identification and evaluation of alternative natural feed additives that can benefit the health of the productive animals and also improve their performance [1,2].

One such feed additive that is being examined as growth promoter, are the mannan oligosaccharides (MOS) of the cell wall of the yeast *Saccharomyces cerevisiae*. MOS can adhere to pathogenic bacteria that have type-I fimbriae and therefore limit their ability to colonize the mucosa of the digestive tract and multiply. Moreover, MOS can benefit the intestinal function by improving the height, the uniformity and the integrity of the intestinal villi. Also, they can exert a positive effect on the immune response of the animal and the production of IgA antibodies [3,4].

Other feed additives are the acidifiers, i.e. organic acids and their salts such as calcium formate (CF) or calcium propionate (CP). The acidifiers can modify the pH of both the feed and the animal's digestive tract and can disrupt the normal cell function and protein synthesis of various gut microorganisms. Moreover, they are natural substances, which have an important role in cell metabolism [5].

The aim of this study has been to examine whether diets supplemented with MOS and acidifiers CF or CP, alone and in combination, would exert an effect on the performance and the native caecal bacteria populations of growing Japanese quail (*Coturnix japonica*).

2. Materials and methods

Four hundred and fifty 1-day-old Japanese quail (*C. japonica*) as hatched were individually weighted and assigned randomly to six treatment groups with three replications of twenty five birds each, which were housed in separate wire suspended cages until the 42nd day of age.

Abbreviations: MOS, mannan oligosaccharides; CF, calcium formate; CP, calcium propionate; CFU, colony forming units.

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To meet the nutrient requirements of growing quail, a complete basal diet (Table 1) in mash form was formulated [6] and analyzed according to AOAC [7]. The basal diet was given to the controls (Group CONTR). The birds of the other three groups were fed the above diet with the addition of 6 g CF/kg (Group CFO6) or 6 g CP/kg (Group CPR6) or 1 g MOS/kg (Group MOS1) or both 1 g MOS/kg plus 6 g CF/kg (Group MOS1-CFO6) or both 1 g MOS/kg plus 6 g CP/kg (Group MOS1-CPR6). The MOS used was "MOS 500" (Ultra Bio-Logics Inc., Canada). The CF used was "Calcium formate feed grade 99%" (Degussa-Huls, Canada). The CP used was "Calcium propionate granules" (Dr. Paul Lohmann, Germany). Feed and drinking water were offered to birds *ad libitum*. The body weight was examined at weekly intervals and mortality was recorded daily. Conventional breeding and management procedures were applied throughout the experimental period. The quail were handled according to the principles of the Greek Directorate General of Veterinary Services for the care of animals in experimentation.

On day 42 of age, one male and one female bird from each replicate were randomly selected, weighted and slaughtered under commercial conditions. After dressing the carcass weight was measured.

On days 21 and 42 of the experimentation, one quail from each subgroup was sacrificed, after anesthetizing with chloroform, by cervical dislocation and the caecal content of each bird was transferred under aseptic conditions into a sterile glass vial. After recording its weight, the material was diluted 1:10 with sterile peptone diluent water (Conda, Spain). Subsequently, the glass vial was shaken 25 times in 7 s over a 30 cm arc. The interval between mixing and removing the test potion did not exceed 3 min [8]. Tenfold serial dilutions for each sample were made with sterile peptone diluent water, until they were diluted to 10^{-7} and plated in duplicate to enumerate the following microorganisms: (1) Total Aerobic Bacteria were enumerated by the pour-plate method using Standard Methods Agar (Conda, Spain). Plates were incubated at 36 °C for 2–3 days [9,10]. (2) Lactic acid bacteria were enumerated by the pour-overlay method using MRS agar (Conda, Spain). Plates were incubated anaerobically at 30 °C for 3 days. (3) *Enterobacteriaceae* were enumerated by the pour-overlay method using Violet Red Bile Glucose agar (Conda, Spain). Plates were incubated at 37 °C for 24 h. Purple colonies surrounded by the purple zone, were enumerated and recorded as *Enterobacteriaceae* [11]. (4) Coliforms were enumerated by the pour-overlay method using Violet Red Bile Lactose agar (Conda, Spain). Plates were incubated at 37 °C for 24 h. Purple colonies surrounded by the purple zone, were enumerated and recorded as coliforms [11]. Average results of duplicate measurements, are presented as $\log_{10}$ colony forming units (CFU)/g of caecal content [12].

The statistical analysis was performed using the SPSS 16.0.1 statistical package (SPSS Inc., Chigaco, IL, USA). The experimentation was based on a factorial model "2x3" (MOS $\times$ acidifiers). In all cases the design was balanced, with three replications per treatment (group). The one-way analysis of variance (ANOVA) for the six groups of the experimentation was performed. Also, the two-way ANOVA was performed, using as main effects the inclusion of MOS in the feed (two levels) and the inclusion of acidifiers in the feed (three levels). Interaction plots were used to measure and explain the possible interaction of the factors [13]. A value of $P \leq 0.050$ was considered significant. Levene's test was applied to test the homogeneity of the variances. Tukey's test was applied to determine statistical differences between the means.

Table 1
Ingredients and composition of basal diet.

	g/kg
Ingredients	
Maize	452.6
Soybean meal	320.0
Wheat	100.0
Corn gluten meal	79.7
Calcium carbonate	14.3
Dicalcium phosphate	11.4
Soybean oil	10.8
Lysine	3.7
Vitamin and trace mineral premix[a]	3.5
Salt	2.1
Sodium bicarbonate	1.9
Chemical analysis	
Dry matter	914
Crude protein	238
Crude fat	28
Crude fiber	36
Ash	62
Calculated analysis	
Calcium	8.5
Total phosphorus	6.5
Lysine	13
Methionine & Cystine	8.7
Metabolisable energy, kcal/kg	2950

[a] Supplying per kg feed: 14000 IU vitamin A, 5000 IU vitamin D_3, 30 mg vitamin E, 13 mg vitamin K, 3 mg vitamin B_1, 8 mg vitamin B_2, 3 mg vitamin B_6, 20 μg vitamin B_{12}, 85 mg vitamin niacin, 20 mg pantothenic acid, 2 mg folic acid, 200 μg biotin, 10 mg vitamin C, 960 mg choline cloride, 100 mg Zn, 116 mg Fe, 120 mg Mg, 20 mg Cu, 0.2 mg Co, 1 mg I, 0.3 mg Se.

3. Results and discussion

At the end of the experimentation no significant ($P > 0.050$) difference was noticed on body weight, carcass weight and mortality. Also, the interaction between MOS and the acidifiers was not significant ($P > 0.050$) for the above measurements. In previous studies involving the dietary use of MOS in bird diets, Ghosh et al. [14] and Sarica et al. [15] did not observe significant improvement, whereas Oguz and Parlat [16] found higher body weight with for birds fed MOS. Moreover, in birds fed acidifiers Cakir et al. [17] did not notice significant improvement, but Ocak et al. [18] found higher body weight. The growth promoter effects of MOS and acidifiers may be attributed to their ability to limit the growth of potential pathogens in the animal's digestive tract [19,20].

Table 2 presents the effect of MOS and acidifiers CF and CP on quail caecal total aerobic bacteria, lactic acid bacteria, *Enterobacteriaceae* and coliforms on the 21st and 42nd days of age. On the 21st day significant ($P = 0.001$) higher total aerobic bacteria count was noticed for the quail of group MOS1 compared to those of groups CONTR, CPR6, MOS1-CFO6 and MOS1-CPR6, and for the quail of groups CFO6 and MOS1-CPR6 compared to those of group CPR6. Also, significantly ($P = 0.012$) higher lactic acid bacteria count was found for the quail of groups CONTR, MOS1 and MOS1-CPR6 compared to those of group CPR6. The dietary addition of MOS resulted in significantly higher total aerobic bacteria ($P = 0.005$) and lactic acid bacteria ($P = 0.029$) counts. Moreover, the dietary addition of CP resulted in significantly lower total aerobic bacteria ($P = 0.014$) and lactic acid bacteria ($P = 0.016$) counts compared to non-addition of acidifier. No significant effect ($P > 0.050$) was noticed for the *Enterobacteriaceae* and coliforms counts. Furthermore, regarding the interaction between MOS and acidifiers, significant ($P = 0.002$) effect was noticed for the total aerobic bacteria count. The combined addition of MOS and CF resulted in increase of the total aerobic bacteria count, whereas the combined addition of MOS and CP resulted in decrease of the total aerobic bacteria count. On the 42nd day of age CF and CP on quail caecal total aerobic bacteria, lactic acid bacteria, *Enterobacteriaceae* and coliforms, no significant ($P > 0.050$) difference was noticed between the six groups. Moreover, no significant ($P > 0.050$) effect was found for MOS or acidifiers and no significant ($P > 0.050$) interaction was noticed.

Table 2
Effect of dietary MOS and acidifiers CF and CP on quail caecal total aerobic bacteria, lactic acid bacteria, *Enterobacteriaceae* and coliforms (log CFU/g) on 21st day and 42nd of age (mean ± s.d.).

Groups[a]	Total aerobic bacteria	Lactic acid bacteria	*Enterobacteriaceae*	Coliforms
21st day of age				
CONTR	$9.50^{ab} \pm 0.24$	$9.36^{a} \pm 0.22$	8.58 ± 0.45	8.71 ± 0.44
CFO6	$9.92^{bc} \pm 0.07$	$8.47^{ab} \pm 0.11$	9.58 ± 0.22	9.49 ± 0.29
CPR6	$9.17^{a} \pm 0.26$	$7.01^{b} \pm 1.09$	9.04 ± 0.41	8.97 ± 0.37
MOS1	$10.41^{c} \pm 0.31$	$9.70^{a} \pm 1.14$	9.33 ± 1.05	9.03 ± 0.86
MOS1-CFO6	$9.59^{ab} \pm 0.33$	$8.68^{ab} \pm 0.64$	9.34 ± 0.24	9.30 ± 0.32
MOS1-CPR6	$9.76^{b} \pm 0.12$	$9.11^{a} \pm 0.66$	9.07 ± 0.71	8.63 ± 1.20
P value	0.001	0.012	0.439	0.616
MOS effect				
0 g/kg	$9.53^{a} \pm 0.37$	$8.28^{a} \pm 1.17$	9.07 ± 0.54	9.06 ± 0.47
1 g MOS/kg	$9.92^{b} \pm 0.44$	$9.16^{b} \pm 0.86$	9.25 ± 0.66	8.98 ± 0.81
P value	0.005	0.029	0.532	0.818
Acidifier effect				
0 g/kg	$9.96^{a} \pm 0.55$	$9.53^{a} \pm 0.76$	8.95 ± 0.83	8.87 ± 0.63
6 g CF/kg	$9.76^{ab} \pm 0.28$	$8.57^{ab} \pm 0.42$	9.46 ± 0.24	9.39 ± 0.29
6 g CP/kg	$9.47^{b} \pm 0.37$	$8.06^{b} \pm 1.40$	9.06 ± 0.52	8.80 ± 0.82
P value	0.014	0.016	0.319	0.285
Interaction (P) MOS × Acidifiers	0.002	0.089	0.355	0.683
42nd day of age				
CONTR	9.85 ± 0.75	9.70 ± 0.79	9.43 ± 0.49	9.72 ± 0.57
CFO6	9.55 ± 0.80	9.11 ± 0.60	8.64 ± 1.70	8.81 ± 1.75
CPR6	9.18 ± 0.24	8.71 ± 0.57	8.59 ± 0.32	8.64 ± 0.32
MOS1	9.52 ± 0.94	8.86 ± 1.16	8.64 ± 0.30	9.11 ± 0.60
MOS1-CFO6	9.50 ± 0.57	9.41 ± 0.99	8.80 ± 1.25	8.86 ± 1.01
MOS1-CPR6	9.17 ± 0.35	8.52 ± 0.18	8.55 ± 0.70	8.62 ± 0.58
P value	0.801	0.472	0.856	0.711
MOS effect				
0 g/kg	9.53 ± 0.63	9.17 ± 0.71	8.89 ± 0.99	9.06 ± 1.06
1 g MOS/kg	9.40 ± 0.60	8.93 ± 0.86	8.66 ± 0.74	8.86 ± 0.69
P value	0.684	0.524	0.622	0.671
Acidifier effect				
0 g/kg	9.69 ± 0.78	9.28 ± 1.00	9.04 ± 0.57	9.41 ± 0.62
6 g CP/kg	9.53 ± 0.62	9.26 ± 0.75	8.72 ± 1.34	8.84 ± 1.28
6 g CF/kg	9.18 ± 0.27	8.62 ± 0.39	8.57 ± 0.49	8.63 ± 0.42
P value	0.415	0.282	0.689	0.351
Interaction (P) MOS × Acidifiers	0.899	0.469	0.662	0.801

Values in the same column with a superscript in common do not differ significantly at $P \leq 0.050$.
[a] Groups: CONTR = Control; CFO6 = 6 g CF/kg; CPR6 = 6 g CP/kg; MOS1 = 1 g MOS/kg; MOS1-CFO6 = 1 g MOS/kg + 6 g CF/kg; MOS1-CPR6 = 1 g MOS/kg + 6 g CP/kg.

In previous studies, Sarica et al. [15] found no significant difference in quail fed MOS compared to controls on total aerobic bacteria or *Escherichia coli* counts in the small intestine. Also, Ghosh et al. [14] did not report significant difference on coliforms or *E. coli* counts in the small intestine, but found decrease of *Clostridium perfringens*. Baurhoo et al. [21,22] noticed increase of lactobacilli and bifidobacteria in the ceca of broilers due to dietary MOS. Spring et al. [23] found decrease of *Salmonella* in the ceca of broilers due to the dietary MOS, but no difference in lactobacilli, coliforms, enterococci and anaerobic bacteria. The addition of MOS in the feed can limit the growth of some pathogens or potential pathogens such as *Salmonella* and *E. coli* [3,4,14]. Also, it can increase the counts of beneficial bacteria, such as lactobacilli and bifidobacteria. Several mechanisms have been proposed to explain this modification in the microflora balance: competition for receptor sites, production of antimicrobial products (e.g. bacteriocins), production of volatile fatty acids or stimulation of the host immune system [24,25].

As far as the acidifiers are concerned, Ghosh et al. [14] reported reduction of coliforms, *E. coli* and *C. perfringens* in the small intestine of quail fed acidifiers. Moreover, Paul et al. [26] found reduction of coliforms and *E. coli* in the small intestine of broilers fed acidifiers. Samanta et al. [27] reported increase of lactobacilli in the small intestine of broilers fed acidifiers, while Pirgozliev et al. [28] described reduction of lactobacilli and coliforms in the crop, small intestine and ceca of broilers. Since the acidifiers are mostly absorbed in the first parts of the digestive tract, their effect on the bacterial populations of the last parts of the digestive tract may be the result of their action in the crop, proventriculus and gizzard. In addition, Dhawale [29] described that the multiplication of pathogens or potential pathogens begins in the crop, because the ingested feed remains there for a large period of time (up to 45 min) and the pH and temperature conditions there benefit the multiplication of such microbes.

The differences in the bacterial counts between the 21st and the 42nd day can be attributed to the different age of quail. Birds at younger age have not completed the development of their digestive tract and the bacterial populations have not stabilized yet. Whereas, adult birds have mature digestive tract and the bacterial populations are much more stable and not so easily influenced by different diets [30,31].

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The Use of Bread Yeast as a Biocontrol Agent for Controlling Seed-Borne Fungi of Faba Bean

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Abstract: Present objective was to study this phenomenon on the common seed-borne fungi of faba bean in soil amended with composted organic wastes and infested with the most commonly isolated fungi from *Vicia faba* seed. *In vitro* studies showed that the yeast was effective in reducing the linear growth of *Cephalosporium* sp., *F. verticillioides*, *F. oxysporum*,. *F. solani*, *R. solani* and *V. dahliae*. Pre- and post-emergence damping-off caused by *Cephalosporium* sp., *F. verticillioides*, *F. oxysporum*, *F. solani*, *R. solani* and *V. dahliae* was reduced significantly when seeds of faba bean were coated with a water suspension (10^9 cfu mL^{-1}) of the yeast before sowing in soil supplemented with compost type (1) (prepared by Mansoura manufacturer with organic waste from city garbage) or type (2) (consisted of 1 ton of horticultural waste and 100 kg sheep manure). Soil was artificially infested with the fungi isolated from faba bean seeds. The above treatment significantly increased plant growth parameters including height, shoot and root length, number of branches/plant, number of pods/plant, pod weight/plant, fresh weight and dry weight. Photosynthetic pigments (chlorophyll A, chlorophyll B and carotenoids) were also increased by the treatments. Total phenols content in the treated plant leaves was higher than in the control plants.

Key words: *Saccharomyces cerevisiae*, bread yeast, biological control, faba bean, composted organic materials

INTRODUCTION

Vicia faba, which has several common names (broad bean, fava bean, faba bean, horse bean, field bean, tic bean), is a species of bean (Fabaceae) native to North Africa and Southwest Asia and is extensively cultivated elsewhere.

This crop is subjected to numerous injuries and stresses which interfere with growth and development. Many fungi are serious pathogens on flowers and seeds, reducing seed yield both qualitatively and quantitatively. Other fungi, including saprophytes and very weak parasites, may lower seed quality. The most common seed-borne fungi on faba bean are: *Ascochyta fabae*, the cause of leaf and pod spot; *Botrytis cinerea*, the cause of grey mould; *Botrytis fabae*, the cause of chocolate spot; *Fusarium* sp., the cause of foot rot and wilts and *Rhizoctonia solani*, the cause of damping-off of seedlings. Seed abortion, shrunken seeds, reduction in seed size, seed rot, seed necrosis, seed discoloration, reduction in germination capacity and physiological alterations in seed are caused by these pathogens (Neergaard, 1979).

Certain yeasts possess many features which make them favourable as biocontrol agents (Wilson and Wisniewski, 1989). Gianluca *et al.* (2006) isolated antagonistic yeasts from the epiphytic flora associated with grape berries. The yeasts showed biological control of *Aspergillus carbonarius* and *A. niger*, which produce Ochratoxin A in grape. Lassois *et al.* (2008) reported the antagonistic activity of two yeast strains, *Pichia anomala* and *Candida oleophila*, on the parasitic complex of banana crown rot.

Composts have been found to be effective as biocontrol agents of various plant pathogens under field conditions (Keener *et al.*, 2000). Ioanna *et al.* (2008) found that no chemical control method was adequate to control the soil-borne fungus *Verticillium dahliae*. Management strategies were focused on preventive measures, utilizing microbes to suppress *V. dahliae*. Eggplants grown in sterilized or non-sterilized compost were transplanted to soil infested with *V. dahliae* microsclerotia, amended or not with sterilized or non-sterilized compost. The most effective treatments were those that included non-sterilized compost; therefore, the observed suppression of *V. dahliae* could be attributed to microbial agents.

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Several microbes were isolated from the root system of eggplants grown in the compost and tested *in vitro* against *V. dahliae*. Two bacterial strains were identified as members of the *Pseudomonas fluorescens* complex and two fungal isolates of *Fusarium oxysporum* were selected for further evaluation under glasshouse conditions. The ability of the microbial agents to reduce the percentage of diseased leaves compared to the control treatment was demonstrated.

This study was undertaken to evaluate the effect of bread yeast in suppressing the most common seed-borne pathogens of faba bean in the presence of soil amended with composted organic household wastes and yard trimmings.

MATERIALS AND METHODS

Isolation and identification of yeast: *Saccharomyces cerevisiae* (Meyen ex E.C. Hansen) was isolated on malt extract agar medium (MEA), identified in the Microbiology Department, Faculty of Agriculture, Mansoura University and tested for its antagonistic effects against the following seed-borne fungi of faba bean: *Cephalosporium* sp., *F. oxysporum*, *F. solani*, *F. verticillioides*, *R. solani* and *V. dahliae*.

***In vitro* agar plate bioassays:** The isolated yeast was assayed *in vitro* for inhibition of the six pathogenic fungi. Initial inhibition assays were conducted on PDA plates with single streaks of yeast drawn across the plate's centers. After 2-3 days growth at 20-25°C, PDA discs of 0.5 cm diameter were taken from the margins of growing pathogen colonies and placed on either sides of the yeast streaks at a distance of 30 mm, then incubated for an additional 4-6 days at 20-25°C. The distance was measured from the fungal colony center to its edge nearest the yeast streak. This was compared to the growth of a non-streaked control to determine the degree of inhibition of pathogen growth (Wang *et al*., 2003). Three replicates were used for each pathogen.

Effect of *S. cerevisiae* combined with composted organic materials on the seed-borne pathogenic fungi of faba bean plants under greenhouse conditions: Two types of compost were used; Compost (1) which was prepared by Mansoura manufacturer for organic manure from city garbage and Compost (2) which consisted of 1 ton of horticultural waste and 100 kg sheep manure. Chemical and physical analyses of these composts were performed at the Mansoura manufacturer for compost (1) and at the Faculty of Agriculture, Mansoura University for compost (2). The results are shown in Table 1.

Table 1: Chemical and physical analysis of the two types of compost

Parameters	Compost type (1)	Compost type (2)
Moisture content (%)	48.20	42.20
Total N (%)	1.05	0.96
Total P (%)	0.19	0.23
Total K (%)	0.91	0.98
Organic carbon (%)	44.82	25.49
C/N ratio	24:1	15:10
pH	8.4	7.50
EC (dS m^{-1})	1.85	1.78

An experiment was conducted to determine the effect of the composted organic materials enriched with the yeast *S. cerevisiae* on the six pathogenic fungi. *S. cerevisiae* was applied as a seed dressing to the faba bean seeds. Three flasks containing two-days-old liquid cultures of *S. cerevisiae* were used. Seeds were coated with an adhesive material (acacia gum) before treatment. Yeast-coated seeds contained about 10^9 cfu $seed^{-1}$ (Weller and Cook, 1983).

Eighteen plastic pots (25 cm diameter) containing sterilized sandy loam soil were used for each fungus. Three replicates (pots) were used for each treatment as follows:

- Control (non-amended soil, non-treated seeds)
- Compost type (1) treated soil, non-treated seeds
- Compost type (2) treated soil, non-treated seeds
- Yeast (non-amended soil, *S. cerevisiae* treated seeds)
- Compost type (1) treated soil, *S. cerevisiae* treated seeds
- Compost type (2) treated soil, *S. cerevisiae* treated seeds

Each type of compost was added to the pots at the rate of 25 g/pot. The treatments were inoculated separately with each fungal preparation at the rate of 5% (w/w). Pots were kept in a greenhouse for 7 days at 22±5°C to allow the fungi to adapt before sowing seeds. During that period, the soil was moistened when necessary. Seeds were coated with a yeast suspension taken from 3-days-old liquid cultures. Five seeds were planted in each pot. Control pots were prepared similarly but were pathogen-free.

The numbers of pre- and post-emergence damping off and stunted seedlings, as well as healthy plants, were recorded after 15, 30 and 45 days. Growth parameters including plant height (cm), root length (cm), shoot length (cm), number of branches (branch/plant), number of pods (pod/plant), pod weight (g), fresh weight (g) and dry weight (g) were recorded after 60 days. Photosynthetic pigments (mg g^{-1} fresh weight) and total phenols (mg catechol/100 g fresh weight) were measured.

Estimation of photosynthetic pigments content: The spectrophotometric method recommended by

Metzner *et al.* (1965) was used in this investigation. A definite weight of fresh leaves was homogenized immediately after harvesting in 5 mL cold aqueous acetone (85%), kept overnight in a refrigerator and then centrifuged. The supernatant, which contained the pigments, was diluted with cold aqueous acetone to an appropriate volume for spectrophotometric measurements. The extract was measured against blanks of pure 85% acetone solution at three wavelengths: 452, 644 and 663 nm. The concentration of each pigment, chlorophyll A (Chl. A), chlorophyll B (Chl. B) and carotenoids (Carot.), was determined by using the following equations:

$$\text{Chl. A} = 10.3\ E_{663}-0.918\ E_{644}$$

$$\text{Chl. B} = 19.7\ E644-3.87\ E663$$

$$\text{Carot.} = 4.2\ E452-(0.0264\ \text{Chl. a} + 0.426\ \text{Chl. b})$$

Estimation of total phenols content: Total phenols were determined using the Folin-Ciocalteau reagent (Singleton and Rossi, 1965). To determine the calibration curve, 1 mL aliquots of 0.02, 0.04, 0.06, 0.08 and 1.00 mg mL^{-1} ethanolic catechol solutions were combined with 0.5 mL Folin-Ciocalteau reagent. After 3 min, 2 mL (200 g L^{-1}) sodium carbonate was added and the contents were mixed thoroughly. The final color change was measured calorimetrically at 650 nm using a SPEKOL11 Carl Zeiss photometer and the calibration curve was drawn.

Fresh leaf samples (2 g) were homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10,000 rpm for 15 min and the supernatant was saved. The residues were re-extracted twice with 80% ethanol and the supernatants were pooled, put in evaporating dishes and evaporated to dryness at room temperature. Residues were dissolved in 5 mL of distilled water. One hundred microlitres of this extract was diluted to 3 mL with water and mixed with the same amount of reagents as described above. The color was developed and absorbance measured after 60 min. The results were expressed as mg catechol/100 g fresh weight material. All determinations were replicated three times. Total content of phenolic compounds in plant ethanolic extracts was calculated as catechol equivalents by the following equation:

$$T = \frac{c \times V}{m} \times 100$$

Where:

T: Total content of phenolic compounds, in mg of catechol/100 g of fresh weight material

c: The concentration of catechol established from the calibration curve, in mg mL^{-1}

V: The volume of extract in mL

m: The weight of pure plant ethanolic extract in g

Statistical analysis: Data were subjected to analysis of variance by Duncan's multiple range tests using SAS version 6.11 (Duncan, 1995).

RESULTS

Effect of *S. cerevisiae* isolated yeast from compost on seed-borne pathogenic fungi of faba bean plants under greenhouse conditions: The following pathogenic fungi were used in this investigation: *Cephalosporium* sp., *F. verticillioides*, *F. oxysporum*, *F. solani*, *R. solani* and *V. dahliae*.

***In vitro* agar plate bioassays:** Data presented in Table 2 show the degree of fungal inhibition due to the effect of isolated yeast; *Cephalosporium* sp. had linear growth of 1.35 cm, *F. verticillioides* had 0.97 cm, *F. oxysporum* had 0.77 cm, *F. solani* had 1.80 cm, *R. solani* had 0.97 cm and *V. dahliae* had 0.87 cm.

Effect of *S. cerevisiae* combined with tested composts on faba bean seeds grown in pathogen-infested soil under greenhouse conditions: Faba bean seeds coated with *S. cerevisiae* were grown in soil supplemented with compost type (1) or type (2). Soil was infested with the fungal isolates. The results showed decreases in pre- and post-emergence damping-off and stunted seedlings (Table 3-5).

Data in Table 3 show that seed treatment with *S. cerevisiae* significantly reduced the amount of pre-emergence damping-off due to the presence of following fungi in soil: *F. oxysporum* and *F. solani* (50.00%), *F. verticillioides* (25.00%), *R. solani* (33.32%) and *V. dahliae* (25.00%).

Data in Table 4 show that the treatment in which compost type (1) was added to soil and seeds were coated

Table 2: Antagonistic effect of isolated yeast on linear growth of tested pathogenic fungi *in vitro*

Fungus	Linear growth (cm)	Inhibition (%)
Control	2.75a [x]	0.00
Cephalosporium sp.	1.35bc	50.90
F. oxysporum	0.77c	72.00
F. solani	1.80b	34.55
F. verticillioides	0.97c	64.73
R. solani	0.97c	64.73
V. dahliae	0.87c	68.36

Control: No antagonist yeast. [x]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 3: Effect of *S. cerevisiae* on seedling survival of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Pre-emergence damping-off (%)			Post-emergence damping-off (%)			Stunted seedlings (%)		
Fungus	Control	Yeast	Reduction (%)	Control	Yeast	Reduction (%)	Control	Yeast	Reduction (%)
Non-infested soil	6.67c[x]	0.00e	100.00	6.67b	0.00b	0.00	6.67b	6.67a	0.00
Cephalosporium sp.	13.33bc	13.30cd	0.00	20.00ab	13.33ab	33.35	33.33a	26.67a	20.00
F. verticillioides	53.33a	40.00a	25.00	6.67b	6.67ab	0.00	26.67ab	20.00a	25.00
F.oxysporum	13.33c	6.67de	50.00	20.00ab	20.00a	0.00	20.00ab	13.33a	33.35
F. solani	40.00ab	20.00bc	50.00	26.67ab	13.33ab	50.00	13.33ab	13.33a	0.00
R. solani	40.00ab	26.67b	33.32	33.33a	20.00a	40.00	20.00ab	13.33a	33.35
V. dahliae	26.67bc	20.00bc	25.00	6.67b	6.67ab	0.00	33.33a	20.00a	40.00

Control: Healthy seeds planted as non-coated seeds. Yeast: Healthy yeast-coated seeds planted in pathogen-infested soil. Reduction (%) = Yeast-Control/Control×100. [x]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 4: Effect of compost type (1) combined with *S. cerevisiae* on seedling survival of faba bean plants grown in pathogen-infested-soil under greenhouse conditions

	Pre-emergence damping-off (%)			Post-emergence damping-off (%)			Stunted seedlings (%)		
Fungus	Control	*S. cerevisiae* +Compost (1)	Reduction (%)	Control	*S. cerevisiae* +Compost (1)	Reduction (%)	Control	*S. cerevisiae* +Compost (1)	Reduction (%)
Non-infested soil	6.67c	0.00b	100.00	6.67b	0.00a	100.00	6.67b	0.00a	100.00
Cephalosporium sp.	13.33bc	6.67ab	50.00	20.00ab	6.67a	66.65	33.33a	6.67a	80.00
F. verticillioides	53.33a	20.00a	62.50	6.67b	6.67a	0.00	26.67ab	6.67a	75.00
F. oxysporum	13.33c	6.67ab	50.00	20.00ab	13.33a	33.35	20.00ab	13.33a	33.35
F. solani	40.00ab	20.00a	50.00	26.67ab	0.00a	100.00	13.33ab	0.00a	100.00
R. solani	40.00ab	13.33ab	66.68	33.33a	13.33a	60.00	20.00ab	6.67a	66.65
V. dahliae	26.67bc	13.33ab	50.00	6.67b	6.67a	0.00	33.33a	13.33a	60.00

Control: Healthy seeds planted in non-treated soil and non-coated seeds. *S. cerevisiae* +compost 1: Healthy yeast coated seeds planted in pathogen-infested and compost type (1) treated soil. Reduction (%) = *S. cerevisiae* +compost type (1)-Control/Control×100. [x]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 5: Effect of compost type (2) combined with *S. cerevisiae* on seedling survival of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Pre-emergence damping-off (%)			Post-emergencedamping-off (%)			Stunted seedlings (%)		
Fungus	Control	*S. cerevisiae* +Compost (1)	Reduction (%)	Control	*S. cerevisiae* +Compost (1)	Reduction (%)	Control	*S. cerevisiae* +Compost (1)	Reduction (%)
Non-infested soil	6.67c[x]	0.00a	100.00	6.67b	0.00a	100.00	6.67b	0.00a	100.00
Cephalosporium sp.	13.33bc	6.67a	50.00	20.00ab	6.67a	66.65	33.33a	6.67a	80.00
F. verticillioides	53.33a	6.67a	88.00	6.67b	0.00a	100.00	26.67ab	6.67a	66.65
F. oxysporum	13.33c	6.67a	50.00	20.00ab	0.00a	100.00	20.00ab	0.00a	100.00
F. solani	40.00ab	6.67a	83.32	26.67ab	6.67a	75.00	13.33ab	6.67a	50.00
R. solani	40.00ab	6.67a	83.32	33.33a	0.00a	100.00	20.00ab	13.33a	33.35
V. dahliae	26.67bc	6.67a	75.00	6.67b	6.67a	0.00	33.33a	6.67a	80.00

Control: Healthy seeds planted in non-treated soil (non-coated seeds). *S. cerevisiae* +compost (2): Healthy yeast-coated seeds planted in pathogen-infested and compost type (2) treated soil. Reduction (%) = *S. cerevisiae* +compost type (2)-Control/Control×100. [x]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

with *S. cerevisiae* significantly reduced pre-emergence damping-off caused by *R. solani* (66.68%), *Cephalosporium* sp. (50.00%), *F. verticillioides* (62.50%), *F. oxysporum* (50.00%), *F. solani* (50.00%) and *V. dahliae* (50.00%).

Data in Table 5 show that the addition of compost type (2) with *S. cerevisiae* significantly reduced pre-emergence damping-off caused by *F. verticillioides* (88.00%), *Cephalosporium* sp. (50.00%), *F. oxysporum* (50.00%), *F. solani* (83.32%), *R. solani* (83.32%) and *V. dahliae* (75.00%).

Data in Table 3 show that seed treatment with *S. cerevisiae* significantly reduced post-emergence damping-off caused by *F. solani* (50.00%), *Cephalosporium* sp. (33.35%) and *R. solani* (40.00%). *F. verticillioides*, *F. oxysporum* and *V. dahliae* were not affected by the yeast.

Data in Table 4 show that compost type (1) in the presence of *S. cerevisiae* significantly reduced post-emergence damping-off caused by *F. solani* (100%), *Cephalosporium* sp. (66.65%), *F. oxysporum* (33.35%) and *R. solani* (60.00%). *F. verticillioides* and *V. dahliae* were resistant to the antagonistic properties of *S. cerevisiae*.

Data in Table 5 show that compost type (2) in the presence of *S. cerevisiae* significantly reduced post-emergence damping-off caused by *F. verticillioides*, *F. oxysporum* and *R. solani* (100%) compared with other pathogenic fungi: *Cephalosporium* sp. (66.65%), *F. solani* (75.00%) and *V. dahliae* did not react to *S. cerevisiae*.

Data in Table 3 show that seed treatment with *S. cerevisiae* significantly reduced the number of stunted seedlings due to *V. dahliae* (40.00%), *Cephalosporium* sp. (20.00%), *F. verticillioides* (25.00%), *F. oxysporum* (33.35%) and *R. solani* (33.35%). *F. solani* was not affected by *S. cerevisiae*.

Data in Table 4 show that compost type (1) in the presence of *S. cerevisiae* significantly reduced the number of stunted seedlings caused by *F. solani* (100%), as compared with *Cephalosporium* sp. (80.00%), *F. verticillioides* (75.00%), *F. oxysporum* (33.35%), *R. solani* (66.65%) and *V. dahliae* (60.00%).

Data in Table 5 show that compost type (2) added to soil in the presence of seed coated with *S. cerevisiae* significantly reduced the number of stunted seedlings caused by *F. oxysporum* (100%), as compared with *Cephalosporium* sp. (80.00%), *F. verticillioides* (66.65%), *F. solani* (50.00%), *R. solani* (33.35%) and *V. dahliae* (80.00%).

Effect of *S. cerevisiae* combined with tested composts on the growth parameters of faba bean plants grown in pathogen-infested soil under greenhouse conditions: In this experiment faba bean seeds coated with *S. cerevisiae* and grown in soil amended with compost type (1) and (2) showed an increase in various growth parameters (plant height, shoot length, root length, number of branches/plant, number of pods/plant, pod weight/plant, fresh weight and dry weight) when compared with non-coated seeds (Table 6-8).

Plant height (cm): Data in Table 6 show an increase in plant height in the treatment where *S. cerevisiae* was used and soil was infested with *R. solani*, with an average height of 88.46 cm, while *Cephalosporium* sp. averaged 79.60 cm, *F. verticillioides* 78.50 cm, *F. oxysporum* 84.33 cm, *F. solani* 82.23 cm and *V. dahliae* 79.10 cm.

Data in Table 7 show an increase in plant height in the treatment where compost type (1) was added to the soil and seeds were coated with *S. cerevisiae* in soil infested with *R. solani*, with an average height of 92.76 cm, while *Cephalosporium* sp. averaged 86.26 cm, *F. verticillioides* 92.26 cm, *F. oxysporum* 87.86 cm, *F. solani* 86.66 cm and *V. dahliae* 88.00 cm.

Data in Table 8 show an increase in plant height in the treatment where compost type (2) was added to the soil and seeds were coated with *S. cerevisiae* in soil infested with *F. oxysporum*, with an average height of 91.80 cm, while *Cephalosporium* sp. averaged 83.50 cm, *F. verticillioides* 95.00 cm, *F. solani* 91.33 cm, *R. solani* 87.70 cm and *V. dahliae* 88.00 cm.

Shoot length (cm): Data in Table 6 show an increase in shoot length in the treatment where faba bean seeds coated with *S. cerevisiae* were sown in soil infested with *R. solani*. The average length was 64.66 cm, while *Cephalosporium* sp. averaged 57.60 cm, *F. verticillioides* 60.00 cm, *F. oxysporum* 61.00 cm, *F. solani* 59.56 cm and *V. dahliae* 59.00 cm.

Data in Table 7 show that the treatment of compost type (1) and *S. cerevisiae* in the presence of *R. solani* significantly increased shoot length to an average

Table 6: Effect of *S. cerevisiae* on growth parameters of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Growth parameters											
	Plant height (cm)			Shoot length (cm)			Root length (cm)			No. of branches		
Fungus	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)
Non-infested soil	64.30a	91.33a	42.03	45.30a	67.00a	47.90	19.00a	24.33a	28.05	1.86a	2.96a	59.14
Cephalosporium sp.	55.56bc	79.60b	43.27	42.80ab	57.60a	34.58	12.76b	22.00bc	72.41	1.67a	2.33a	39.52
F. verticillioides	59.63ab	78.50b	31.65	45.30a	60.00a	32.45	14.33ab	18.50bc	29.10	1.83a	2.16a	18.03
F. oxysporum	54.30bc	84.33ab	55.30	38.70b	61.00a	57.62	15.60ab	23.33a-c	49.55	1.67a	2.00a	19.76
F. solani	58.33bc	82.23ab	40.97	41.56ab	59.56a	43.31	16.76ab	22.66a-c	42.24	1.77a	2.66a	50.28
R. solani	52.93c	88.46ab	67.13	38.33b	64.66a	68.69	14.60ab	23.80ab	35.20	1.33a	2.33a	75.19
V. dahliae	57.60bc	79.10b	37.33	44.30a	59.00a	33.18	13.30b	20.10a-c	51.12	1.43a	2.63a	83.92
	Growth parameters											
	No. of pods (pods/plant)			Pod weight (g)			Fresh weight (g)			Dry weight (g)		
Fungus	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)	Control	*S. cerevisiae*	Increase (%)
Non-infested soil	4.67a	6.66a	42.61	14.73a	16.00a	8.62	60.30a	65.00a	7.80	18.86a	19.60a	3.92
Cephalosporium sp.	2.33bc	4.33a	85.84	6.10bc	7.40a-c	21.31	31.37c	38.10de	21.45	6.63c	10.53d	58.82
F. verticillioides	3.33ab	6.33a	90.00	9.40b	12.00ab	27.66	30.00c	50.20bc	67.33	7.40bc	13.10b-d	77.00
F. oxysporum	4.33a	5.00a	15.47	6.00bc	10.86ab	81.00	33.60bc	52.23ab	55.45	14.80ab	16.96ab	14.59
F. solani	2.00bc	5.00a	100.00	3.60cd	8.00a-c	122.22	34.60bc	44.10cd	27.46	7.80bc	11.20cd	43.59
R. solani	1.33c	4.00a	200.00	5.00bc	8.10a-c	62.00	45.17ab	50.66bc	12.15	9.26bc	15.66-c	69.11
V. dahliae	2.00bc	5.00a	150.00	6.00bc	6.66bc	11.00	34.90bc	52.00ab	49.00	8.90bc	10.00d	12.36

Control: Non-coated seeds. *S. cerevisiae*: Yeast-coated seeds planted in pathogen-infested soil. Increase (%) = *S. cerevisiae*-Control/Control×100. ^{X}Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 7: Effect of compost type (1) combined with *S. cerevisiae* on growth parameters of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Growth parameters											
	Plant height (cm)			Shoot length (cm)			Root length (cm)			No. of branches		
Fungus	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)
Non-infested soil	64.30a	97.86a	52.19	45.30a	71.00a	56.73	19.00a	26.86a	41.36	1.86a	3.20a	72.04
Cephalosporium sp.	55.56bc	86.26b	55.26	42.80ab	66.93a-c	56.38	12.76b	19.33b	51.49	1.67a	2.66a-c	59.28
F. verticillioides	59.63ab	92.26ab	54.72	45.30a	68.26a-c	50.68	14.33ab	24.00ab	67.48	1.83a	2.93ab	60.11
F. oxysporum	54.30bc	87.86b	61.80	38.70b	63.20bc	63.30	15.60ab	24.66ab	58.08	1.67a	2.33bc	39.52
F. solani	58.33bc	86.66b	48.57	41.56ab	61.83c	48.77	16.76ab	24.83ab	48.15	1.77a	2.86ab	61.58
R. solani	52.93c	92.76ab	75.25	38.33b	68.80ab	79.49	14.60ab	23.96ab	64.11	1.33a	2.53a-c	90.22
V. dahliae	57.60bc	88.00b	52.78	44.30a	61.46c	38.74	13.30b	26.60a	84.96	1.43a	2.86ab	100.00

	Growth parameters											
	No. of pods (pods/plant)			Pod weight (g)			Fresh weight (g)			Dry weight (g)		
Fungus	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)
Non-infested soil	4.67a	6.00a	28.48	14.73a	18.66a	26.68	60.30a	72.13a	19.62	18.86a	24.00a	27.25
Cephalosporium sp.	2.33bc	4.66a	100.00	6.10bc	14.00b	129.50	31.37c	53.06b	69.14	6.63c	10.00c	50.82
F. verticillioides	3.33ab	5.33a	60.00	9.40b	14.00b	48.93	30.00c	68.00a	126.67	7.40bc	14.90ab	101.35
F. oxysporum	4.33a	5.66a	30.72	6.00bc	12.00b	100.00	33.60bc	57.06b	69.82	14.80ab	17.43ab	17.77
F. solani	2.00bc	5.00a	150.00	3.60cd	11.33b	214.72	34.60bc	43.00c	24.28	7.80bc	12.00bc	53.85
R. solani	1.33c	5.66a	325.56	5.00bc	12.93b	158.60	45.17ab	70.50a	56.08	9.26bc	16.83ab	81.75
V. dahliae	2.00bc	6.00a	200.00	6.00bc	14.73ab	145.50	34.90bc	43.23c	23.87	8.90bc	14.70ab	65.17

Control: Non-coated seeds planted in non-treated soil. Compost (1) + *S. cerevisiae*: Healthy yeast-coated seeds planted in pathogen-infested and compost type (1) treated soil. Increase (%) = Compost (1)+ *S. cerevisiae*-Control/Control×100. [X]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 8: Effect of compost type (2) combined with *S. cerevisiae* on growth parameters of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Growth parameters											
	Plant height (cm)			Shoot length (cm)			Root length (cm)			No. of branches		
Fungus	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)
Non-infested soil	64.30a	96.30a	49.77	45.30a	68.70a	51.66	19.00a	31.23a	64.37	1.86a	3.23a	73.66
Cephalosporium sp.	55.56bc	83.50a	50.29	42.80ab	65.13ab	52.17	12.76b	19.00b	48.90	1.67a	2.66ab	59.28
F. verticillioides	59.63ab	95.00a	59.32	45.30a	65.33ab	44.22	14.33ab	29.66a	106.98	1.83a	3.23a	76.50
F. oxysporum	54.30bc	91.80a	69.06	38.70b	64.46ab	66.56	15.60ab	27.33a	75.19	1.67a	2.66ab	59.28
F. solani	58.33bc	91.33a	56.57	41.56ab	64.46ab	55.10	16.76ab	26.86a	60.26	1.77a	2.73ab	54.24
R. solani	52.93c	87.70a	65.69	38.33b	66.00a	72.19	14.60ab	27.60a	89.04	1.33a	2.73ab	105.26
V. dahliae	57.60bc	88.00a	52.78	44.30a	60.00b	35.44	13.30b	28.00a	110.53	1.43a	2.73ab	90.90

	Growth parameters											
	No. of pods (pods/plant)			Pod weight (g)			Fresh weight (g)			Dry weight (g)		
Fungus	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)
Non-infested soil	4.67a	5.66a	21.20	14.73a	15.60a	5.90	60.30a	80.33a	33.21	18.86a	25.00a	2.56
Cephalosporium sp.	2.33bc	5.00a	114.59	6.10bc	11.06a-c	81.31	31.37c	48.53bc	54.40	6.63c	10.53bc	58.82
F. verticillioides	3.33ab	5.33a	60.00	9.40b	13.70ab	45.74	30.00c	66.00ab	120.00	7.40bc	14.70bc	98.65
F. oxysporum	4.33a	5.00a	15.47	6.00bc	12.60ab	110.00	33.60bc	66.20ab	97.02	14.80ab	20.50ab	38.51
F. solani	2.00bc	5.66a	183.00	3.60cd	12.20ab	238.89	34.60bc	40.00c	15.60	7.80bc	10.20bc	30.77
R. solani	1.33c	5.33a	300.00	5.00bc	10.00bc	100.00	45.17ab	56.30b	24.64	9.26bc	15.80b	70.63
V. dahliae	2.00bc	5.66a	183.00	6.00bc	12.70ab	111.67	34.90bc	49.50bc	41.83	8.90bc	12.86bc	44.49

Control: Non-coated seeds planted in non-treated soil. Compost (2) + *S. cerevisiae*: Healthy yeast-coated seeds planted in pathogen-infested and compost type (2) treated soil. Increase (%) = Compost (2) + *S. cerevisiae*-Control/Control×100. [X]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

68.80 cm, while *Cephalosporium* sp. averaged 66.93 cm, *F. verticillioides* 68.26 cm, *F. oxysporum* 63.20 cm, *F. solani* 61.83 cm and *V. dahliae* 61.46 cm.

Data in Table 8 show that the treatment of compost type (2) in the presence of *S. cerevisiae* significantly increased average shoot length where soil was infested

with *R. solani* to 66.00 cm, while *Cephalosporium* sp. averaged 65.13 cm, *F. verticillioides* 65.33 cm, *F. oxysporum* 64.46 cm, *F. solani* 64.46 cm and *V. dahliae* 60.00 cm length.

Root length (cm): Data in Table 6 show an average increase in root length in the treatment where *S. cerevisiae* was used and soil infested with *Cephalosporium* sp. of 22.00 cm, while *F. verticillioides* averaged 18.50 cm, *F. oxysporum* 23.33 cm, *F. solani* 22.66 cm, *R. solani* 23.80 cm and *V. dahliae* 20.10 cm.

Data in Table 7 show an average increase in root length of faba bean plants grown in soil supplemented with compost type (1) when seeds were coated with *S. cerevisiae* and soil was infested with *V. dahliae* of 26.60 cm, while *Cephalosporium* sp. averaged 19.33 cm, *F. verticillioides* 24.00 cm, *F. oxysporum* 24.66 cm, *F. solani* 24.83 cm and *R. solani* 23.96 cm.

Data in Table 8 show an average increase in root length in the treatment where compost type (2) combined with *S. cerevisiae* was used with soil infested with V. dahliae of 28.00 cm, while *Cephalosporium* sp. averaged 19.00 cm, *F. verticillioides* 29.66 cm, *F. oxysporum* 27.33 cm, *F. solani* 26.86 cm and *R. solani* 27.60 cm.

Number of branches (branches/plant): Data in Table 6 show an increase in the average number of branches in the treatment where *S. cerevisiae* was used and soil infested with *V. dahliae* of 2.63 branches/plant, while *Cephalosporium* sp. averaged 2.33 branches/plant, *F. verticillioides* 2.16 branches/plant, *F. oxysporum* 2.00 branches/plant, *F. solani* 2.66 branches/plant and *R. solani* 2.33 branches/plant.

Data in Table 7 show an increase in the average number of branches in the treatment where compost type (1) combined with *S. cerevisiae* was used in soil infested with *V. dahliae* of 2.86 branches/plant, while *Cephalosporium* sp. averaged 2.66 branches/plant, *F. verticillioides* 2.93 branches/plant, *F. oxysporum* 2.33 branches/plant, *F. solani* 2.86 branches/plant and *R. solani* 2.53 branches/plant.

Data in Table 8 show an increase in the average number of branches in the treatment where soil was supplemented with compost type (2), *S. cerevisiae* was used and the soil infested with *R. solani* of 2.73 branches/plant, while *Cephalosporium* sp. averaged 2.66 branches/plant, *F. verticillioides* 3.23 branches/plant, *F. oxysporum* 2.66 branches/plant, *F. solani* 2.73 branches/plant and *V. dahliae* 2.73 branches/plant.

Number of pods (pods/plant): Data in Table 6 show an increase in the average number of pods in the treatment where *S. cerevisiae* was used and soil infested with *R. solani* of 4.00 pods/plant, while *Cephalosporium* sp. averaged 4.33 pods/plant, *F. verticillioides* 6.33 pods/plant, *F. oxysporum* 5.00 pods/plant, *F. solani* 5.00 pods/plant and *V. dahliae* 5.00 pods/plant.

Data in Table 7 show an increase in the average number of pods in the treatment of compost type (1) and *S. cerevisiae* with *R. solani* infested soil of 5.66 pods/plant, while *Cephalosporium* sp. averaged 4.66 pods/plant, *F. verticillioides* 5.33 pods/plant, *F. oxysporum* 5.66 pods/plant, *F. solani* 5.00 pods/plant and *V. dahliae* 6.00 pods/plant.

Data in Table 8 show an increase in the average number of pods in the treatment where compost type (2) and *S. cerevisiae* were used in soil infested with *R. solani* of 5.33 pods/plant, whild *Cephalosporium* sp. averaged 5.00 pods/plant, *F. verticillioides* 5.33 pods/plant, *F. oxysporum* 5.00 pods/plant, *F. solani* 5.66 pods/plant and *V. dahliae* 5.66 pods/plant.

Pod weight (g/plant): Data in Table 6 show an increase in average total pod weight in the treatment where *S. cerevisiae* was used and soil infested with *F. solani* of 8.00 g/plant, while *Cephalosporium* sp. averaged 7.40 g, *F. verticillioides* 12.00 g, *F. oxysporum* 10.86 g, *R. solani* 8.10 g and *V. dahliae* 6.66 g.

Data in Table 7 show an increase in the average total pod weight in the treatment where compost type (1) and *S. cerevisiae* were used with soil infested with *F. solani* of 11.33 g/plant, while *Cephalosporium* sp. averaged 14.00 g, *F. verticillioides* 14.00 g, *F. oxysporum* 12.00 g, *R. solani* 12.93 g and *V. dahliae* 14.73 g.

Data in Table 8 show an increase in average total pod weight in the treatment where compost type (2) and *S. cerevisiae* were used in soil infested with *V. dahliae* of 12.70 g/plant, while *Cephalosporium* sp. averaged 11.06 g, *F. verticillioides* 13.70 g, *F. oxysporum* 12.60 g, *F. solani* 12.20 g and *R. solani* 10.00 g.

Fresh weight (g/plant): Data in Table 6 show an average increase in the fresh weight of faba bean plants in the treatment where *S. cerevisiae* was used and soil infested with *F. verticillioides* of 50.20 g, while *Cephalosporium* sp. averaged 38.10 g, *F. oxysporum* 52.23 g, *F. solani* 44.10 g, *R. solani* 50.66 g and *V. dahliae* 52.00 g.

Data in Table 7 show an average increase in fresh weight in the treatment where compost type (1) and *S. cerevisiae* were used with soil infested with

F. verticillioides of 68.00 g, while *Cephalosporium* sp. averaged 53.06 g, *F. oxysporum* 57.06 g, *F. solani* 43.00 g, *R. solani* 70.50 g and *V. dahliae* 43.23 g.

Data in Table 8 show an average increase in fresh weight in the treatment where compost type (2) and *S. cerevisiae* were used in soil infested with *F. verticillioides* of 66.00 g, while *Cephalosporium* sp. averaged 48.53 g, *F. oxysporum* 66.20 g, *F. solani* 40.00 g, *R. solani* 56.30 g and *V. dahliae* 49.50 g.

Dry weight (g/plant): Data in Table 6 show an increase in average dry weight in the treatment where *S. cerevisiae* was used and soil infested with *F. verticillioides* of 13.10 g, while *Cephalosporium* sp. averaged 10.53 g, *F. oxysporum* 16.96 g, *F. solani* 11.20 g, *R. solani* 15.66 g and *V. dahliae* 10.00 g.

Data in Table 7 show an increase in average dry weight in the treatment where compost type (1) and *S. cerevisiae* were used in soil infested with *F. verticillioides* of 14.90 g, while *Cephalosporium* sp. averaged 10.00 g, *F. oxysporum* 17.43 g, *F. solani* 11.20 g, *R. solani* 16.83 g and *V. dahliae* 14.70 g.

Data in Table 8 show an increase in average dry weight in the treatment where compost type (2) and *S. cerevisiae* were used in soil infested with *F. verticillioides* of 14.70 g, while *Cephalosporium* sp. averaged 10.53 g, *F. oxysporum* 20.50 g, *F. solani* 10.20 g, *R. solani* 15.80 g and *V. dahliae* 12.86 g.

Effect of *S. cerevisiae* separately and combined with each compost on photosynthetic pigments and total phenols content of faba bean plants grown in fungi infested soil under greenhouse conditions.

Faba bean seeds coated with *S. cerevisiae* separately, as well as combined with compost type (1) and type (2) treated soil, were grown in soil infested with each fungus. The plants showed an increase in photosynthetic pigments (chlorophyll A, chlorophyll B and carotenoids) and total phenols content when compared with the control (Table 9-11).

Data in Table 9 show that chlorophyll A content increased when *S. cerevisiae*-coated seeds were planted in soil infested with *R. solani* by 70.11%, while the increase was 32.43% with *F. solani*, 17.69% with *Cephalosporium* sp., 15.87% with *F. verticillioides*, 14.84% with *F. oxysporum* and 11.28% with *V. dahliae*.

Data in Table 10 show that chlorophyll A content increased in the *S. cerevisiae*+compost type (1) treatment in soil infested with *R. solani* by 65.52%, while the increase was 39.64% with *F. solani*, 19.23% with *Cephalosporium* sp., 15.87% with *F. verticillioides*, 13.28% with *F. oxysporum* and 12.78% with *V. dahliae*.

Data in Table 11 show that chlorophyll A content increased in the *S. cerevisiae*+compost type (2) treatment in soil infested with *R. solani* by 72.41%, while the increase was 30.63% with *F. solani*, 20.00% with *Cephalosporium* sp., 19.84% with *F. verticillioides*, 13.53% with *V. dahliae* and 13.28% with *F. oxysporum*.

Data in Table 9 show that chlorophyll B content increased when *S. cerevisiae*-coated seeds were planted in soil infested with *R. solani* by 73.53%, while the increase was 24.04% with *Cephalosporium* sp., 18.81% with *F. verticillioides*, 12.50% with *F. oxysporum*, 51.80% with *F. solani* and 11.93% with *V. dahliae*.

Data in Table 10 show that chlorophyll B content increased in the *S. cerevisiae*+compost type (1) treatment in soil infested with *R. solani* by 73.53%, while the increase was 51.80% with *F. solani*, 24.04% with *Cephalosporium* sp., 18.81% with *F. verticillioides*, 12.50% with *F. oxysporum* and 11.93% with *V. dahliae.*

Data in Table 11 show that chlorophyll B content increased in the *S. cerevisiae*+compost type (2) treatment in soil infested with *R. solani* by 85.29%%, while the increase was 48.19% with *F. solani*, 26.92% with *Cephalosporium* sp., 26.92% with *F. oxysporum*, 14.85% with *F. verticilloides and 11.93% with V. dahliae*.

Data in Table 9 show that carotenoids content increased when *S. cerevisiae*-coated seeds were planted in soil infested with *R. solani* by 68.42%, while the increase was 35.05% with *F. solani*, 11.11% with *Cephalosporium* sp., 9.01% with *V. dahliae*, 5.08% with *F. oxysporum* and 5.00% with *F. verticillioides*.

Data in Table 10 show that carotenoids content increased in the *S. cerevisiae*+compost type (1) treatment in soil infested with *R. solani* by 77.63%, while

Table 9: Effect of *S. cerevisiae* on photosynthetic pigments and total phenols content of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Chl A (mg g^{-1} fresh wt.)			Chl B (mg g^{-1} fresh wt.)			Carotenoids (mg g^{-1} fresh wt.)			Total phenols (mgcat/100 g fresh wt.)		
Fungus	Control	*S. cerevisiae*	Increase	Control	*S. cerevisiae*	Increase	Control	*S. cerevisiae*	Increase	Control	*S. cerevisiae*	Increase
Non-infested soil	1.44a	1.60a	11.11	1.16a	1.31a	12.93	1.25a	1.50a	20.00	98.94e	112.10d	13.30
Cephalosporium sp.	1.30b	1.53ab	17.69	1.04b	1.29ab	24.04	1.17a	1.30b	11.11	116.85d	129.19bc	10.56
F. verticillioides	1.26b	1.46ab	15.87	1.01b	1.20b-d	18.81	1.20a	1.26b	5.00	113.13d	114.35cd	1.08
F. oxysporum	1.28b	1.47ab	14.84	1.04b	1.17cd	12.50	1.18a	1.24b	5.08	127.23c	149.10a	17.19
F. solani	1.11c	1.47ab	32.43	0.83c	1.26a-c	51.80	0.97b	1.31b	35.05	133.00bc	147.31a	10.76
R. solani	0.87d	1.48ab	70.11	0.68d	1.18cd	73.53	0.76c	1.28b	68.42	178.95a	190.62a	6.52
V. dahliae	1.33ab	1.48ab	11.28	1.09ab	1.22b-d	11.93	1.22a	1.33b	9.01	138.33b	142.94ab	3.33

Control: Non-coated seeds. *S. cerevisiae*: Healthy yeast-coated seeds planted in pathogen-infested soil. Increase (%) = *S. cerevisiae*-Control/Control x 100.
[X]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 10: Effect of compost type (1) combined with *S. cerevisiae* on photosynthetic pigments and total phenols content of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Chl A ($mg\ g^{-1}$ fresh wt.)			Chl B ($mg\ g^{-1}$ fresh wt.)			Carotenoids ($mg\ g^{-1}$ fresh wt.)			Total phenols (mgcat/100 g fresh wt.)		
Fungus	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)	Control	Compost (1) +*S. cerevisiae*	Increase (%)
Non-infested soil	1.44a	1.67a	15.97	1.16a	1.36a	17.24	1.25a	1.49a	19.20	98.94e	114.57bc	15.80
Cephalosporium sp.	1.30b	1.55ab	19.23	1.04b	1.32ab	26.92	1.17a	1.43ab	22.22	116.85d	146.70a	25.55
F. verticillioides	1.26b	1.46b	15.87	1.01b	1.16d	14.85	1.20a	1.30cd	8.33	113.13d	126.19b	11.54
F. oxysporum	1.28b	1.45b	13.28	1.04b	1.32ab	26.92	1.18a	1.42a-c	20.24	127.23c	149.09a	17.18
F. solani	1.11c	1.55ab	39.64	0.83c	1.23cd	48.19	0.97b	1.30cd	34.02	133.00bc	151.95a	14.25
R. solani	0.87d	1.44b	65.52	0.68d	1.26bc	85.29	0.76c	1.35bc	77.63	178.95a	190.56a	6.49
V. dahliae	1.33ab	1.50b	12.78	1.09ab	1.22cd	11.93	1.22a	1.37bc	12.30	138.33b	149.40a	8.00

Control: Non-coated seeds. Compost (1) + *S. cerevisiae*: Healthy yeast-coated seeds planted in compost (1) treated soil. Increase (%) = Compost (1)+ *S. cerevisiae*-Control/Control×100. [X]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

Table 11: Effect of compost type (2) combined with *S. cerevisiae* on photosynthetic pigments and total phenols of faba bean plants grown in pathogen-infested soil under greenhouse conditions

	Chl A ($mg\ g^{-1}$ fresh wt.)			Chl B ($mg\ g^{-1}$ fresh wt.)			Carotenoids ($mg\ g^{-1}$ fresh wt.)			Total phenols (mgcat/100 g fresh wt.)		
Fungus	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)	Control	Compost (2) +*S. cerevisiae*	Increase (%)
Non-infested soil	1.44a	1.60a	11.11	1.16a	1.36a	17.24	1.25a	1.45a	16.00	98.94e	124.47c	25.80
Cephalosporium sp.	1.30b	1.56ab	20.00	1.04b	1.16b	11.54	1.17a	1.34ab	14.53	116.85d	143.00c	22.38
F. verticillioides	1.26b	1.51ab	19.84	1.01b	1.20b	18.81	1.20a	1.35ab	12.50	113.13d	170.39a-c	50.61
F. oxysporum	1.28b	1.45b	13.28	1.04b	1.20b	15.38	1.18a	1.35ab	14.40	127.23c	156.75bc	23.20
F. solani	1.11c	1.45b	30.63	0.83c	1.25ab	50.60	0.97b	1.31bc	35.05	133.00bc	195.08ab	46.68
R. solani	0.87d	1.50ab	72.41	0.68d	1.26ab	85.29	0.76c	1.29bc	69.74	178.95a	203.68a	13.82
V. dahliae	1.33ab	1.51ab	13.53	1.09ab	1.23b	12.84	1.22a	1.34ab	9.83	138.33b	169.12a- c	22.26

Control: Non-coated seeds. Compost (2) + *S. cerevisiae*: Healthy yeast-coated seeds planted in compost (2) treated soil. Increase (%) = Compost (2)+ *S. cerevisiae*-Control/Control×100. [X]Mean within a column followed by the same letter(s) is not significantly different according to Duncan's multiple range tests

the increase was 34.02% with *F. solani*, 22.22% with *Cephalosporium* sp., 20.24% with *F. oxysporum*, 12.30% with *V. dahliae* and 8.33% with *F. verticillioides*.

Data in Table 11 show that carotenoids content increased in the *S. cerevisiae*+compost type (2) treatment in soil infested with *R. solani* by 69.74%, while the increase was 35.05% with *F. solani*, 14.53% with *Cephalosporium* sp., 14.40% with *F. oxysporum*, 12.50% with *F. verticilloides* and 9.83% with *V. dahliae*.

Data in Table 9 show that total phenols content increased when *S. cerevisiae*-coated seeds were planted in soil infested with *F. oxysporum* by 17.19%, while the increase was 10.76% with *F. solani*, 10.56% with *Cephalosporium* sp., 6.52% with *R. solani*, 3.33% with *V. dahliae* and 1.08% with *F. verticillioides*.

Data in Table 10 show that total phenols content increased in the *S. cerevisiae*+compost type (1) treatment in soil infested with *Cephalosporium* sp. by 25.55%, while the increase was 17.18% with *F. oxysporum*, 14.25% with *F. solani*, 11.54% with *F. verticillioides*, 8.00% with *V. dahliae* and 6.49% with *R. solani*.

Data in Table 11 show that total phenols content increased in the *S. cerevisiae*+compost type (2) treatment in soil infested with *F. verticilloides* by 50.61%, while the increase was 46.68% with *F. solani*, 22.38% with *Cephalosporium* sp., 22.26% with *V. dahliae*, 22.20% with *F. oxysporum* and 13.82% with *R. solani*.

DISCUSSION

The present study shows that the application of yeast as a bio-control agent affected all tested fungi *in vitro*. The percentage of inhibition reached 72% with *Fusarium oxysporum* when compared with the other pathogenic fungi and the control. These results are in agreement with Hassanein *et al*. (2002), who reported that yeasts were effective producers of antifungal metabolites. In addition, it was found that isolates of actinomycetes produced chitinase and β-1, 3 glucanase and caused extensive plasmolysis and cell wall lysis of *Cephalosporium maydis in vitro*. Since the cell wall of *C. maydis* consists largely of chitin and β-glucanase (Bartnicki-Garcia and Lippman, 1982), there is a voluminous body of literature on the ability of actinomycetes and fungi to parasitize spores, hyphae and other fungal structures. Many of these observations are linked with plant disease bio-control (Jeffries and Young, 1994; Van de Boogert and Deacan, 1994; Davanlou *et al*., 1999).

Weller (1988), Lee *et al*. (1991), Yuan and Crawford (1995), Valois *et al*. (1996), El-Tarabily *et al*. (1997),

Youssef *et al*. (2001) and Gianluca *et al*. (2006) reported also that a microorganism that colonizes roots is ideal for use as a biocontrol agent against soil-borne diseases and, consequently, improving plant growth. Yeasts applied for the control of plant pathogens were found to produce proteinaceous killer toxins lethal to susceptible yeast and fungi strains (Hodgson *et al*., 1995; Abranches *et al*., 1997; Marquina *et al*., 2002; Santos *et al*., 2004).

Results obtained from this study showed that the addition of yeast as an antagonist improved disease suppression due to the compost and decreased disease severity (pre- and post-emergence damping-off and stunted seedlings). This result is supported by the finding of Postma *et al*. (2003) in which they concluded that the antagonist may enrich composts to increase the reliability of the beneficial effects of compost in disease suppressiveness.

In this study, the treatment of compost plus yeast increased plant growth. This result is supported by Kleifield and Chet (1992) and El-Mehalawy *et al*. (2004) who reported that the growth increase caused by rhizosphere microorganisms depended mainly on the ability of those microorganisms to survive and develop in the rhizosphere. In addition, several plant-microbe interactions were developed which benefited plant growth through different mechanisms, such as the production of plant regulators, siderophores, phosphate solubilization, nutrient uptake and availability (Hoflich and Kuhn, 1996; Gupta *et al*., 1998; Bowen and Rovira, 1999).

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Biocontrol of *Fusarium* Moulds and Fumonisin B_1 Production

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Abstract: The present study was carried out to investigate maize seeds infected by *Fusarium* moulds and fumonisin B_1 production. In order to suppress the growth of fungal infection, and/or fumonisin B_1 production in maize seeds; applicability of *Saccharomyces cerevisiae* as a biocontrol agent as well as detoxification of fumonisin B_1 was investigated. Out of 100 samples of maize investigated 65 (65%) were fungal infection. Of them 38 (58.46%) were *Fusarium moniliforme* others were *F. graminearum* 19 (29.23%) and *F. oxysporum* 8 (12.31%). Growth of *Fusarium moniliforme* and fumonisin B_1 detoxification were negatively correlated with different doses of *S. cerevisiae* while detoxification was positively correlated with the doses. At dose 1, 3, 5 and 7 g of *S. cerevisiae, Fusarium* dry weight and detoxification percent of fumonisin B_1 were 5.8, 40.56, 4.3, 77.63, 2.8, 89.52, 0.9 and 100, respectively. The effect of water content as well as different temperatures on fumonisin B_1 productions was investigated. At water content of 50 %, a higher level of fumonisin (16.3 µg/g) was detected in ground maize while in Corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively. At temperature 21°C level of FB_1 was 19.3 µg/g after incubation period for 4 weeks while at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate. For growth $r = -0.993$, $p = 0.001$; for detoxification $r = 0.927$, $p = 0.024$.

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1. Introduction

Maize (*Zea mays*) is a cereal crop grown throughout the world. Maize plays an important role in the diet of millions of African people due to its high yields per hectare, its ease of cultivation and adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Fandohan *et al*., 2003). The total production of Africa in 2001 was estimated to be about 42 million tons (FAO, 2002). In the field as well as in the store, many pests and parasites attack maize during the storage period. Insects are most often considered as the principal cause of grain losses (Gwinner *et al*., 1996). Surveying harvested maize grains done by many investigators (Samson, 1991; Ominski *et al*., 1994; Munkvold and Desjardins, 1997; Desjardins *et al*., 2000; Orsi *et al*., 2000; El-Shabrawy, 2001), who reported that *Fusarium spp.* is the major fungal population recovered from the rotted maize ears. *F. verticillioides* and *F. moniliforme* are the most dominant species and earlier colonist of the pre-harvest maize ears before other moulds such as *Penicillium* spp. and *Aspergillus* spp. Kossou and Aho (1993) reported that fungi could cause about 50 – 80 % of damage on farmers' maize during the storage period if conditions are favorable for their development. Experts at the food and Agriculture Organization (FAO) at the United Nation have estimated that over 25 % of worlds food crops are lost each year due to mycotoxin contamination with the *Fusarium* species (Chelkowski, 1998). Fumonisins are mycotoxin produced by a variety of fungi of genus *Fusarium*; *Fusarium monilforme* and other species, fumonisins are natural contaminants of cereal grains worldwide and rare mostly found in corn and products derived from corn (Wan Norhasima *et al*., 2009). Nowadays, a great attention has been focused on the possibility of using natural and safe agents as a biocontrol against different diseases.

Biological control of different plant diseases was focused primarily using bacteria or filamentous fungi (Whipps, 2001). So, application of yeasts as biocontrol agents acts as a new trend against different pathogens. Potential use of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters were recent investigated by El-Tarabily and Sivasithamparam (2006). El-Tarabily (2004) reported that the fungal activities of *Rhizoctonia solani* diseased

sugar beet plants were well suppressed by using different types of yeast. Wide variety of yeasts has been used extensively for the biological control of post-harvest diseases of fruits and vegetables (Punja, 1997; Zheng *et al*., 2003), against moulds of stored grains (Petersson *et al*., 1999) and to control powdery mildews (Urquhart and Punja, 1997). The most investigated fumonisin is FB_1, which can cause severe disorders in animals such as Equine Leukoencephalomalacia (Marasas *et al*., 1988), pulmonary edema syndrome and hydrothorax in pigs (Haschek *et al*., 1992) and it is nephrotoxic, hepatotoxic and hepatocarcinogenic in rats. Furthermore, FB_1 has been implicated to be associated with high rates of human esophageal cancer in South Africa, the United States and China (Rheeder *et al*., 1992). The aim of this study was to suppress the soil-borne pathogenic fungus *Fusarium moniliforme* by using *S. cerevisiae* as biocontrol agent and as fumonisin B_1 production control in maize.

2. Material and Methods

The bio-compound used in this study is active dry yeast of *S. cerevisiae*. Yeast application was conducted as different doses amended to autoclaved, ground maize, using four concentrations of 1, 3, 5 and 7 g/100g.

2.1. Samples

A total of 100 samples of maize were collected from different fields in Al-Dakahliya governorate, Egypt. These samples were kept at -4ºC until *Fusarium* moulds were isolated and fumonisin B_1 analysis was carried out.

Figure 1. Infected maize samples

2.2. Isolation and identification of *Fusarium* moulds

Fusarium moulds in maize seeds were determined by plating seeds on 15 ml of sterile Czapek-Dox agar containing Iprodion and Dichloral (CZID-agar) in Petri-plates (Abildgren *et al.,* 1987). The seeds were surface sterilized in 10% sodium hypochlorite for 2 minutes, rinsed in sterilized distilled water and air dried on sterile filter paper. Ten plates of potato dextrose agar media (PDA) were seeded (10 seeds/plate) and incubated at 25ºC for five to seven days. All *Fusarium* isolates were subcultured on PDA, Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), using a single spore technique (Leslie and Summerell, 2006, and Siddiquee *et al*., 2010). PDA cultures were incubated at 25ºC and CLA and SNA cultures were incubated at 25ºC under near UV light for two to four weeks. Cultural characters were assessed by eye and by microscopic examination. Colony morphology was recorded from cultures grown on PDA. The morphology of macroconidia, microconidia, conidiogenous cells and the chlamydospores was assessed from cultures grown on SNA and CLA. Morphological identifications of isolates were made using the criteria of (Nelson *et al*., 1983, and Leslie and Summerell, 2006).

2.3. Production of fumonisin B_1 by *Fusarium moniliforme*

Mycotoxin production in culture was tested on autoclaved, ground maize (Muthomi *et al*, 2000). One hundred grams of the course ground maize in 250 ml Erlenmeyer flasks were moistened with 75 ml distilled water and autoclaved at 121°C for 20 min in two consecutive days. After cooling, each flask was inoculated with 10 mm diameter agar disks cut from 7 to 14 day-old cultures. One flask used as a control sample while others were amended with *S. cerevisiae* at different concentrations (1, 3, 5, and 7 g). The incubation period was 4 weeks.

2.4. Fumonisin B_1 extraction

The samples were extracted using the method of Muthomi *et al* (2002). In brief, samples were homogenized in methanol-water; 3:1(v/v) for 3 min. After centrifugation, an aliquot of the supernatant was cleaned-up, which were preconditioned with methanol and methanol-water (3:1). After washing with methanol, the fumonisins were eluted with acetic acid-methanol; 1:99 (v/v), then stored at 4ºC to analysis.

2.5. Fumonisin B_1 analysis

Fumonisin B_1 was analyzed by the method of Katta *et al*. (1997) with HPLC. The fumonisin B_1 standard was purchased from Sigma Chemical Company (St. Loius, MO). A standard curve was constructed with levels ranging from 0.02 to 2 ng/µl for fumonisin B_1 and a new standard curve was constructed on each day of analysis and only standard curves with correlation coefficients >0.999 were accepted. Fumonisin B_1 (FB_1) was quantified by correlating peak areas of the sample extracts to that of the standard curves. The sensitivity of the method was 0.025 µg /g for fumonisin B_1.

2.6. Influence of some physical parameters on FB_1 production

Effect of different temperature on FB_1 production by *F. moniliforme* was investigated at different incubation period. Also water content of the medium was investigated to test substrate influence on fumonisin B_1 production according to ASTM D 2216 - Standard Test Method for Laboratory Determination of water (moisture) content of soil, rock, and soil-aggregate mixtures.

2.7. Statistical analysis

The statistical SPSS version 15 was uses in data analysis. The Chi^2 was uses to compare observed and expected frequencies. The Pearson correlation coefficient was used to study the doses of *S. cerevisiae* and fungal growth as well as the detoxification percent of fumonisin B_1. Linear or polynomial regressions were used.

3. Results

Isolation and identification of the fungal strain

The collected seeds were washed in running tap water for 20 minutes, surface sterilized in 10 % sodium hypochlorite for 2 minutes, rinsed in sterilized distilled water and air dried on sterile filter paper. The disinfected seeds were placed on CZID-agar and incubated at 25ºC in the dark. After five to seven days the fungal colonies were observed under a microscope and hyphal tips of *Fusarium*-like fungi were transferred to PDA. The pure cultures of the isolates were obtained using a single-spore culture technique. Species identifications were determined following the descriptions in the manual of Nelson *et al*. (1983) and Leslie and Summerell (2006). Three strains of *Fusarium* viz. *F. moniliforme, F. graminearum* and *F. oxysporum* was isolated from infected seeds. Occurrence of isolated *Fusarium* species was 58.46, 29.23 and 12.31 %, respectively (Table 1).

Table 1. Number of *Fusarium* spp. isolates and their occurrence in maize seeds

Fusarium spp. identified	Maize	
	No. of isolates	Occurrence (%)
F. moniliforme	38	58.46
F. graminearum	19	29.23
F. oxysporum	8	12.31
Total	65	100
p-value (Chi^2)	0.001	

Fumonisin B_1 (FB_1) detection

The percentage of *Fusarium* isolates which have the ability to produce fumonisin B_1 was summarized in table 2. Sixty five isolates was collected in the study, of all the isolates two fungal isolates not produce detectable level of FB_1 whereas only one isolate produced FB_1.

Table 2. Toxigenic Fusarium isolates collected from infected maize seeds

Fusarium spp.	No. of collected isolates	No. of toxigenic isolates	Toxigenic isolated (%)
F. moniliforme	38	33	86.8
F. graminearum	19	0	0
F. oxysporum	8	0	0
Total	65	33	86

Effect of *S. cerevisiae* on fungal growth

The effect of different *S. cerevisiae* doses/g on the growth of *Fusarium moniliforme* was detected. Our study revealed that, there was a reverse relationship between increasing *S. cerevisiae* concentration and growth of *Fusarium moniliforme*. Fresh and dry weights of Fusarium *moniliforme* were decreased to 1.9 mg and 0.9 mg in the presence of 7 gm of yeast. Generally, addition of *S. cerevisiae* decreased fresh and dry weight of *F. moniliforme* gradually in comparison to zero dose (control) as shown in table 3 and fig 2.

Table 3. Influence of different S. *cerevisiae* doses on the growth of *Fusarium moniliforme*

S. cerevisiae dose/ (g)	*F. moniliforme*	
	Fresh weight (mg)	Dry weight (mg)
Zero (control)	12.3	7.5
1	8.9	5.8
3	6.1	4.3
5	4.0	2.8
7	1.9	0.9

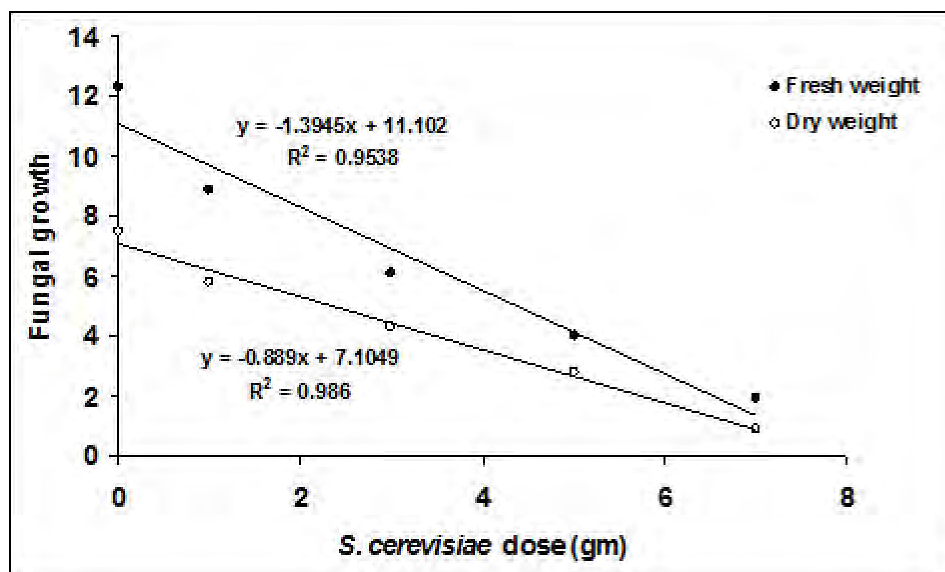

Figure 2. Influence of different *S. cerevisiae* doses on the growth of Fusarium *moniliforme*

Biocontrol of fumonisin B_1

Table 4 and fig 3 represent the effect of *S. cerevisiae* on the production of fumonisin B_1. Application of *S. cerevisiae* at different doses reduces FB_1 production gradually in relation to zero dose (control). The detoxification percent at doses 3 and 5 were 77.63 and 89.52, respectively. Also dose 7 was sufficient for complete detoxification of fumonisin B_1. Table 5 showed the dry weight, fresh weight and detoxification and their correlation with *S. cerevisiae* dose.

Influence of some physical parameters on FB_1 production Effect of different water content

Effect of different water content (30, 40 and 50 %) on production of fumonisin B_1 (FB_1) in sterile ground maize by *Fusarium moniliforme* isolate was determined over period of 6 weeks as shown in table 6. Generally, *F. proliferatum* grew faster with increasing water content. Fumonisin B_1 production depends upon water content and type of substrate. At water content of 50%, a higher level of fumonisin (16.3 µg/g) was detected in ground maize, while in corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively.

Effect of different temperatures on FB_1 production by *F. moniliforme* was investigated as showed in table 7. *F. moniliforme* incubated at different temperatures ranges from 17 to 28°C. Generally our results indicated that level of fumonisin B_1 was increased gradually by increasing temperatures degrees until reach a high level of production at temperature 21°C and then decreased by increasing

temperature degrees. At temperature 21°C level of FB_1 was 19.3 µg/g after incubation period 4 weeks while at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate.

Table 4. Effect of *S. cerevisiae* on the production of fumonisin B_1

***S. cerevisiae* dose (g)**									
0		1		3		5		7	
O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)	O. (µg/g)	D. (%)
14.3	0	8.5	40.56	3.2	77.63	1.5	89.52	ND	100

Table 5. Correlation between the doses of S. *cerevisiae* and fungal growth as well as the detoxification percent of fumonisin B_1

	Dry weight	Fresh weight	Detoxification
S. cerevisiae dose (g)	r = - 0.993	r = - 0.977	r = 0.927
	p = 0.001	p = 0.004	p = 0.024

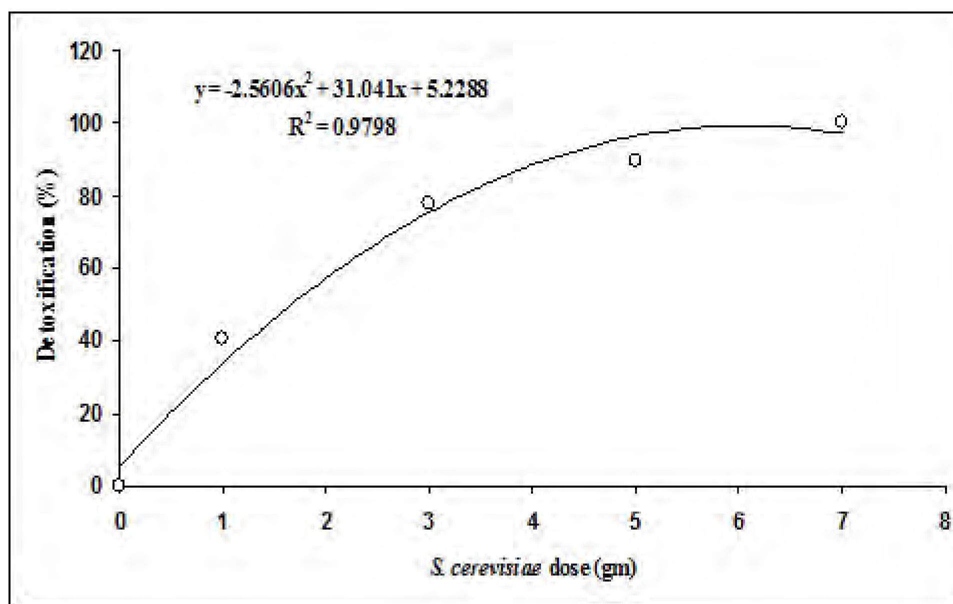

Figure 3. Effect of *S. cerevisiae* on the production of fumonisin B_1

Table 6. Influence of water content on FB_1 production by *F. moniliforme*

	Water content		
	30%	40%	50%
Corn flour	0.70	0.90	0.50
Coarse ground maize	13.25	14.20	16.30
Intact grain	3.43	6.75	8.66

Table 7. Influence of different temperatures on FB_1 production by *F. moniliforme*

Incubation period	Temperatures			
	17 °C	21 °C	25°C	28°C
Week 2	2.5	8.6	4.1	2.2
Week 3	3.9	10.3	5.8	4.1
Week 4	6.4	15.0	11.1	8.7
Week 5	8.5	19.3	14.8	9.7
Week 6	8.3	10.5	9.1	4.9

4. Discussion

Mycotoxins are fungal metabolites capable of having acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic and oestrogenic effects to man and animals (Castella *et al*., 1999). Fumonisins are a group of naturally occurring Mycotoxins produced by *Fusarium moniliforme*, *Fusarium proliferatum* and other related species (Bacon *et al*., 2001 and Miller, 2001). Common mycotoxins in maize include aflatoxins, fumonisins, moniliformin, deoxynivalenol and zearalenone. *Fusarium verticillioides* is the leading fungal species in producing fumonisins that have been shown to cause equine leukoencephalomalacia, pulmonary edema, human esophageal cancer and rat liver cancer (Van Egmond, *et al*., 2007).

Similar to our work Shephard *et al*. (1996) showed that, *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers. Also Rheeder *et al*. (2002), recorded that, *F. verticillioides* and *F. proliferatum* are produce the highest amounts of toxins: up to 17900 µg/g of FB_1 have been recorded in cultures for the former, and 31000 µg/g FB_1 for the latter.

Our study showed that, *F. moniliforme* produced highest amounts of fumonisin 19.3 µg/g after five weeks incubation period. Also detoxification percent of FB_1 was significantly increased gradually by addition of *S. cerevisiae* while growth of *Fusarium moniliforme* was significantly decreased. Linear regression analysis between *S. cerevisiae* doses and fresh weight and dry weight showed that significant negative correlation was detected. The regression equations for fresh and dry weights were: $Y = -1.3945x + 11.102$, $R^2 = 0.9538$ and $Y = -0.889x + 7.1049$, $R^2 = 0.986$, respectively.

The detoxification percent at doses 3 and 5 were 77.63 and 89.52, respectively. Also it clear that dose 7 was sufficient for complete detoxification of fumonisin B_1. A similar result was obtained by Paola *et al*. (2010). He showed that, seed treatment with *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* reduced the infection by the fungus and FB_1 contents. Also these results are similar as of El-Tarabily (2004), Madi *et al*. (1997), Moustafa and Mohamed (2008); they reported that *R. solani* and *S. rolfsii* were effectively suppressed by yeasts.

Both temperature and water content of the substrate are factors that are of particular importance for the growth and the mycotoxinogenesis of *Fusarium* species that are mainly mesophilic and hydrophilic fungal strains usually developing on living plants, acting as parasites (Marin *et al*., 2004). Generally our results indicated that, level of fumonisin B_1 was increased gradually by increasing temperatures degrees until reach a high level of production at temperature 21°C and then decreased by increasing temperature degrees. At temperature 21°C level of FB_1 was 19.3 µg/g after incubation period 4 weeks. While at temperature 28°C was 9.7 µg/g after the same incubation period and on the same substrate.

Similarly, Mogensen *et al*. (2009) found that *Fusarium* spp. had the maximal production of FB_1 and FB_2 at 20-25°C. Also, Marin *et al*. (1995 and 1999) showed that both *F. moniliforme* and *F. proliferatum* grew faster with increasing water activity. Similarly, our work showed that, at water content of 50% higher levels of fumonisin (16.3 µg/g) was detected in ground maize, while in corn flour and intact grain level of fumonisin was 0.50 and 8.66 µg/g, respectively. Those results are in agreement with data obtained in other studies (Samapundo *et al*., 2005; Bailly *et al*., 2005 and Narasimha *et al*., 2010). FB_1 production optimal conditions taken off for the end of the study were; 5 weeks at 21°C on sterile coarsely cracked maize with water content of 50 %.

In conclusion, the current work indicates that maize seeds are contaminated to various degrees with

Fusarium moulds and fumonisin B_1 and the use *S. cerevisiae* as a biocontrol agent can control the *Fusarium* moulds growth as well as fumonisin B_1 production in these seeds. In the present study, we suggest that caution should be used whenever, maize seeds are to be stored prior to use because of the normal occurrence of mycotoxinogenic moulds as part of the natural seed microflora.

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Survival of genetically-engineered and wild-type strains of the yeast *Saccharomyces cerevisiae* under simulated environmental conditions: a contribution on risk assessment

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H. FUJIMURA, Y. SAKUMA AND E. AMANN. 1994. A genetically-engineered strain of *Saccharomyces cerevisiae* employed for the industrial production of the human coagulation Factor XIIIa (rhFXIIIa) was used for a survival study under simulated environmental conditions. The homologous strain devoid of the recombinant plasmid and the homologous strain bearing the 2 μm-based vector plasmid without the rhFXIIIa-encoding DNA insert were compared. The strains were introduced into natural soil/water suspension, into soil/medium suspension and into waste water. After intervals, samples of cell suspensions were taken and viable cell numbers were determined by plating on antibiotic-containing medium. In addition, a non-radioactive technique involving enhanced chemiluminescence was employed to detect plasmid-bearing yeast cells. The rhFXIIIa expression plasmid showed a high stability during the simulated environmental condition. No differences in survival rates, however, could be detected for the plasmid-bearing and plasmid-less strains under the three conditions tested, suggesting that the presence of plasmid does not confer selective advantages on the survival of the yeast cells. It is concluded that, even after accidental release of the engineered yeast cells into the environment, elimination rates would be comparable to those for non-recombinant yeast strains.

INTRODUCTION

During the past 10 years there have been rapid developments in genetic engineering techniques, involving increasing numbers of laboratories. Apart from the deliberate release of genetically-engineered micro-organisms (GEMs) and plants into the environment, for example for purposes of pest control, pollution abatement and frost protection, there is an increasing chance of accidental release of GEMs, used for the 'closed system' production of pharmaceutical products, into the surrounding environments. To assess the risks posed by accidentally-released GEMs into the environment, research is necessary on their survival and growth and on the transfer of genetic information between model micro-organisms under safe, controlled environmental conditions simulated in the laboratory.

The model studies on the survival of genetically-engineered bacteria provide some indications. For example, Chao and Feng (1990) observed no significant difference in survival between plasmid-bearing and plasmid-less *Escherichia coli* cells in natural waters. Devanas *et al.* (1986) reported good survival of plasmid-containing *E. coli* compared with plasmid-less cells in nutrient-rich sediment. Cruz-Cruz *et al.* (1988) observed that the presence of plasmid did not confer a selective disadvantage upon *Pseudomonas aeruginosa*. Israeli *et al.* (1993) reported that genetically-engineered *Ps. syringae* strains are more sensitive than their wild-type parent strain to air-exposure in the dry state. Caldwell *et al.* (1989) and Jain *et al.* (1987) demonstrated that *Pseudomonas* spp. stably maintained plasmid genotypes through an 8-week period.

In spite of the increased industrial utilization of genetically-engineered yeast, only a few studies have been performed so far on the survival of such strains. Bröker (1990) studied survival of genetically-engineered *Saccharomyces cerevisiae* and observed no differences in the survival kinetics of genetically-engineered and wild-type yeast strains.

In the present study, the survival of a genetically-engineered yeast strain bearing a recombinant human coagulation Factor XIIIa (rhFXIIIa) expression plasmid was compared with that of plasmid-less cells.

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The usefulness of a non-radioactive technique for colony hybridization analysis of yeast cells was also investigated. There are several studies of a radioactive method for detecting genetically-engineered bacteria (Saylor and Layton 1990). The present work indicates that the non-radioactive method with enhanced chemiluminescence (ECL) is useful for the analysis of plasmid retention rates in genetically-engineered yeast cells.

MATERIALS AND METHODS

Yeast strain, plasmids and media

Saccharomyces cerevisiae CL3ABYS86 (*MATα ura3 leu2 his3 pra1 prb1 prc1 cps*) (Schuller and Entian 1988), obtained from M. Bröker of Behringwerke, was used. Plasmid pEMBLyex4 (2 μm + *LEU2d* + *URA3* + pUC18) (Cesareni and Murray 1987) or rFXIII expression plasmid pMB330A (pEMBLyex4 containing the FXIIIa cDNA inserted under the control of the $GAL1_{UAS}$-*CYC1* hybrid promoter and 2 μm terminator) (Bröker *et al.* 1991) was introduced into CL3ABYS86 according to the lithium method (Ito *et al.* 1983). Plasmid-bearing or plasmid-less cells were cultured in SD medium (containing g l^{-1}: glucose, 20; yeast nitrogen base without amino acid, 6·7) supplemented with appropriate bases and amino acids (Sherman *et al.* 1983) for 3 d at 30°C and then inoculated into rich YEPD medium (containing (g l^{-1}): glucose, 20; peptone, 20; yeast extract, 10) and cultured at 30°C for 2 d.

Survival determination

The procedure was carried out as described by Bröker (1990). Cells from 10 ml of stationary cultures were harvested from YEPD medium and washed twice with sterile water and added: (1) to 500 ml of water supplemented with 100 g of soil from the company ground of Hoechst Japan Ltd in Kawagoe, Saitama; (2) to 500 ml of YEPD medium supplemented with the 100 g soil and (3) to 500 ml of waste water taken from the company's waste water processing plant. The suspensions were incubated in 2 l flasks at room temperature. At time intervals, aliquots of cell suspension were diluted with sterile water and spread on YEPD plates containing streptomycin, ampicillin, neomycin, gentamycin, kanamycin, tetracyclin and chloramphenicol (0·2 mg each ml^{-1}). When the colony-forming units (cfu) ml^{-1} were below 100, a total of 1 ml (10 times, 0·1 ml each) of the original culture was spread on the plates. The plates were incubated at 30°C for 3 d and the colonies counted.

Plasmid stability

he colonies on the antibiotic-containing plates were transferred to SD supplemented with 20 mg l^{-1} histidine(SD + His) plates and incubated at 30°C for 3 d. Plasmid-bearing cells grew on SD + His plates, but plasmid-less cells did not. On the other hand, wild-type yeast cells derived from soil or waste water grew on minimal SD plates and may therefore be excluded from the following calculation. Plasmid retention is the ratio of the number of plasmid-bearing colonies (his^-) to the number of total colonies (his^- and his^- ura^- leu^-) except wild yeast colonies.

Colony hybridization

The procedures for culturing single colonies and for preparation of nylon membranes were carried out according to the standard procedures (Sherman *et al.* 1983) with slight modifications as follows: colonies on antibiotic-containing YEPD plates were transferred to a Hybond-N^+ nylon membrane (Amersham International, UK) on a YEPD plate and incubated at 30°C overnight. The membrane was then treated on filter paper (3MM, Whatmann International, UK) saturated with the following solutions: (1) SET buffer (1 mol l^{-1} sorbitol, 50 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris-HCl, pH 7·5) containing 1% (v/v) 2-mercaptoethanol at room temperature for 15 min; (2) SET containing Zymolyase 100T (1 mg ml^{-1}) (Seikagaku Kohgyo, Tokyo, Japan) at 37°C for 2 h; (3) 0·5 mol l^{-1} NaOH for 7 min; (4) 0·5 mol l^{-1} Tris-HCl, pH 7·5, 10 × SSC (1·5 mol l^{-1} NaCl, 0·15 mol l^{-1} sodium citrate) for 4 min, twice; (5) 2 × SSC for 4 min. The membrane was then air-dried on paper and baked at 80°C for 2 h. The membrane was soaked with TE (10 mmol l^{-1} Tris-HCl, pH 7·5, 1 mmol l^{-1} EDTA, pH 8·0), and then yeast-derived debris was removed by attaching the membrane with a dry filter paper. A 1·4 kb *Bam*HI-*Hin*dIII fragment carrying a cDNA segment of human FXIIIa derived from pMB330A (Bröker *et al.* 1991) was used as a probe. 'Random prime' labelling of the probe, hybridization and the non-radioactive detection procedures were carried out using Amersham's ECL system kit as described by Pollard-Knight *et al.* (1990).

RESULTS

Survival kinetics of yeast strains

Survival kinetics of yeast cells bearing the rhFXIIIa expression plasmid pMB330A (CL3ABYS86[pMB330A]), of cells bearing the vector plasmid pEMBLyex4 (CL3ABYS86[pEMBLyex4]) and of plasmid-less cells (CL3ABYS86) in soil/water suspensions are shown in Fig. 1. After incubation at room temperature for 20 d, the numbers of plasmid-bearing and plasmid-less cells were reduced to less than 1 cfu ml^{-1}. This experiment was carried out three times. Under this condition, no significant

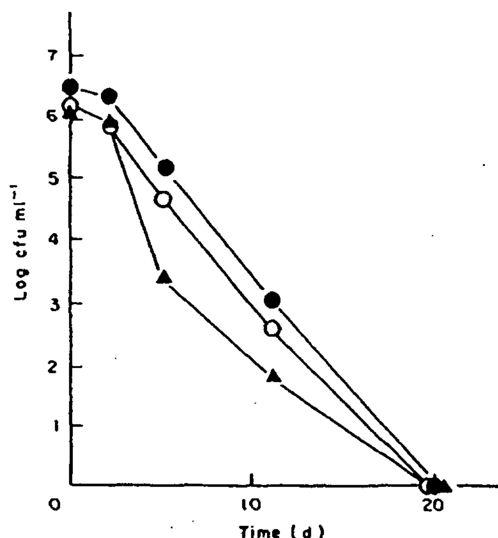

Fig. 1 Survival of plasmid-bearing and plasmid-less cells of *Saccharomyces cerevisiae* in non-sterile soil/water suspension. ○, CL3ABYS86; ●, CL3ABYS86[pMB330A]; ▲, CL3ABYS86[pEMBLyex4]

differences in survival rates were reproducibly observed between pMB330A-bearing cells, pEMBLyex4-bearing cells and plasmid-less cells, although there were some differences between three experiments.

The survival of the plasmid-bearing yeast cells and plasmid-less cells in waste water was then investigated (Fig. 2). After incubation for 20 d, less than 0·0001% of plasmid-bearing cells and plasmid-less cells survived. Compared with the above case (soil/water suspensions), a more drastic decline in the number of surviving cells was observed in the waste water.

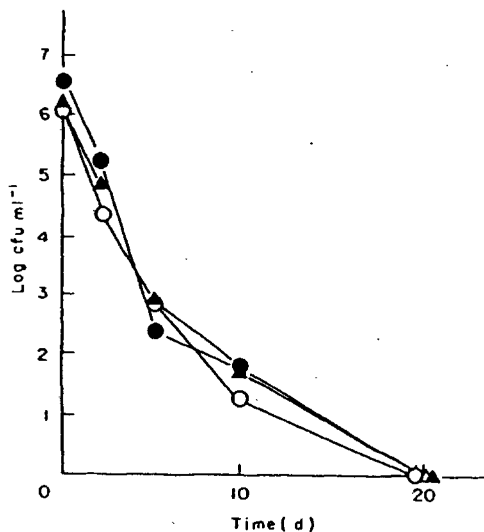

Fig. 2 Survival of plasmid-bearing and plasmid-less cells of *Saccharomyces cerevisiae* in waste water. ○, CL3ABYS86; ●, CL3ABYS86[pB330A]; ▲, CL3ABYS86[pEMBLyex4]

Finally, survival kinetics of yeast cells in soil/medium suspensions were investigated. Plasmid-bearing and plasmid-less cells were inoculated into YEPD rich medium containing the ground soil and incubated at room temperature. In this experiment, the number of cfu ml^{-1} of all three types of cells increased at first, and decreased to less than 1 cfu ml^{-1} after 20 d (Fig. 3a). In soil/medium suspensions, the growth of autochtonic fungi and mould was noticed. Excessive growth of fungi and bacteria may have inhibited the survival of yeast cells. In sterile YEPD medium not containing soil, the numbers of yeast cells remained constant during the first 20 d (Fig. 3b).

In summary, the plasmid-bearing and plasmid-less yeast cells died at comparable frequencies, and no significant differences in survival rates were reproducibly observed under three different conditions.

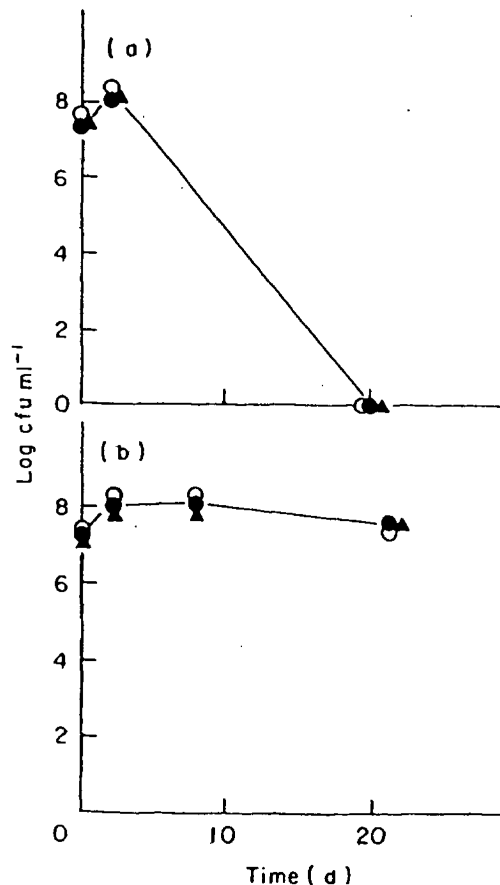

Fig. 3 Survival of plasmid-bearing and plasmid-less cells of *Saccharomyces cerevisiae* in non-sterile soil/rich medium and in sterile YEPD medium. (a) Non-sterile soil/rich medium suspension; (b) sterile YEPD medium. ○, CL3ABYS86; ●, CL3ABYS86[pMB330A]; ▲, CL3ABYS86[pEMBLyex4]

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Survival of Genetically Modified and Self-Cloned Strains of Commercial Baker's Yeast in Simulated Natural Environments: Environmental Risk Assessment

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Although genetic engineering techniques for baker's yeast might improve the yeast's fermentation characteristics, the lack of scientific data on the survival of such strains in natural environments as well as the effects on human health prevent their commercial use. Disruption of acid trehalase gene (*ATH1*) improves freeze tolerance, which is a crucial characteristic in frozen-dough baking. In this study, *ATH1* disruptants constructed by genetic modification (GM) and self-cloning (SC) techniques were used as models to study such effects because these strains have higher freeze tolerance and are expected to be used commercially. Behavior of the strains in simulated natural environments, namely, in soil and water, was studied by measuring the change in the number of viable cells and in the concentration of DNA that contains *ATH1* loci. Measurements were made using a real-time PCR method during 40 days of cultivation. Results showed that the number of viable cells of GM and SC strains decreased in a time-dependent manner and that the decrease rate was nearly equal to or higher than that for wild-type (WT) yeast. For all three strains (SC, GM, and WT) in the two simulated natural environments (water and soil), the DNA remained longer than did viable cells but the decrease patterns of either the DNA or the viable cells of SC and GM strains had tendencies similar to those of the WT strain. In conclusion, disruption of *ATH1* by genetic engineering apparently does not promote the survival of viable cells and DNA in natural environments.

Molecular genetic engineering techniques for breeding of commercial baker's yeast are well established. Such techniques could improve the yeast's characteristics, such as fermentation ability and stress tolerance, and could decrease the cost for baker's yeast production and for bakery processes (16, 18, 22, 23, 25). Genetic engineering techniques produce two categories of yeasts: genetically modified (GM) yeast, which contains a heterologous DNA segment derived from organisms taxonomically different from their host cells, and self-cloning (SC) yeast, which does not contain any DNA derived from other organisms and does not produce any additional proteins except for proteins originally produced in the yeast (2, 10, 29). SC processes are considered the same as naturally occurring gene conversion, such as recombination, deletion, and transposition, and thus SC yeast is not considered a GM organism. For this reason, SC yeast might be more acceptable for consumers than GM yeast. However, genetically engineered baker's yeasts, not only GM yeasts but also SC yeasts, are currently not used commercially. One reason for the hesitation in commercial use of GM or SC strains of yeast is the lack of scientific data on the survival of such strains in natural environments as well as the effects on human health (5, 12, 14).

Assessment of the viability of yeasts constructed by GM and SC techniques in natural environments is important because such yeast might be inadvertently or intentionally released into natural environments, such as soil and water environments, during propagation processes of yeast products in factories or during baking processes in bakeries. It is important to provide the general public with accurate information about the behavior of genetically engineered yeast under natural conditions so that consumers can comfortably accept such techniques and the resultant products, resulting in a boost of the commercial use of GM or SC yeasts in the food industry. The aim of this study was to clarify the survival of viable cells and DNA of SC and GM yeast at the molecular level in natural environments.

In this study, gene disruptants of acid trehalase gene (*ATH1*) derived from commercial baker's yeast were constructed by using GM or SC techniques and then used as models of genetically engineered yeast. In *ATH1* disruptants, trehalose is highly accumulated and functional as a cryoprotectant under freezing conditions (22). Because disruption of *ATH1* improves the freeze tolerance of commercial baker's yeast, the commercial use of *ATH1* disruptants is expected in frozen-dough baking (22).

Despite the increased studies on the genetic engineering techniques of microorganisms, only a few studies on the survival of GM and SC yeasts under natural environments have been reported previously (3, 8). For example, Fujimura et al. (8) showed that under simulated environmental conditions, *Saccharomyces cerevisiae* that overproduces human coagulation factor XIIIa showed the same survival rate as the strain that harbors an empty vector. Specific methods for detecting genetically engineered yeast, however, have not

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TABLE 1. Strains and oligonucleotide primers used in this study

Strain or primer	Genotype or sequence	Source or reference
Strains		
T7	*MAT*a *ATH1 CYH2 URA3*	22
T7CR	*MAT*a *ATH1 cyh2 URA3*, derived from T7	This study
T7CRu	*MAT*a *ATH1 cyh2 ura3*, derived from T7CR	This study
T7CR-SC	*MAT*a *ath1::URA3 cyh2 ura3*, derived from T7CRu	This study
T7CR-GM	*MAT*a *ath1::kanMX4 cyh2 URA3*, derived from T7CR	This study
T18	*MAT*α *ATH1 CYH2 URA3*	22
T18CR	*MAT*α *ATH1 cyh2 URA3*, derived from T18	This study
T18CRu	*MAT*α *ATH1 cyh2 ura3*, derived from T18CR	This study
T18CR-SC	*MAT*α *ath1::URA3 cyh2 ura3*, derived from T18CRu	This study
T18CR-GM	*MAT*α *ath1::kanMX4 cyh2 URA3*, derived from T18CR	This study
T118	*MAT*a/α *ATH1/ATH1 CYH2/CYH2 URA3/URA3*, obtained by mating T7 and T18	22
T118CR-WT	*MAT*a/α *ATH1/ATH1 cyh2/cyh2 URA3/URA3*, obtained by mating T7CR and T18CR, defined as WT strain	This study
T118CR-SC	*MAT*a/α *ath1::URA3/ath1::URA3 cyh2/cyh2 ura3/ura3*, obtained by mating T7CR-SC and T18CR-SC, defined as SC strain	This study
T118CR-GM	*MAT*a/α *ath1::kanMX4/ath1::kanMX4 cyh2/cyh2 URA3/URA3*, obtained by mating T7CR-GM and T18CR-GM, defined as GM strain	This study
BY4741Δ*ath1*	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ath1::kanMX4*	EUROSCARF
Primers		
P1	5′-CAGTCCTTTATGAGAGCCTGC-3′	
P2	5′-AGAAGCCTCCGCATTGAACCA-3′	
P3	5′-CAATGAAGGCGTCGTTGT-3′	
P4	5′-ATGCTGGTCGCTATACTGC-3′	
P5	5′-GTAGATTCACGGACCAGGA-3′	
P6	5′-ACAATCTGAGTCAGCAAGGCA-3′	
P7	5′-CAGGAAGATCAAATTGGTAGG-3′	
P8	5′-TAGAGGTATGGCCGGTGGTA-3′[a]	
P9	5′-GCTTCCAGAAATGAGCTTGTTGC-3′	

[a] A 3′-terminal adenine base (underlined) corresponding to the *cyh2* mutation position.

yet been established. In contrast to only a few studies on genetically engineered yeasts, many studies on genetically engineered bacteria have been reported, such as *Pseudomonas* strains used for bioremediation and lactic acid bacteria used for probiotics (1, 7, 19, 21). Specific methods for the detection of genetically engineered bacteria have been reported previously (11, 26, 27, 30).

The goal of this current study was to clarify the survival of cells and specific DNA fragments of GM and SC yeasts in natural environments. Soil and water were chosen as models of natural environments because deliberate or accidental releases to such natural environments might occur. Diploid strains derived from commercial strains were used to simulate industrial baker's yeast in this study. First, a system to detect GM and SC yeasts in natural environments was constructed using quantitative real-time PCR (RTm-PCR) technology recently used to rapidly quantify genes and microorganisms in complex environments (6, 9, 24, 30). Then, the changes in the number of viable cells and in the concentration of DNA during 40 days in the two simulated natural environments (soil and water) were measured and compared for these three strains, namely, GM type of *ATH1* disruptants that harbor an antibiotic resistance marker gene derived from bacteria (28), SC type of *ATH1* disruptant constructed using an auxotrophic marker gene that was originally cloned from yeast (20), and wild-type (WT) strain.

MATERIALS AND METHODS

Construction of strains. Table 1 summarizes the strains and oligonucleotide primers used in this study. Strains T7 and T18 are haploid strains that are derivatives of commercial baker's yeast (22). T118 is a diploid strain obtained by mating haploid strains T7 and T18. The strain T118 has high fermentation ability (22). To discriminate these strains from indigenous yeast strains, diploid strains that harbor cycloheximide resistance were constructed. The spontaneous cycloheximide-resistant mutants (13) were isolated from strains T7 and T18, yielding T7CR and T18CR. T118CR-WT was constructed by mating T7CR and T18CR. T118CR-WT was resistant to 10 μg/ml of cycloheximide. Because T118CR-WT had an intact *ATH1* locus, T118CR-WT was defined as the WT strain in this study. The diploid strain of the SC type of *ATH1* disruptant was constructed as follows. To allow for utilization of the *URA3* gene as a selective marker, spontaneous *ura3* mutants from T7CR and T18CR were obtained by 5-fluoroorotic acid selection (4), yielding T7CRu and T18CRu. Gene disruption of *ATH1* with *URA3* was carried out as described previously (22), yielding T7CR-SC and T18CR-SC. T118CR-SC was constructed by mating T7CR-SC and T18CR-SC, and T118CR-SC was defined as the SC strain in this study. The diploid strain of GM type of the *ATH1* disruptant was constructed as follows. The *ath1::kanMX4* fragment was obtained by PCR using primers P6 and P7 (Table 1 and Fig. 1A) and genomic DNA of BY4741Δ*ath1* as a template. Gene disruption of the *ATH1* locus in strains T7CR and T18CR was achieved using the PCR fragment of *ath1::kanMX4*, yielding T7CR-GM and T18CR-GM. T118CR-GM was constructed by mating strains T7CR-GM and T18CR-GM, and T118CR-GM was defined as the GM strain in this study.

Cocultivation systems in soil and water environments. Model soil and water environments were inoculated with WT, SC, and GM yeast cells grown at 30°C for 48 h in YPD medium that contained 10 g of yeast extract (Difco, Detroit, Mich.), 20 g of peptone (Difco), and 20 g of glucose (per liter). Nonsterile river sand (Matsuzaki, Japan) for horticulture was used as the model soil, where the water content was 7.2% (wt/wt) and pH was 6.5. Sterile distilled water was used as the model water.

Two cocultivation systems were used: a series I cocultivation system, which harbored WT and GM strains, and series II, which harbored SC and GM strains. For the soil environment, two strains (either WT and GM or SC and GM) were coinoculated into 70 g of soil in a 125-ml plastic bottle at a cell density of 10^6 cells (each strain) per 1 g of dry soil and then immediately mixed. For the water environment, the strains were coinoculated into 500 ml of sterile distilled water

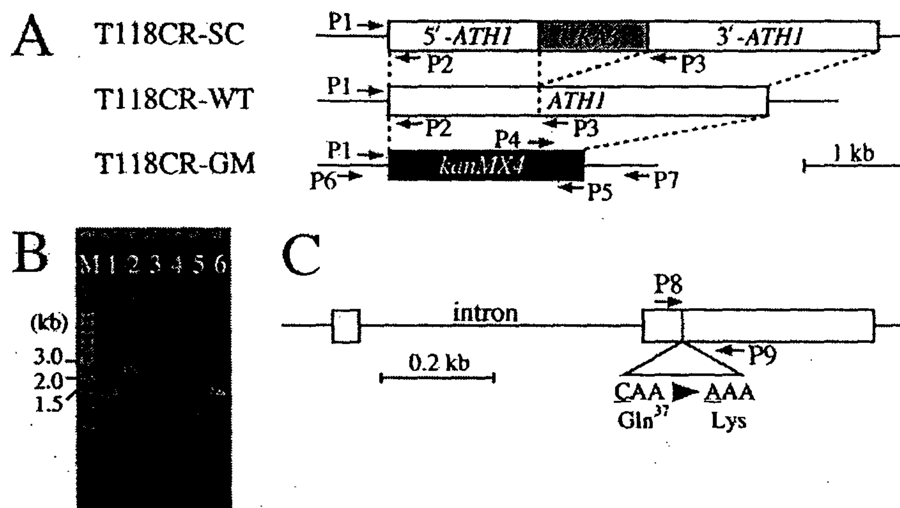

FIG. 1. (A) Schematic model of *ATH1* loci and PCR primers. Coding region of the *ATH1* gene is indicated by open boxes. Regions of *URA3* and *kanMX4* genes are indicated by shaded and filled boxes, respectively. Positions and directions of PCR primers are indicated by arrows. (B) *ATH1* loci of WT, SC, and GM strains were confirmed by PCR amplification. Lane M shows the molecular size marker. Lanes 1 to 6 show PCR product amplified using primers P1 and P3 from genome of T118CR-WT (lane 1), P1 and P3 from that of T118CR-SC (lane 2), P1 and P3 from that of T118CR-GM (lane 3), P1 and P5 from that of T118CR-WT (lane 4), P1 and P5 from that of T118CR-SC (lane 5), and P1 and P5 from that of T118CR-GM (lane 6). (C) Schematic model of *cyh2* locus and PCR primers. Coding region of the *CYH2* gene is indicated by open boxes. Position and direction of PCR primers are indicated by arrows. Mutation position of *cyh2* (CAA to AAA) is indicated by a large open triangle.

in a 1-liter flask at a cell density of 10^6 cells (each strain) per 1 ml of water and then immediately mixed. These soil and water cocultivation systems were then incubated at 25°C for 40 days and 42 days, respectively, under dark conditions without shaking. During incubation, samples were taken for measurement of the number of viable cells and the DNA concentration. At the time of sampling, the cocultivation systems were mixed to ensure homogenous samples.

Measurement of the number of viable cells remaining in soil and water environments. The number of viable cells of inoculated yeast cells remaining in the soil and water environments (series I and II cocultivation systems) were measured using the cultivation method as follows. The soil environment samples (0.5 g) were suspended in distilled water, and total volume was adjusted to 5 ml. Serial 10-fold dilutions of the soil suspension and the water environment samples were prepared using distilled water. The dilutions (200 μl) were then individually plated onto CPS agar medium, which is YPD medium containing 5 mg of cycloheximide (Sigma-Aldrich, St. Louis, Mo.), 0.75 g of sodium propionate, 0.3 g of streptomycin (Sigma-Aldrich), 5 g of lactic acid, and 20 g of agar (per liter). The colonies that appeared after incubation for 3 days at 30°C on CPS medium were considered total viable cells (VCt) of the inoculated yeast cells. These total survivors appearing in CPS agar medium (20 to 200 colonies) were then replica plated onto YPD-G418 agar medium (i.e., YPD agar medium supplemented with an aminoglycoside antibiotic, G418 [Sigma], at a concentration of 0.3 g/liter) and incubated at 30°C for 1 day. The colonies that appeared on YPD-G418 agar were considered cells that harbored *ath1::kanMX4* locus (VCg). The number of viable cells that harbored either wild-type *ATH1* (VCw) or self-cloned *ath1::URA3* locus (VCs) were calculated by subtraction of VCg from VCt. The number of viable cells was expressed as the mean from triplicate experiments.

To measure the number of indigenous bacteria, portions of each suspension of the soil and water environment samples were plated onto tryptic soy broth agar (Difco) and then incubated at 37°C for 3 days. The colonies that appeared on the agar were counted as the number of indigenous bacteria.

DNA extraction from soil and water environments and from yeast cells. DNA contained in 0.5-g soil environment samples was extracted using a FastDNA spin kit for soil (Q-Biogene, Carlsbad, Calif.) according to the manufacturer's instructions. The extracted DNA was then dissolved in 50 μl of distilled water and used for RTm-PCR analysis.

DNA contained in 1-ml water environment samples was extracted according to a protocol described by Philippsen et al. (17). The extracted DNA was then dissolved in 60 μl of distilled water and used for RTm-PCR analysis.

Yeast DNA used as standard DNA in RTm-PCR analysis was extracted from yeast cells, which were grown in 2 ml of YPD medium at 30°C for 48 h, using a FastDNA spin kit for soil according to the manufacturer's instructions. The extracted DNA was dissolved in 50 μl of distilled water. The DNA concentration was measured by using a spectrophotometer (Ultraspec UV2100 pro; Amersham Biosciences, Piscataway, N.J.).

Measurement of yeast DNA concentration in soil and water environments. The concentration of DNA from WT, SC, and GM strains in the soil or water environments was quantified using the RTm-PCR method (15, 24). RTm-PCR was done using a hot-start PCR kit (LightCycler FastStart DNA Master SYBR Green I; Roche, Mannheim, Germany) and LightCycler instrument (Roche), and the analysis was done using the LightCycler software version 3.5 (Roche). For RTm-PCR, 20 μl of the reaction mixture was used, consisting of 0.05% (wt/vol) of bovine serum albumin, 1 μM of each respective primer (Table 1) (also discussed in the next paragraph), 3 mM of $MgCl_2$, 0.29 μl of LightCycler FastStart enzyme (included in the kit), 1.71 μl of LightCycler FastStart reaction mix (included in the kit), and 2 μl of template DNA solution.

Primers for the detection of *ATH1* and *cyh2* loci were as follows. *ATH1* locus in the WT strain and *ath1::URA3* locus in the SC strain were detected as the 105 bp of PCR product using primers P1 and P2 (Table 1 and Fig. 1A). *ATH1* locus replaced by *kanMX4* in the GM strain was detected as the 101 bp of PCR product using primers P4 and P5 (Table 1 and Fig. 1A). *cyh2* locus of the WT, SC, and GM strains was detected as the 119 bp of PCR product using primers P8 and P9 (Table 1 and Fig. 1C). Primer P8 was designed based on the mutation position in the *CYH2* gene to enable the detection of the *cyh2* locus.

Thermal cycling conditions for detection of *ATH1* and *cyh2* loci were as follows. The thermal cycling conditions for *ATH1* and *ath1::URA3* locus in the WT and SC strains consisted of initially heating samples to 95°C and storage at 95°C for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 63°C and storage at 63°C for 4 s, and heating at 10°C/s to 72°C and storage at 72°C for 5 s. The thermal cycling conditions for detection of the *kanMX4* locus in the GM strain consisted of initially heating samples to 95°C and storage at 95°C for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 61°C and storage at 61°C for 5 s, and heating at 10°C/s to 72°C and storage at 72°C for 6 s. The thermal cycling conditions for detection of the *cyh2* locus of the WT, SC, and GM strains consisted of initially heating samples to 95°C and storage there for 10 min, followed by 40 cycles of heating at 20°C/s to 95°C and storage at 95°C for 15 s, cooling at 20°C/s to 65°C and storage at 65°C for 4 s, and heating at 20°C/s to 72°C and storage at 72°C for 5 s. Fluorescence of double-stranded DNA-SYBR Green I complex was measured at the end of each 72°C cycle (extension process).

Melting curve analysis was done to confirm that the correct amplification of *ATH1* or *cyh2* loci occurred. The condition for this analysis consisted of heating

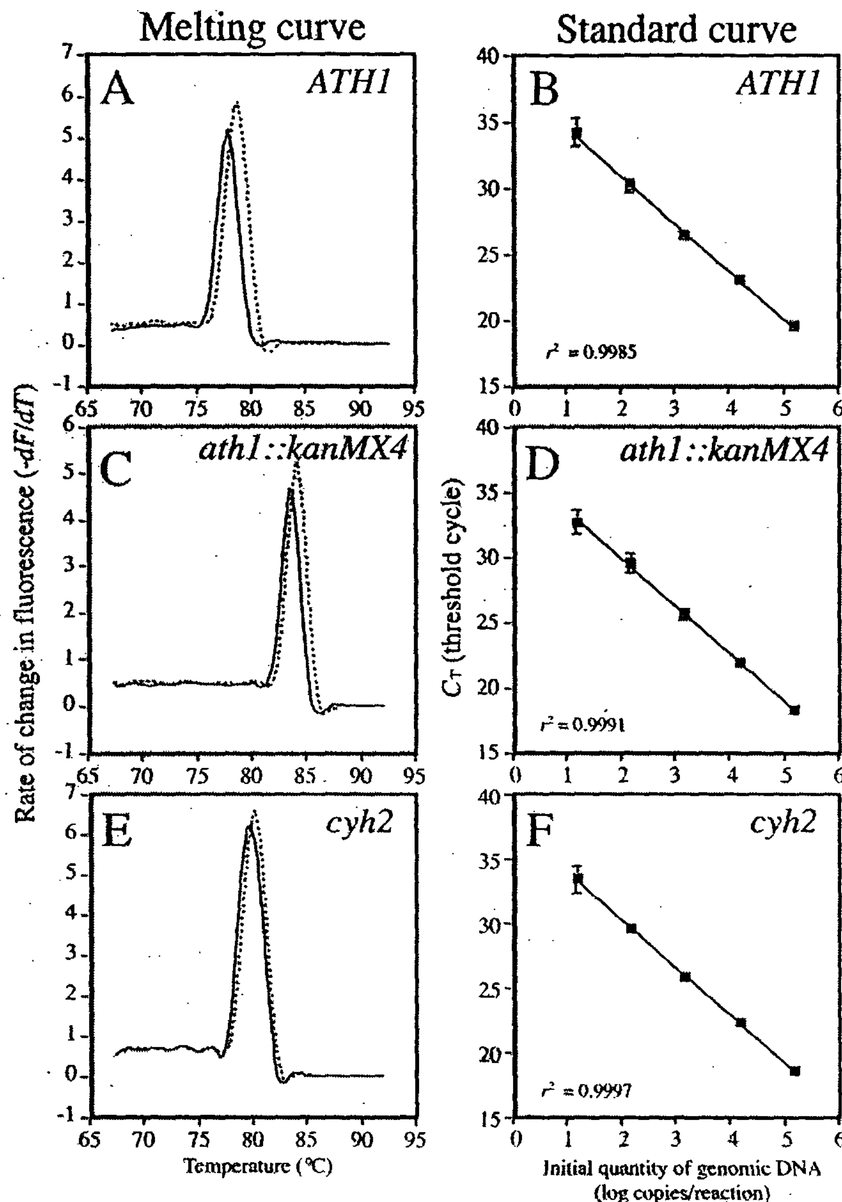

FIG. 2. Melting curves and standard curves of RTm-PCR products. (A, C, and E) Melting curves generated by plotting the negative first derivative of fluorescence against temperature ($-dF/dT$), which is a parameter for the rate of change in fluorescence based on DNA melting. (B, D, and F) Standard curves and correlation coefficients (r^2) by plotting C_T versus DNA quantity. C_T values are expressed as means ± standard deviations from triplicate experiments. Panel A is the melting curve of the *ATH1* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P1 and P2. Panel B is the standard curve for the *ATH1* locus. Panel C is the melting curve of the *ath1::kanMX4* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P4 and P5. Panel D is the standard curve for the *ath1::kanMX4* locus. Panel E is the melting curve of the *cyh2* locus amplified from control DNA (solid line) and from DNA extracted from soil (dotted line) using primers P8 and P9. Panel F is the standard curve for the *cyh2* locus.

at 20°C/s to 95°C, cooling at 20°C/s to 65°C and storage at 65°C for 15 s, and heating at 0.1°C/s to 95°C with continuous monitoring of the fluorescence. A melting curve was obtained by plotting the negative first derivative of fluorescence against temperature (i.e., $-dF/dT$). The melting temperature (T_m) of the double-stranded DNA products was represented by a peak in the melting curve.

Concentrations of the DNA contained in the soil and water environment samples were determined using the standard curves. The standard curves for measurement of *ATH1* and *cyh2* loci were generated by plotting the log of the number of copies in a 10-fold dilution series of the standard DNA extracted from he WT, SC, and GM strains against the C_T value, in which C_T was defined as the fractional cycle number (calculated using the LightCycler software) where the fluorescence increased above the detection threshold. The standard curve was then represented by a linear regression line of these C_T values. The quality of the standard curve was confirmed by the correlation coefficient (r^2) of C_T and DNA quantity. The DNA concentration was normalized based on the copy number per cell (two copies of *ATH1* loci and two copies of *cyh2* locus were contained in a diploid cell). DNA concentration was expressed as an equivalent of cell number and was the mean from triplicate experiments.

RESULTS

Construction of discriminative measurement systems of viable GM, SC, and WT strains. Homozygous SC and GM

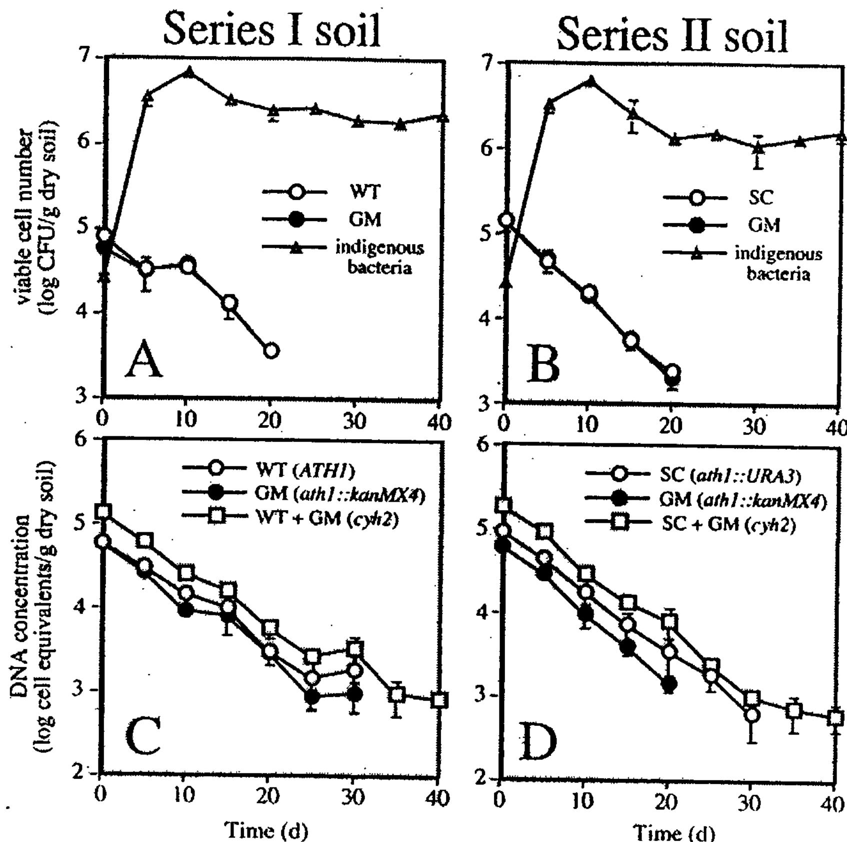

FIG. 3. Number of viable cells (A and B) and concentration of DNA containing the *AHT1* loci and *cyh2* locus (C and D) during 40 days (d) of cultivation in the soil environment. Panel A shows the number of viable cells in series I cocultivation (WT+GM) and panel B in series II cocultivation (SC+GM). Panel C shows DNA concentration of the *ATH1*, *ath1::kanMX4*, and *cyh2* loci in series I cocultivation (WT+GM) and panel D, that of the *ath1::URA3*, *ath1::kanMX4*, and *cyh2* loci in series II cocultivation (SC+GM). Number of viable cells and DNA concentration are expressed as means ± standard deviations from triplicate experiments.

strains of *ATH1* disruptants were constructed in the genetic background of T118. The expected *ATH1* loci of these strains are illustrated in Fig. 1A. Disruption of the *ATH1* loci of SC and GM was confirmed by PCR using primers P1 and P3 designed for the amplification of both intact *ATH1* and *ath1::URA3* and using primers P1 and P5 designed for the amplification of *ath1::kanMX4*, respectively (Fig. 1A). Amplifications of expected lengths of DNA from the WT, SC, and GM strains were observed (Fig. 1B), thus confirming that *ATH1* loci of the strains were correctly constructed.

Spontaneous mutations for cycloheximide resistance were introduced into the WT, SC, and GM strains to distinguish these strains from indigenous yeast cells under simulated soil environments. Sequence analysis of *CYH2* locus of the WT strain revealed that mutation occurred at position 623 (C to A) in the *CYH2* gene, suggesting the replacement of Gln^{37} by Lys (Fig. 1C). These strains grew on CPS agar plates, which contained 5 μg/ml of cycloheximide. Indigenous yeast included in the soil used in this study could not grow on CPS agar medium (data not shown). These results suggest that the three strains constructed here (WT, SC, and GM) are suitable for discriminative measurements from indigenous yeast using CPS agar nedium.

The G418 resistance of strains constructed here was examined to determine suitable conditions for discriminative measurement between the GM strain that harbors *kanMX4* and the WT and SC strains. The GM strain was resistant to 300 μg/ml of G418, whereas the WT and SC strains did not grow on agar medium containing G418 (data not shown). These data suggest that discriminative measurement between the GM strain and the SC and WT strains is possible by using agar medium containing 300 μg/ml of G418.

Molecular detection of *ATH1* and *cyh2* loci in soil and water environments. To construct a quantification system using RTm-PCR for the DNA fragments of the *ATH1* locus that survived in the soil and water samples, we designed specific PCR primers (P1 and P2) for amplification of the *ATH1* locus in both the WT and SC strains and specific primers (P4 and P5) for that in the GM strain (Table 1). The positions of the primers are indicated in Fig. 1A. The *cyh2* locus that was not modified by genetic engineering techniques was used for the neutral control to confirm the analysis results of the *ATH1* locus. Specific primers (P8 and P9) for amplification of the *cyh2* locus were designed based on the nucleotide sequence of *cyh2* in these strains (Table 1 and Fig. 1C).

Figure 2 shows the RTm-PCR profiles of DNA fragments amplified from the WT (*ATH1*) strain and those from the GM (*ath1::kanMX4*) strain. To confirm the correct amplification of each target locus, T_m of the PCR products was measured using melting curve analysis (Fig. 2A, C, and E). Figure 2A shows the melting curve analysis results for the *ATH1* locus amplified

from the WT strain using primers P1 and P2. The T_m of the PCR fragment amplified from DNA directly extracted from the soil samples was identical with that from standard DNA of the WT strain (Fig. 2A). In our assay system, the *ATH1* fragment amplified from the WT (*ATH1*) strain was expected to be the same as that from the SC (*ath1*::*URA3*) strain. Consistent with this expectation, both the T_m of the *ATH1* fragment amplified from DNA directly extracted from the soil samples and that from standard DNA of the SC strain were identical to that from the WT strain (data not shown). The T_m of the DNA fragments of *ath1*::*kanMX4* locus amplified from the soil samples was confirmed to be identical to the T_m from standard DNA of the GM strain using primers P4 and P5 (Fig. 2C). The T_m of the DNA fragments of *cyh2* locus amplified from the soil samples was confirmed to be identical to the T_m from standard DNA of the WT strain using primers P8 and P9 (Fig. 2E). The T_m of the amplified fragments from standard DNA of the SC and GM strains was identical to that of the WT strain (data not shown). Melting curve analysis using the genomic DNA of strain T118 (*CYH2*) using primers P8 and P9 as a template revealed that specific amplification did not occur (data not shown), suggesting that the *cyh2* locus is correctly detected by using primers P8 and P9. The molecular sizes of the PCR products from the *ATH1*, *ath1*::*kanMX4*, and *cyh2* loci measured by agarose gel electrophoresis were consistent with predicted DNA sizes (105, 101, and 119 bp, respectively) (data not shown). These results of T_m and molecular size analyses confirm the accuracy and high specificity of the PCR amplification of *ATH1* loci with *cyh2* locus as the neutral control.

To quantify the DNA fragments of *ATH1*, *ath1*::*kanMX4*, and *cyh2* in the water and soil environment samples, standard curves were constructed by using standard DNA (Fig. 2B, D, and F, respectively). The standard curves for each DNA fragment had a high r^2 (>0.99) and were not affected by other components, except for the concentration of DNA included in the samples directly extracted from the soil (data not shown). These curves shown in Fig. 2 confirm the reliability of the measurements of DNA containing the *ATH1*, *ath1*::*kanMX4*, and *cyh2* loci contained in a soil environment. The same experiments were done using DNA extracted from water samples. The RTm-PCR profiles for these water samples were identical to those for the standard DNA (data not shown), confirming the reliability of quantification of DNA in a water environment.

Survival rates of viable yeast cells and DNA concentration in the soil environment. To compare the survival rates of viable yeast cells and DNA concentrations of the three strains under strictly identical conditions, we constructed two series of cocultivation assay systems, namely, cocultivation of GM and WT strains (series I) and that of GM and SC strains (series II). In brief, two different strains (either GM and WT or GM and SC) were inoculated into soil and water samples and then cultured for 40 days and 42 days, respectively. During the culturing, the number of viable cells and the DNA concentration were measured using the discriminative methods described above (see Materials and Methods).

Figure 3 shows the measured number of viable cells and DNA concentration of the GM, SC, and WT strains in the soil environment. Figure 3A shows the results from the series I cocultivation system containing GM and WT strains. The number of viable cells of both strains logarithmically decreased in a similar time-dependent manner. The DNA fragment of the *ATH1* and *ath1*::*kanMX4* loci specified WT and GM strains, respectively, in the soil. The DNA concentration decreased more slowly than did the number of viable cells (Fig. 3C). The DNA concentration of *cyh2*, which was the neutral control for DNA quantification, decreased similarly to the concentrations of *ATH1* and *ath1*::*kanMX4* and was nearly equal to the sum of the concentrations of these two *ATH1* loci (Fig. 3C). Figure 3B and D show data from the series II cocultivation system containing GM and SC strains. Although the decrease rate of DNA concentration was lower than that of the number of viable cells, the DNA of both strains contained in the soil decreased logarithmically with the decrease in the number of viable cells (Fig. 3D). The data shown in Fig. 3 indicate that viable cells of both the GM and SC strains decreased in the same manner as those of the WT strain.

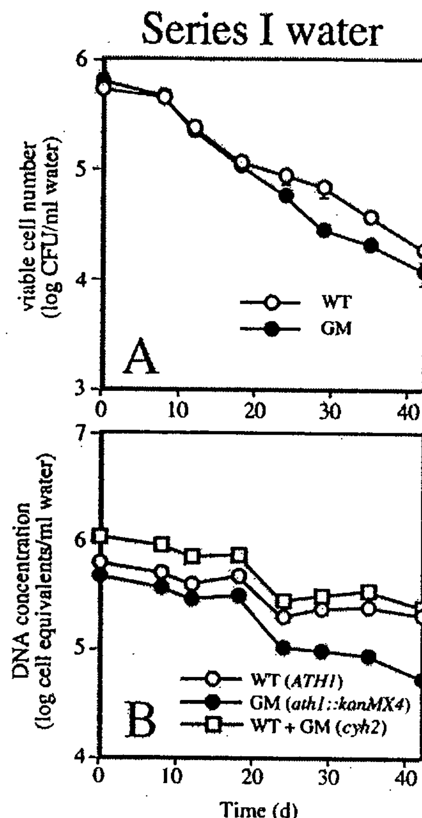

FIG. 4. Number of viable cells (A) and concentration of DNA containing *AHT1* loci and *cyh2* locus (B) of series I cocultivation (WT+GM) during 42 days (d) of cultivation in the water environment. Number of viable cells and DNA concentration are expressed as means ± standard deviations from triplicate experiments.

Based on these results, there was no significant difference in the survival of viable cells and DNA in the soil environment among the GM, SC, and WT strains.

Survival rates of viable yeast cells and DNA concentrations in the water environment. Figure 4 shows the survival rates of viable yeast cells and the concentrations of DNA from the GM and WT strains (series I) in the water environment. Although the decrease rates of both strains in water were lower

than those in soil, the number of viable cells and the DNA concentration of the GM strain decreased in a time-dependent manner but the decrease rate of the GM strain was significantly higher than that of the WT strain.

DISCUSSION

In summary, the number of viable cells and DNA concentration of the GM and SC yeast strains in simulated natural environments, namely, in soil and water, were compared with that of the WT strain. The survival rate of yeast cells was not related to the presence or absence of genetically engineered *ATH1* loci in the soil environment because all three strains tested (GM, SC, and WT) showed similar kinetics of survivability. Decrease in the *ATH1* loci and the neutral gene *cyh2* were also investigated under the same conditions. The decrease in DNA that contained either *ATH1* or *cyh2* loci was correlated with the decrease in viable cells. For all three strains in both simulated environments, DNA remained longer than did viable cells, although the decrease rates of the SC and GM strains were the same as or higher than that of the WT strain.

The effectiveness of techniques using an RTm-PCR method to quantitatively measure a specific yeast DNA contained in soil was tested and demonstrated. Under the experimental soil conditions used here, the sensitivity of the RTm-PCR method was the same as that of the viable-cell-count method. After inoculation of yeast cells at a concentration of 10^6 cells/g of dry soil (Fig. 3C and D), the measured DNA concentration was approximately 10^5 cell equivalents/g of dry soil whereas the number of viable cells was approximately 10^5 cells/g of soil. The recovery rates of DNA and viable cells were approximately 10%. Although the behavior of the nonrecoverable cells and DNA remains unclear at present, the behavior of the recoverable cells and DNA is assumed to represent that of the nonrecoverable cells and DNA because we obtained similar data from three independent experiments with reproducibility. Under the experimental conditions for the water environment used in our study, the sensitivity of the RTm-PCR method was almost the same as that of the viable-cell-count method (Fig. 4). The RTm-PCR method therefore should be a useful tool for the detection of yeast cells in natural environments as well because the method can measure the number of specific yeast cells more rapidly and more easily than other detection methods, including the viable-cell-count method. We attempted to apply the RTm-PCR method to yeast detection in more complicated environments such as kitchen garbage, but we could not obtain specific amplification of the yeast DNA (data not shown). Although further research into the detection of DNA in such complicated environments is necessary, the RTm-PCR method should be a useful tool for detection in natural environments.

Bröker (3) and Fujimura et al. (8) reported that no differences could be detected in the survival rate of either recombinant or wild-type yeast cells under either sterile conditions (in water) or nonsterile conditions (in soil). Although in this study the presence of the genetically engineered loci of *ATH1* might not directly affect the survival of GM and SC types of commercial baker's yeast in nonsterile soil conditions, our results suggested that the GM type of baker's yeast was less stable than the WT in the sterile water condition.

The RTm-PCR assay showed that the DNA fragment derived from yeast strains decreased at a slower rate than did the viable cells under soil and water environments. The rates of decrease in the concentration of DNA of the GM and SC strains were not significantly different from that of the WT strain. These results suggest that the disruption of *ATH1* by genetic engineering does not promote the survival of viable cells and DNA in natural environments.

Although the survivability of baker's yeast constructed by GM and SC techniques was clarified here, the effects of any release of GM and SC yeasts on indigenous microflora remain unknown. Further research is planned to study these effects by determining the effect of yeast inoculation on the microflora of indigenous bacteria, fungi, and yeast.

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Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development

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Abstract

This minireview summarizes the present state of knowledge concerning the importance of yeasts in fish gut. Yeasts have been commonly isolated in the gastrointestinal tract, and high population densities were sometimes noted in healthy fish, but the data were quite variable in terms of colony counts and taxonomical diversity. *Rhodotorula* sp. seemed relatively frequent in both marine and freshwater fish, and *Debaryomyces hansenii* has been found to be dominant in rainbow trout. Some other dominant strains have been described, such as *Metschnikowia zobelii*, *Trichosporon cutaneum*, and *Candida tropicalis* in marine fish, and *Candida* sp., *Saccharomyces cerevisiae*, and *Leucosporidium* sp. in rainbow trout. The natural proliferation of yeasts in fish mucus may be generally considered as commensalism, in spite of a few cases of pathological infections mainly due to opportunistic strains.
Several strains were documented to settle and grow in fish intestine after experimental introduction, particularly *S. cerevisiae* and *D. hansenii* in rainbow trout. There have been a few instances of competition among yeasts in fish intestine, while the effect of yeast on associated bacteria is still unclear.
Yeasts can stimulate the immune response in fish. ß-glucans is likely the most important compound in this regard, but some other cell-wall components or soluble factors may also play a role. Both cellular and humoral responses have been educed by dietary yeast, depending on the experimental conditions. Other benefits may be expected for the host, especially the intestinal colonisation of early feeding fry with yeast, which may have some effect on development, e.g. by accelerating the maturation of the digestive system. In older fish, dietary yeast may stimulate metabolism and growth.
Such beneficial effects need to be further investigated, either in cases of natural colonisation or after dietary introduction, while trying to elucidate the mode of action, and determine whether cellular viability is a prerequisite for efficacy.

Keywords: yeast; probiotics; gastrointestinal microbiota; gut maturation; growth promotion; immune system

1. Introduction

Yeasts are ubiquitous microorganisms, which disseminate with animals, air and water currents, and which can grow in various environments where organic substrates are available. Their presence has been noted in fish guts for some time in wild, as well as farmed animals, but this natural occurrence has been generally considered as incidental. Industrial yeast is commonly used in aquaculture, either alive to feed live food organisms, or after processing, as a feed ingredient (Stones and Mills, 2004). Some extracts, like β-glucans, are used as immuno-stimulants, and more recently, living yeasts have been proposed as probiotics. It may be time to inventory the state of knowledge concerning yeasts in fish intestinal microbiota, with the view to evaluate their possible effects on fish health and metabolism, and ways to monitor natural and artificial colonisation.

2. Natural occurrence in healthy fish

The investigation of yeasts as components of fish microbiota does not necessarily require specific culture media, and many microbiological broths and agars are suitable. There are however dedicated media whose selectivity may be improved by adding antibiotics. It appears that yeasts can constitute a significant part of the microbiota in fish gut (Table 1). In a few cases, yeasts may be more numerous than cultivable bacteria, like in the deep-sea eel *Synaphobranchus kaupi* (Ohwada et al., 1980), or in mature masu salmon in freshwater (Yoshimizu et al., 1976b). These examples seem exceptional, but yeast may be of physiological importance even when accounting for less than 1% of the total microbial isolates. It should be kept in mind that yeasts have cell volumes that may be larger than those of bacteria by a hundredfold. For instance, the cell volume of brewer's yeast averages 200-300 μm^3 (Cahill et al., 1999), while growing cells of *Pseudomonas fluorescens* reach a volume of ca. 1 μm^3 (Wilhelm et al., 1998). Therefore, an apparently negligible number of colony forming units (CFU) may correspond to a population size sufficient to act upon the host. For instance, anticipating the effects of live yeasts on fish metabolism (section 7), *Debaryomyces hansenii* was efficient in European sea bass larvae at 10^4 CFU g^{-1}, on a body weight basis (Tovar et al., 2002), whereas the bacterial counts may range from 10^7 to 10^9 CFU g^{-1} (Gatesoupe et al., 1997). It remains that highly variable levels of the natural yeast population have been observed, from below the detection threshold up to 10^7 CFU g^{-1}, on a gut content basis (Table 1).
This variability appeared also when the yeasts were characterised. Valente et al. (1999) emphasized the difficulty to define species of microorganisms, and this seemed particularly arduous with yeast. The Merriam-Webster Medical Dictionary spoke of "a unicellular chiefly ascomycetous fungus (as of the family *Saccharomycetaceae*) that has usually little or no mycelium, that typically reproduces asexually by budding". The morphological determination may be sometimes difficult, when the budding cells form pseudomycelium. The classification of yeasts has been constantly undergoing change, based first on morphological and physiological criteria (Pelczar and Reid, 1972), then on the development of molecular taxonomy (Valente et al., 1999). Yeasts occurring in fish microbiota may be classified in two distinct phyla of the fungal kingdom: *Ascomycota*, among which *Saccharomycetaceae* are probably the most important family, but also *Basidiomycota*, which include the genus *Rhodotorula* – these red yeasts are commonly observed in fish microbiota. Table 2 summarises tentatively the genera that have been reported in fish intestine. The classification was based on Patterson and McGinnis (2006), with complementary information from Ericksson (2006) and NCBI Taxonomy Browser (Wheeler et al., 2000). It is noteworthy that

the latter database proposed several orders for strains characterised as *Cryptococcus, Rhodotorula*, and *Sporobolomyces*, unlike Patterson and McGinnis (2006). Some phylogenetic revision among these genera may therefore be expected (Biswas et al., 2001; Takashima et al., 2001; Gadanho and Sampaio, 2002). This database was also used to identify the genera of four strains isolated from rainbow trout, *Yarrowia* sp., *Kluyveromyces* sp., *Filobasidium* sp., and *Bulleromyces* sp. (Gatesoupe, unpublished), by using partial sequences of PCR amplicons from primers ITS 1-4 (Aubin et al., 2005a). Among the 14 references of yeast characterisation listed in Table 1, *Rhodotorula* sp. was found dominant six times, both in marine and freshwater fish. *Metschnikowia zobelii*, *Trichosporon cutaneum*, and *Candida tropicalis* were also dominant in some marine fish. Most studies in freshwater concerned rainbow trout, or other *Oncorhynchus* spp. *Debaryomyces hansenii* was found dominant in four of these references, but *Candida* sp., *Saccharomyces cerevisiae*, and *Leucosporidium* sp. were also dominant in some rainbow trout intestinal samples. *Cryptococcus* sp., *Pichia anomala*, and *Saccharomycodaceae* were mentioned as minor occasional components of fish microbiota. Few colonies of *Sporobolomyces* sp. were also isolated from the intestine of healthy European sea bass fry (Gatesoupe, unpublished). The effort to characterise yeasts in fish is still meagre, and new studies will likely expand the diversity of taxons reported in fish microbiota.

3. Pathogens

Black yeast-like *Exophiala* spp. are parasites of fish, but their incidence is not discussed here, since they are clearly distinct from the yeasts hosted by healthy fish (Alderman, 1982; Reuter et al., 2003). Few types of yeast have been reported as responsible for disease in fish (Table 3). *Metschnikowia bicuspidata* var. *bicuspidata* caused mortality in chinook salmon fry fed infected *Artemia franciscana*, while the fry fed a commercial dry diet were not affected (Moore and Strom, 2003). These authors succeeded to reproduce the disease by intraperitoneal injection of the pathogen. This is an example of the risk of emergence of new diseases due to rearing practices, under artificial conditions and confrontation of living organisms that are not normally encountered in the wild. There were three cases of infection by *Candida* spp., two on salmon, and one on gilthead sea bream. The genus has also been found in healthy fish, even dominant in the gut of rainbow trout (Sakata et al., 1993; Gatesoupe et al., 2005a; Table 1). In their report of candidiasis in gilthead sea bream, Galuppi et al. (2001) emphasized that the yeast and other fungi may be potentially harmful in an immuno-compromised host, or in adverse environmental conditions. *Sporidiobolaceae* like *Cryptococcus* spp., *Sporobolomyces salmonicolor* and *Trichosporon* sp. also seem to be opportunistic, as they occasionally cause disease in fish. Most yeasts are likely harmless to healthy fish reared in good conditions, but new candidate probiotics should be carefully tested, while watching for any sign of casual mycosis. Even *Saccharomyces cerevisiae* var. *boulardii* may cause fungemia in diseased organisms, though the yeast is generally recognized as safe (Lherm et al., 2002; Cassone et al., 2003; Riquelme et al., 2003; Herbrecht and Nivoix, 2005). However, the risk of disease caused by candidate probiotics appears to be much more limited with yeast than with bacteria.

4. Experimental colonisation of the intestine

Andlid et al. (1995) demonstrated the ability of yeasts to colonise the intestine of rainbow trout and turbot. This was particularly evident after single administration either in the rearing

water, or by force feeding (Table 4). More than 10 days after one introduction of *Rhodotorula glutinis* or *D. hansenii* HF1, the amount of yeast was much higher in the intestine than in the surrounding water. Most cells were counted in the transient content, but some adhered to the intestinal mucosa. The counts were particularly high in the feces, which likely contributed to establishing the yeast in the rearing system. The affinity of yeast for fish intestine was further studied by the same authors, who showed that autochthonous *S. cerevisiae* CBS 7764 and *D. hansenii* HF1 could adhere and grow on the intestinal mucus of rainbow trout as the sole food source (Vázquez-Juárez et al., 1997; Andlid et al., 1998). Vázquez-Juárez et al. (1993) showed that three strains isolated from flatfish adhered to several substrates (*Rhodotorula rubra*, *R. glutinis*, and *Candida zeylanoides*). Cell surface hydrophobicity was suspected to play a role in adhesion and colonisation (Vázquez-Juárez et al., 1994), as well as cell surface glycoproteins (Vázquez-Juárez, 1996) and heat shock proteins (Andlid et al., 1999). Allochthonous strains of *S. cerevisiae* were also retrieved associated with intestinal mucus in rainbow trout after overnight starvation, when the dietary yeast was daily supplied. The colonisation was maximum during the first month of feeding (Aubin et al., 2005a; Gatesoupe et al., 2005a; Waché et al., 2006). After five months of feeding, the yeast was still counted in high numbers in the intestinal content, but few were detected after overnight starvation (Gatesoupe et al., 2005b).

Allochthonous yeasts were tested on marine fish larvae, either *S. cerevisiae* var. *boulardii* CNCM I-1079 on pollack (Gatesoupe, 2002), or *S. cerevisiae* X2180 and *D. hansenii* HF1, isolated from rainbow trout, on European sea bass (Tovar-Ramírez et al., 2002, 2004). The counts of yeast retrieved in the larvae were ca. 10^4-10^6 CFU g^{-1}, on a body weight basis. These amounts were considerable in comparison with the results expressed on an intestinal weight basis for rainbow trout, but it was not possible to know whether the yeast cells were only transient in the gut, or whether the mucosa was actually colonised. In European sea bass larvae, microscopic observations of washed intestinal sections revealed the adherence of *S. cerevisiae* X2180 and *D. hansenii* HF1 in vitro (Tovar-Ramírez et al., 2002). The dietary yeast *S. cerevisiae* var. *boulardii* CNCM I-1079 was not detected in the intestine of European sea bass fingerlings, and the level of colonisation after 9 months was low (Gatesoupe, unpublished).

5. Microbial competition

After settlement in fish intestine, the yeasts have to compete with other microorganisms. Vázquez-Juárez et al. (1993) investigated putative factors for such competition in *R. rubra*, *R. glutinis*, and *C. zeylanoides*. The yeasts produced extracellular proteases and siderophores, and they bound lactoferrin. Iron availability is a key issue for fish microbiota (Gatesoupe et al., 1997), and such features may play a role for antagonism to some pathogens, the virulence of which is iron-dependent (Calvente et al., 1999). Yeasts are also known to produce killer toxins that can be used to control pathogenic fungi (e.g. Llorente et al., 1997; Marquina et al., 2001; Schmitt and Breinig, 2002).

Yeasts have been used as biocontrol agents for plant fungal diseases (e.g. Filonow et al., 1996; Petersson et al., 1999; Payne and Bruce 2001; Santos et al., 2004), and it would be worth investigating their potential against the numerous fungal diseases affecting fish (Alderman, 1982). Ducluzeau and Bensaada (1982) showed the antagonism of *S. cerevisiae* var. *boulardii* to *Candida* spp. settlement in murine intestine. Such observations in fish microbiota are still scarce, but there are two examples of competition among yeasts. The concomitant supplementation of *D. hansenii* HF1 and *S. cerevisiae* CBS 7764 in the diet of rainbow trout resulted in fecal colonisation by *D. hansenii* HF1, which corresponded to 95%

of the isolates, though both strains were autochthonous (Andlid et al., 1995). Natural colonisation by *D. hansenii* was maximum in rainbow trout fry at day 20 post start feeding (Waché et al., 2006). In case of artificial colonisation by dietary allochthonous *S. cerevisiae*, which was maximum at day 10, the later colonisation by *D. hansenii* was limited by a hundredfold, likely due to competition.
Yeast may be antagonistic to entero-pathogenic bacteria, e.g. due to adhesion of bacterial cells (Gedek, 1999) or by secreting proteases that inhibit toxins (Castagliuolo et al., 1999). Andlid et al. (1995) observed reduced numbers of bacteria when yeast colonisation peaked in rainbow trout intestine, though the effect of yeasts on bacteria associated in fish intestine is not clear. The proportions of the dominant strains was not the same with or without dietary addition of *S. cerevisiae* var. *boulardii* CNCM I-1079, but it was not possible to draw any general trend, due to high variability in time, and between locations (Aubin et al., 2005a; Gatesoupe et al., 2005a,b; Waché et al, 2006).

6. Modulation of the immune response

Yeast glucans have been extensively used as immunostimulants in fish (Sakai, 1999). Ortuño et al. (2002) suggested that lyophilised whole cells of a commercial strain of *S.* cerevisiae BMA64-1A could produce a more general immune response, due to other cell wall compounds besides ß-glucans, like mannoproteins, and chitin as a minor component. RNA extracts and partially autolysed brewers yeast may be also efficient (Sakai et al., 2001; Li and Gatlin, 2004). Siwicki et al. (1994) tested several immunostimulants on rainbow trout: lyophilised *Candida utilis*, lyophilised *S. cerevisiae*, ß-glucans (MacroGard®, ß-1,3/1,6-linked polymers of D-glucose, extracted and purified from baker's yeast, *S. cerevisiae*), deacylated chitin (Chitosan®, ß-1,4-linked polymer of D-glucosamine), a premix of selenium and vitamins C and E, and a premix of betaine and amino acids. The additives increased cellular immune response and immunoglobulin serum titres, and the most significant stimulations were generally observed with the two yeasts. After challenge with *Aeromonas hydrophila*, the best protection was obtained in rainbow trout fed either *S. cerevisiae*, ß-glucans, or deacylated chitin, while *C. utilis* seemed less efficient. Quentel et al. (2005) did not observe a humoral response in rainbow trout fed dried active *S. cerevisiae* var. *boulardii* CNCM I-1079, but the treatment protected the trout against *Yersinia ruckeri*, while decreasing the number of asymptomatic carriers after challenge (Quentel et al., 2004). The response of channel catfish seemed different, when Duncan and Klesius (1996) compared the effects of ß-D-glucan from barley and dried baker's yeast, *S. cerevisiae*. Cellular immunity was increased by both feed additives, but to a lesser extent with the yeast. In particular, glucan from barley, but not the yeast, stimulated the chemotactic response to *Edwarsiella ictaluri* exoantigen by macrophages and neutophils, and the chemiluminescent response to *E. ictaluri* by peritoneal exudate phagocytes . However, the fish were not protected against edwardsiellosis in a challenge experiment, even when they were fed glucan. In gilthead sea bream, cellular immunity was stimulated by dietary lyophilised *S. cerevisiae* in a dose-dependent manner, but the complement was not (Ortuño et al., 2002). Serum immunoglobulin M was also stimulated (Cuesta et al., 2004). The response of gilthead sea bream was modulated differently by a mutant strain *S. cerevisiae* fks-1, the cell wall of which was modified due to the inhibition of ß-1,3-D-glucan synthase, and thus enriched in mannoproteins and chitin (Rodríguez et al., 2003). The lysozyme activity was significantly increased after 2-4 weeks of feeding the modified yeast, while serum peroxidase and complement activity were depressed after 6 weeks. The effect of dietary yeast on cellular immunity was confirmed, but with some differences depending on whether the yeast was modified or not. Esteban et al. (2004)

demonstrated that a glucan receptor was involved in phagocytosis by gilthead sea bream leucocytes, unlike the mannose receptor, which was required with the glucan receptor for maximal phagocytosis by murine macrophages (Giaimis et al., 1993). All these data seemed to confirm that ß-glucans may be the most important components to account for immunostimulation of fish by yeast. The specific interest of feeding whole yeast rather than cell wall extract seemed limited for immunostimulation, but that should be evaluated with further challenge experiments, as well as with field studies in fish farms. To my knowledge, the effect of yeast viability on fish immunity has not been tested, whereas it was demonstrated that immune responses of rainbow trout were different when probiotic bacteria were supplied either active or inactivated (Irianto and Austin, 2003; Brunt and Austin, 2005; Panigrahi et al., 2005). Taoka et al. (2006a) confirmed recently that the viability of probiotics affected the immune response of Nile tilapia fed a commercial preparation including *S. cerevisiae*, *Bacillus subtilis*, *Lactobacillus acidophilus*, and *Clostridium butyricum* (Alchem Poseidon®; Alchem-Korea Co. Ltd., Wonju, Korea), but the specific importance of yeast viability was not considered.

7. The effects of live yeasts on fish metabolism

A commercial preparation of live *S. cerevisiae* and *Lactobacilllus coagulans* (Bioboost Forte®, Lyka Labs. Ltd., Bombay) was used as a growth promoter for Indian carp fry. Mohanty et al. (1993) introduced the preparation in an experimental diet, but it was not possible to conclude any effect of the probiotic on *Labeo rohita*, due to the lack of a suitable control diet. The effect of growth promotion was shown in later experiments on *Catla catla* and *Cirrhinus mrigala* (Mohanty et al., 1996; Swain et al., 1996), though it was impossible to demarcate the respective efficiencies of the yeast and *L. coagulans*. *S. cerevisiae* was also tested on *Paralichthys olivaceus*, with Alchem Poseidon® (Taoka et al., 2006b). Again, it was not possible to discriminate the contribution of the yeast among the effects observed on water quality, growth, survival, immune response, or stress and disease resistance. However, the microorganisms might work synergistically in such consortia, and that may be worth further investigating. An example of synergetic effect appeared when pollack larvae were fed *Artemia* nauplii, which were treated first with live *S. cerevisiae* var. *boulardii* CNCM I-1079, then with *Pediococcus acidilactici* MA185 M (Gatesoupe, 2002). The pollack larvae grew better than those fed nauplii treated with one or no probiotic. All combinations are not necessarily efficient, and Quentel et al. (2004) noted that the double supplementation of a diet with *S. cerevisiae* var. *boulardii* CNCM I-1079 and *P. acidilactici* MA185 M lowered the protective effect on rainbow trout challenged with *Y. ruckeri*, in comparison with single treatments.

When *S. cerevisiae* was tested alone, growth and feed efficiency were improved in Israeli carp (*S. cerevisiae* cultured on grain media, Noh et al., 1994) and Nile tilapia (Biosaf®, SafAgri, Minnapolis, MN, Lara-Flores et al., 2003). In tilapia fed a control diet, survival and digestibility were reduced by increasing the population density, while this stress did not affect the groups treated with the yeast (Lara-Flores et al., 2003). Such effects were not observed in rainbow trout, but some increase in lipid accretion and red pigmentation of the flesh was noted by Aubin et al. (*S. cerevisiae* var. *boulardii* CNCM I-1079, 2005b).

Tovar-Ramírez et al. (2002) compared the effects of two yeasts on European sea bass larvae fed compound diets. *D. hansenii* HF1 improved survival, and vertebral conformation of the larvae, possibly due to the observed acceleration of the maturation of the digestive system. These effects were not observed with *S. cerevisiae* X2180, and the larvae grew better when they were fed a control diet, likely due to an inadequate way of introduction of the yeasts after

feed processing, which deteriorated the physical properties of the pellets. Tovar-Ramírez et al. (2004) remedied this defect by introducing *D. hansenii* HF1 in the compound diet before pelletization. The new probiotic diet improved the growth of larval European sea bass, in addition to intestinal maturation, survival, and conformation of the larvae. These improvements were obtained with a dose of ca. 10^6 CFU g^{-1} of diet, whereas a higher dose of 6×10^6 CFU g^{-1} was less efficient. Waché et al. (2006) observed that the maturation of the digestive system took place before day 20 post start feeding in rainbow trout fry, and the natural colonisation by *D. hansenii* was too late to accelerate the onset. In such conditions, the dietary supplementation of *S. cerevisiae* var. *boulardii* CNCM I-1079 since start feeding stimulated at day 10 the activity of three enzymes in the brush border membrane of the enterocytes (alkaline phosphatase, γ-glutamyl-transpeptidase, and leucine-amino-peptidase N), but without any effect on growth. Another strain of *S. cerevisiae* NCYC Sc 47/g did not stimulate this early maturation. The effect of *S. cerevisiae* var. *boulardii* (Laboratoires Biocodex, Montrouge, France) on the digestive maturation in weanling rats was likely mediated by the endoluminal release of spermine and spermidine (Buts et al., 1994). Dietary spermine also induced intestinal maturation in European sea bass larvae (Péres et al., 1997). Consequently, polyamine secretion was a possible mediator for the effect of the yeast on rainbow trout fry (Waché et al., 2006). *D. hansenii* HF1 produced much more spermidine than *S. cerevisiae* X2180, and that might also account for the effects observed in European sea bass (Tovar-Ramírez et al., 2002).
More generally, the polyamines play a fundamental role in proliferating, fast growing and regenerating tissues, while their endogenous synthesis appears somewhat insufficient in healthy animals (Peulen et al., 2002). It is therefore possible that polyamine production by yeasts may explain at least partly the effect observed on fish growth and metabolism. That will need further research.

8. Perspectives

In summary, fish intestine appear to be an occasional niche for yeast colonisation. The risk of harmful invasion seems low, as long as fish are reared in good conditions. Yeast may be generally considered as commensal in fish gut, and possible benefits can be expected for the immune and the digestive systems of the host, though many questions remain open to optimise the effect on fish health.
The first strategic choice is either to favour the development of autochthonous yeast, or to introduce probiotic strains. Natural settlement appears somewhat unpredictable, even in rainbow trout, the most documented species for frequent occurrence of yeast in the intestine. Further investigation is required to clarify the conditions for such colonisation. Some prebiotics might be tested with a view to stimulate growth and activity of yeasts, either native, or deliberately introduced (Selvakumar and Pandey, 1999; Mitterdorfer et al., 2001; Vranesic et al., 2002).
When yeasts are introduced with feed, an actual intestinal colonisation may not be necessary in every case. For instance, Durand-Chaucheyras et al. (1998) noted that *S. cerevisiae* did not colonise lamb rumen, where the probiotic yeast stimulated the activity of some autochthonous microbes. Such microbial interactions have not been evidenced in fish, where yeasts rather act directly on the host. The probiotic dietary yeast was efficient in rainbow trout at five months, in spite of poor association with the intestine (Aubin et al., 2005a; Gatesoupe et al., 2005b). Even if the intestinal transit is relatively short in fish, it is sufficient for digestion, and likely for the release of active compounds from yeast cells.

The question remains whether viability is a prerequisite for the beneficial effects on fish. Autoclaved yeast lost the effect on nutrient utilization by turkey (Bradley and Savage, 1995), but microbial metabolism was not affected in simulated ruminal fermentation (Oeztuerk et al., 2005). Yeast viability is not likely to affect immunomodulation, as long as it seems mediated by cell-wall components. The answer is less obvious if other compounds are involved, like polyamines (ter Steege et al., 1999), enzymes (García-González and Ochoa, 1999) and other soluble factors (Freitas et al., 2005). The issue is crucial towards better understanding of the modes of action, especially those targeting fish metabolism.

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Table 1
Natural occurrence of yeasts in the gastrointestinal tract of healthy fish

Fish	Environment[§]	log(CFU/g)	% microbial isolates	Yeast species*	Reference
Synaphobranchus kaupi	Deep sea	5-7	50-90	-	Ohwada et al. (1980)
Pleuronectes platessa, *Platichthys flesus*	Coastal	2	-	***Rhodotorula* sp.**	Andlid et al. (1995)
Atherinopis affinis littoralis	Coastal	0-2	-	***M.z.***, *K.a.*	Van Uden and Castello-Branco (1963)
Beryx splenedens	Coastal	5	-	-	Sugita et al. (1989)
Girella punctata	Coastal	2	-	-	Sugita et al. (1988)
Pomatomus saltatrix	Coastal	-	3	***Rhodotorula* sp.**	Newman et al. (1972)
Tachurus symmetricus	Coastal	1-5	-	***M.z.***, *D.*	Van Uden and Castello-Branco (1963)
Pagrus major, Evynnis japonica	Sea farm	2-3	3-19	-	Sera and Kimata (1972)
Scophthalmus maximus	Sea farm	-	4	-	Toranzo et al. (1993)
Scophthalmus maximus	Sea farm	-	-	*Ca. zeylanoides*	Vázquez-Juárez et al. (1993)
Other species	Tropical island	-	-	***C.t.***, ***R.***, *Ca.*	Roth et al. (1962)
Other species	Estuar., coastal	-	-	***T.c.***, *Ca.*, *R.*, *H.a.*, *H.v.*, *D.*	Roth et al. (1962)
Marine fish	Marine	-	-	***R.***, *Ca.*, *Cr.*, *D.*, *T.*	Bruce and Morris (1973)
Pseudorasbora parva	River	6	-	-	Sugita et al. (1983)
Oncorhynchus keta	F.w. farm	-	1	-	Trust (1975)
Oncorhynchus keta	F.w. farm	5	34	-	Yoshimizu et al. (1976b)
Oncorhynchus masou	F.w. farm	4-5	84-100	-	Yoshimizu et al. (1976b)
Oncohrynchus masou	F.w. farm	2	20	-	Yoshimizu et al. (1980)
Oncorhynchus mykiss	F.w. farm	5-7	3-18	***Ca.***	Sakata et al. (1993)
Oncorhynchus mykiss	F.w. farm	2-4	-	***S.c.***, ***D.h.***, ***R.***, *Cr.*, *L.*	Andlid et al. (1995)
Oncorhynchus mykiss	F.w. farm	0-5	-	***D.h.***, ***R.***, *T.*	Aubin et al. (2005a)
Oncorhynchus mykiss	F.w. farm	0-2	-	***S.c.***	Gatesoupe et al. (2005b)
Oncorhynchus mykiss	F.w. farm	0-4	-	***Ca.***, ***L.***, ***D.h.***	Gatesoupe et al. (2005a)
Oncorhynchus mykiss	F.w. farm	0-4	-	***D.h.***	Waché et al. (2006)
Oncorhynchus tschawytscha	F.w. farm	3	17	-	Yoshimizu et al. (1976a)
Oncorhynchus tschawytscha	F.w. farm	0-4	0-80	-	Moffitt and Mobin (2006)
Salvelinus alpinus	F.w. farm	3	-	-	Ringø and Olsen (1999)

[§] Environment: estuar. Estuarine; f.w. fresh water

* Yeast species (dominant in bold): *Ca. Candida* sp.; *C.t. Candida tropicalis*; *Cr. Cryptococcus* sp.; *D. Debaryomyces* sp.; *D.h. Debaryomyces hansenii*; *M.z. Metschnikowia zobelii*; *H.a. Hansenula* (*Pichia*) *anomala*; *H.v. Hanseniaspora valbyensis*; *K.a. Kloeckera apiculata*; *L. Leucosporidium* sp.; *S.c. Saccharomyces cerevisiae*; *R. Rhodotorula* sp.; *T. Trichosporon* sp.; *T.c. Trichosporon cutaneum.*

Table 2
Tentative taxonomy of yeast genera reported to occur in fish microbiota

Phylum	Class	Order	Family	Genus	First report of occurence
Ascomycota	*Saccharomycetes*	*Saccharomycetales*	*Dipodascaceae*	*Yarrowia*	Gatesoupe (unpublished)
			Metschnikowiaceae	*Metschnikowia*	Van Uden and Castello-Branco (1963)
			Saccharomycetaceae	*Candida*	Ross and Morris (1965)
				Debaryomyces	Ross and Morris (1965)
				Kluyveromyces	Gatesoupe (unpublished)
				Pichia[§]	Roth et al. (1962)
				Saccharomyces	Andlid et al. (1995)
			Saccharomycodaceae	*Hanseniaspora*	Roth et al. (1962)
				*Kloeckera**	Van Uden and Castello-Branco (1963)
	Chaetothyriomycetes	*Chaetothyriales*	*Herpotrichiellaceae*	*Exophiala*	Carmichael (1966)
Basidiomycota	*Basidiomycetes*	*Filobasidiales*	*Filobasidiaceae*	*Filobasidium*	Gatesoupe (unpublished)
		Tremellales	*Tremellaceae*	*Bulleromyces*	Gatesoupe (unpublished)
	Urediniomycetes	*Leucosporidiales*		*Leucosporidium*	Andlid et al. (1995)
		Sporidiales	*Sporidiobolaceae*	*Cryptococcus*	Bruce and Morris (1973)
				Sporobolomyces	Muench et al. (1996)
				Rhodotorula	Bruce and Morris (1973)
				Trichosporon	Bruce and Morris (1973)

[§] formerly *Hansenula*; * anamorph of *Hanseniaspora*

Table 3

Fish diseases caused by yeast infection

Pathogen	Fish	Symptoms	Reference
Candida sake	*Oncorhynchus rhodurus*	Stomach distended with fluid	Hatai and Egusa (1975)
Candida sp.	*Oncorhynchus tshawytscha*	Internal lesions	Mueller and Whisler (1994)
Candida sp.	*Sparus aurata*	Swimbladder swollen with dense material	Galuppi et al. (2001)
Metschnikowia bicuspidata	*Oncorhynchus tshawytscha*	Mortality, systemic infection, necrosis	Moore and Strom (2003)
Cryptococcus spp.	*Tinca tinca*	Exophthalmos	Pierotti (1971)
Cryptococcus sp.	*Oncorhynchus tshawytscha*	Surface and internal lesions (swimbladder, kidney)	Mueller and Whisler (1994)
Sporobolomyces salmonicolor	*Oncorhynchus tshawytscha*	Ascites, visceral mycosis	Muench et al. (1996)
Trichosporon sp.	*Oncorhynchus tshawytscha*	Internal lesions (swimbladder, kidney)	Mueller and Whisler (1994)

Table 4
Experimental colonisation of fish intestine by yeast

Fish	Introduction	Yeast	Colonisation peak		Reference
			log (CFU/g)*	(days post start)	
Rainbow trout	Single, in water	*Rhodotorula glutinis*	3 (i.c.)	17	Andlid et al. (1995)
Rainbow trout	Single, force feeding	*Debaryomyces hansenii*	6 (feces)	13	Andlid et al. (1995)
Turbot	Single, force feeding	*Debaryomyces hansenii*	3 (s.c., i.c.)	11	Andlid et al. (1995)
Rainbow trout	Feeding every 3 days	*Debaryomyces hansenii*	9 (feces)	53	Andlid et al. (1995)
Rainbow trout	Daily feeding	*Saccharomyces cerevisiae*	4 (i.s.)	10	Waché et al. (2006)
Rainbow trout	Daily feeding	*Saccharomyces cerevisiae*	4 (i.s.)	20	Aubin et al. (2005a)
Rainbow trout	Daily feeding	*Saccharomyces cerevisiae*	3 (i.s.)	31	Gatesoupe et al. (2005a)
Rainbow trout	Daily feeding	*Saccharomyces cerevisiae*	0-1 (i.s.)	152	Gatesoupe et al. (2005b)
Rainbow trout	Daily feeding	*Saccharomyces cerevisiae*	5 (i.c.)	152	Gatesoupe et al. (2005b)
Pollack	Continuous feeding	*Saccharomyces cerevisiae*	5-6 (b.w.)	16	Gatesoupe (2002)
Sea bass	Continuous feeding	*Debaryomyces hansenii*	4 (b.w.)	31	Tovar-Ramírez et al. (2002)
Sea bass	Continuous feeding	*Saccharomyces cerevisiae*	4 (b.w.)	31	Tovar-Ramírez et al. (2002)
Sea bass	Continuous feeding	*Debaryomyces hansenii*	5-6 (b.w.)	20	Tovar-Ramírez et al. (2004)
Sea bass	Daily feeding	*Saccharomyces cerevisiae*	3 (i.c., i.s.)	278	Gatesoupe (unpublished)

* i.c. intestinal content; s.c. stomacal content; i.s. emptied intestinal section ; b.w. body weight

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Effect of dietary yeast on some productive and physiological aspects of growing Japanese quails

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Abstract: Two hundred and twinty five, one-day, unsexed growing Japanese quail were performed to evaluate the effect of dietary Yeast culture (*Saccharomyces cerevisiae*) supplementation on growth performance, carcass characteristics, some physiological responses, and economical efficiency of growing Japanese quails. The chicks were distributed into 3 groups (control and 2 other groups). Each group included three replicates of 25 birds each. The 3 treatment groups were as follows: Birds in group 1 (T1) fed the basal diet (control), birds in groups 2 (T2) fed basal diet with 1% yeast culture spplementation and birds in group 3 (T3) fed the basal diet with 2% yeast supplemention. The experiment was terminated when birds were 6 weeks old. Body weight, weight gain and feed intake were recorded. Feed conversion (g, feed /g, gain) was calculated. At the end of the experiment carcass characteristics were measured, blood samples were taken to determine some blood plasma constituents. The economic efficiency values of dietary treatments were calculated.

The data revealed that, birds fed diets contain yeast culture at levels of 1 or 2% recorded slightly (P>0.05) improvement in body weight and body gain compared with control diet. While, feed intake was diminished (P<0.05) with adding yeast to the control diet. Feed conversion was enhanced (P<0.05) with increasing yeast in the control diet. The dressing percentage and the proportions of the carcass, edible giblets and offals had no effect (P>0.05) when birds fed dietary yeast. Blood plasma showed an improvement (P<0.05) in total protein, albumin, globulin, GOT and GPT when birds fed dietary yeast. The inclusion of yeast culture in Japanese quail diets recorded the higher economical efficiency (expressed as % net revenue/feed cost) compared with control diet..

Keywords: Yeast culture, Japanese quail, performance, physiological responses)

Introduction

Chickens are commonly stressed by various factors affecting growth and feed utilization such as over-crowding, vaccination, chilling, and/or overheating. Many attempts have been undertaken in order to improve the utilization of diet nutrients by adding dietary supplementation of several growth feed additives from different sources (Boulos *et al*., 1992, El-Gendi *et al*., 1994, Ibrahim *et al*., 1998 and Abdel-Azeem, 2002).

Probiotics, such as yeast, have the ability to stimulate digestion and aid in maintaining microbial equilibrium in the gut. Live yeast, such as *Saccharomyces cerevisiae*, contains numerous enzymes that could be released into the intestine and aid existing enzymes in the digestive tract in the digestion of feed. Also, yeast contains vitamins and other nutrients that may produce beneficial production responses (Kornegay et al., 1995). Moreover, yeast supplementation can inhibit pathogenic bacteria and increase the number of anaerobic and cellulytic bacteria as reported by Abdel Azeem (2002) and Soliman *et al.,* (2003). In addition, Celik *et al.,* (2001), Churchil *et al.,* (2001) and Celik *et al.,* (2003) showed that yeast additives reduce the toxic effects of Alfatoxin. While, Spring (2002) and Santin *et al.,* (2003) revealed that yeast can improve immune response of birds.

Performance of birds fed diets containing active dried yeast was improved *(*Abd El-wahed *et al., 2003 and* Soliman *et al., 2003).* Subrat*a et al., (1997)* studied the effect of feeding yeast on the performance of broilers. They reported that carcass parameters did not differ between treatments. Also Abdel-Azeem *(2002)* indicated that carcass traits and internal organs were not affected due to addition of yeast culture at lg/Kg broiler diet.

On the other hand, Omar (2006) revealed that addition of 2% yeast + 0.8% DL-methionine to local strain hens diet increased egg production, egg weight and egg mass, while reduced feed consumption and improved feed conversion (Kg feed/Kg egg mass).

The present study was undertaken to determine the performance, some physiological responses and economical efficiency of growing Japanese quail as affected by dietary yeast culture supplementation (0, 1 and 2 kg/ton) .

Material and Methods

Two hundred and twenty five, one-day old, unsexed, Japanese quails were maintained in electrically heated battery cages housed in light and temperature controlled room. Free access water and feed were available during all time. The birds were divided into 3 groups (75 bird each) according to levels of yeast culture (0, 1 and 3%) . Each group contained 3 replicates of 25 birds.

Diets:-

The basal diet contained adequate levels of nutrients for growing Japanese quail as recommended by the National Research Council, (NRC, 1994) with no yeast culture additions representing the control. Two additional diets were obtained by incorporating two levels (1 and 2%) of yeast culture. The sequence of the 3 dietary treatments was as follow:- Control (without additives), 1.0% yeast

culture and 2.0% yeast culture. The composition of the basal diet is shown in Table 1.

Table 1: The chemical and proximate analyses of the control diet

Ingredients	%
Ground corn, yellow	52.20
Broiler concentrate (52% CP) *	10.00
Soybean meal (44% CP)	35.00
Poultry fat	2.00
Vitamins minerals mixture **	0.50
DL-Methionine	0.30
Total	100.00
Calculated analysis:	
Metabolizable energy K cal/kg	3011
Crude protein, %	24.05
Calcium, %	1.15
Available phosphorus, %	0.51
Methionine and cystine, %	0.80
Lysine, %	1.03

*Each2.5 kg of vitamins and minerals mixture contain: 12000.000 IU vitamin A acetate; 2000.000 IU vitamin D_3; 10.000 mg vitamin E acetate; 2000 mg vitamin K_3; 100 mg vitamin B_1; 4000 mg vitamin B_2; 1500 mg vitamin B_6; 10 mg vitamin B_{12}; 10.000 mg pantothenic acid; 20.000 mg Nicotinic acid; 1000 mg Folic acid; 50 mg Biotin; 500.000 mg chorine; 10.000 mg Copper; 1000 mg Iodine; 300.00 mg Iron; 55.000 mg Manganese; 55.000 mg Zinc, and 100 mg Selenium.

Measurements and determinations:

Body weight and feed intake were recorded for birds biweekly and feed conversion values (g, feed /g, gain) were calculated. At 6 weeks of age, blood samples from randomly five birds of each treatment, were collected from the wing vein in heparinized tubes and centrifuged at 3000rpm /15minutes. The plasma was obtained and immediately stored at -20° C till analysis. Total protein, albumin, total lipids, and GOT and GPT were determined according to methods of Weischelbaum (1946), Doumas (1971), Frings et al., (1972), and (Reitman and Frankel. 1957), respectively. The total globulin values were calculated by subtracting the values of total albumin from those of total protein for each sample.

At the end of the experiment (6 weeks of age), three birds from each replicate were scarified after 12 hours fasting. After bleeding out, the birds were scalded, plucked with electrical cyclomatic picker and eviscerated. Eviscerated carcasses were individually weighed. The percentages of dressing, edible giblets (liver, gizzard, heart, and abdominal fat) and offals (blood, head, legs, and feathers) were calculated in relation to the live body weight.

Cost of one-kilogram feed and cost of feed/ kg gain were calculated based on the prices of feed ingredients and yeast culture prevailing 2007.

ANOVA and LSD procedures were performed on data obtained herein as outlined be Snedecor and Cochran, (1980).

Results and Discussion

Productive performance:

The effects of dietary yeast culture on body weight, body weight gain, feed intake and feed conversion (feed/gain) are shown in Table (2). It was observed that adding yeast at levels of 1 or 2% to the basal diet improved (P>0.05) body weight and body gain. The positive response on body weight and body gain as a result of adding yeast culture may be due to that mannan oligosaccharides (**MOS**) from yeast cell walls have been researched with respect to their value in immune modulation (Newman and Newman, 2001; O'Quinn et al., 2001) and in reduction of intestinal pathogen colonization (Newman, 1994). Some research studies suggest that MOS may improve growth performance in young pigs (Davis et al., 1999; Pettigrew, 2000). Furthermore, yeast can inhibit pathogenic bacteria as reported by Line *et al.,* (1998) and Soliman *et al.,* (2003). In addition, Abdel-Azeem (2002) found that the number of anaerobic and cellulytic bacteria were increased when the experimental diet was supplemented with yeast which enhanced lactate utilization and moderates pH of the media, Therefore, yeast improves the nutrients digestibility and growth performance. Furthermore, Vogt and Matthes (1991) reported that supplemented yeast increased weight gain of broilers.

In the present study, quails fed basal diet presented the greatest (P<0.05) feed intake compared with others fed dietary yeast culture (Table 2). As a result of the decline in feed intake and the improvement in body gain for birds fed dietary yaest culture, the feed conversion (feed, g / gain, g) efficiency was enhanced (P<0.05) by adding yeast at all levels. The best (P<0.05) feed conversion value was noticed when birds fed dietary 2% yeast. This positive enhancement in feed conversion efficiency confirmed that stated by Zeweil (1997), who reported that feed conversion ratios were improved (P<0.05) by increasing level of Yea-Sacc in growing Japanese quail diets, from 1 to 1.5%, by about 2.1 to 6.28% respectively, as compared to the control diet.

Slaughter data

The absolute and proportional weights of carcass, edible giblets, and offals of quaiks fed yeast culture are presented in Table 3. The data showed that no significant differences (P>0.05) were observed on carcass, giblets and offalls weights and/or dressing and giblets percentages as a result of adding yeast culture to growing Japaneses Quail diet. However, higher (P>0.05) values of the previous items were observed when quails fed dietary yeast culture. This result confirm the results of body weight and body gain (Table 2). Also, it gives an approve to the critical role of yeast culture in the metabolic functions as a strong stimulating action on the activity of certain important bacteria, which are actively involved with the digestive processes, protein synthesis and nutrient absorption in the gastro-intestinal tract (Stockland, 1993). These improvements attributed to yeast cultures could be due to decreased proliferation of pathogenic bacteria (Miles, 1993). Moreover, these results coincided with those obtained by Kumprechtova *et al.,* (2000), Naik *et al.,* (2000), Abdel-Azeem (2002) and El-Ghamry *et al.,* (2002), who indicated that slaughter yield was not significantly influenced by feeding diet treated with yeast culture.

In the present study, there was no significant (P>0.05) difference in the proportion of offals as affected by

adding yeast culture to the growing Japanese quail diet.

Table 2: Effect of dietary treatments on performance of growing Japanese quails

Item	Age / weeks	Treatments		
		control	1% yeast culture	2% yeast culture
Body weight (g, bird)	0	10.51[a] ± 0.05	10.63[a] ± 1.79	10.54[a] ± .06
	2	56.31[a] ± 2.59	50.62[a] ± 4.33	46.36[a] ± 2.59
	4	131.70[a] ± 5.57	132.7[a] ± 12.92	126.83[a] ± 5.56
	6	215.80[a] ± 1.76	219.50[a] ± 8.42	219.10[a] ± 1.76
Body gain (g, bird)	0 to 2	45.80[a] ± 2.52	39.99[b] ± 2.95	35.70[b] ± 2.52
	2 to 4	75.45[a] ± 2.52	82.12[a] ± 8.55	80.48[a] ± 3.19
	4 to 6	84.01[a] ± 3.19	86.75[a] ± 10.02	92.57[a] ± 6.06
	0 to 6	205.30[a] ± 6.069	208.8[a] ± 1.30	208.9[a] ± 1.76
Feed intake(g)	0 to 2	43.86[a] ± 3.59	46.02[a] ± 8.47	45.86[a] ± 1.67
	2 to 4	138.95[a] ± 13.88	134.58[a] ± 18.02	110.53[a] ± 13.38
	4 to 6	376.34[a] ± 27.37	341.7[a] ± 13.61	316.83[a] ± 27.73
	0 to 6	559.15[a] ± 34.18	522.54[b] ± 19.22	481.62[c] ± 43.18
Feed conversion (g, feed/g, gain)	0 to 2	1.001[a] ± 0.006	1.157[a] ± 0.007	1.282[a] ± 0.058
	2 to 4	1.924[c] ± .009	1.638[a] ± 0.002	1.470[a] ± 0.054
	4 to 6	4.479[a] ± 0.050	4.049[a] ± 0.036	3.489[a] ± 0.076
	0 to 6	2.723[a] ± 0.022	2.506[b] ± 0.004	2.308[c] ± 0. 009

[a-----g] Means ± standard error in the same row with different superscripts are significantly different (P≤ 0.05).

Table 3: Effect of dietary treatments on carcass characteristics of growing Japanese quails

Items	Treatments		
	control	1% yeast culture	2% yeast culture
Body weight, g	202.0[a] ± 16.03	206.5[a] ± 2.08	205.1[a] ± 15.431
Carcass weight, g	132.5[a] ± 7.916	136.8[a] ± 10.72	139.8[a] ± 12.26
Edible weigh, g	12.30[a] ± 0.091	13.60[a] ± 1.578	12.88[a] ± 0.09
Offal weight, g	68.32[a] ± 4.758	69.68[a] ± 6.07	65.57[a] ± 4.70
Dressing, %	65.59[a] ± 1.33	66.29[a] ± 2.03	68.06[a] ± 1.33
Edible giblets, %	6.08[a] ± 0.019	6.59[a] ± 0.06	6.29[a] ± 0.09
Offal , %	33.92[a] ± 4.105	33.71[a] ± 2.04	31.94[a] ± 3.33

[a-----g] Means ± standard error in the same row with different superscripts are significantly different (P≤ 0.05).

Physiological responses:

Changes in blood plasma total protein, albumin, globulin, total lipids, GOT and GPT as affected by dietary yeast culture are listed in Table 4.

Total protein, albumin and globulin:

Data showed that, adding 1% yeast culture to Japanese quail diet enhanced (P<0.05) plasma total protein and albumin compared with the control diet. Moreover, the highest (P<0.05) plasma albumin value was noticed when birds fed diet contain 1% yeast culture. The greatest improvement (P<0.05) in globulin was recorded when birds fed dietary yeast culture with a highest level (2%). These results contribute with the improving of performance and dressing percentage (Tables 3 and 5). This may explain the significant effects of dietary yeast in improving metabolic process.

White et al., (2002), reported that, increased IgG (*P* < 0.01) and IgA (not significant) levels occurred in pigs fed brewers yeast alone or in combinations with citric acid compared with pigs fed the basal diet. The trends resulting from the feeding of yeast in our study are in agreement with studies involving turkeys (Savage et al., 1996a) and sows fed manna oligosaccharide (MOS) products (Newman and Newman, 2001; O'Quinn et al., 2001). In addition, Santin *et al.,* (2003) and Soliman *et al.,* (2003) indicated that active yeast improves immune response of birds.

Total lipids GOT and GPT:

Data in Table 4 revealed that the lowest (P<0.05) values of total lipids and GOT were observed when birds fed diets contain 1% yeast culture However, no difference (P>0.05) in total lipids and GOT were detected by adding 2% yeast culture to quail diet compared to the control. Plasma GPT increased (P<0.05) by adding yeast culture to the control diet up to 2%. The positive effect on plasma total lipids , GOT and GPT my be due to that the number of anaerobic and cellulytic bacteria were increased when the experimental diet was supplemented with yeast which enhanced lactate utilization and moderates pH of the media, therefore, yeast improves the nutrients digestibility and growth performance (Abdel-Azeem, 2002). Moreover, Churchil *et al.,* (2001) and Celik *et al.,* (2003) revealed that using yeast culture (S. cerevisiae) in broiler diets reduced the toxic effects of Alfatoxin B_1. In addition, Santin *et al.,* (2003) and Soliman *et al.,* (2003) indicated also that active yeast improves immune response of birds.

Economical efficiency:

The economical efficiency of dietary treatments are recorded in Table 5. The profitability of using yeast supplementation depends upon the yeast price and the growth performance of birds fed these dietary additives. The feed cost of 1 kg weight gain was reduced to 5.363 and 5.077 L.E by adding 1 and 2% yeast to the control diet, respectively, compared with 5.665 for the control diet. As a result of improvement in feed conversion

efficiency of dietary yeast, these diets recorded the highest depression in feed cost of 1kg weight gain and the greatest percent of relative economic efficiency (percent of net revenue) compared to that of the control diet. These results agree with Abd El-Malak *et al*. (1995) reported that increasing Bio-Tonic level, from 500 or 750 to 1000 g/ton diet as a supplement, in broiler chicks diet improved the relative economical efficiency expressed as percent of feed cost/kg body gain. The values were 100.00, 98.73, 98.10 and 98.10% for the experimental diets (control, 500, 750 and 1000 gm, Biotonic/Ton diet), respectively.

Table 4: Effect of dietary treatments on some physiological responses of growing Japanese quails

Items	Treatments		
	Control	1% yeast culture	2% yeast culture
Total protein, g/100ml	3.45^{c} ± 0.03	4.503^{a} ± 0.08	3.617^{b} ± 0.02
Albumin, g/100ml	2.280^{b} ± 0.009	2.637^{a} ± 0.45	1.415^{c} ± 0.25
Globulin, g/100m	1.170^{b} ± 0.007	1.867^{b} ± 0.054	2.202^{a} ± 0.24
Total lipids, mg/dl	702.7^{a} ± 0.05	568.7^{b} ± 1.02	697.0^{a} ± 4.71
GOT, U/L	124.6^{a} ± 1.147	105.3^{b} ± 2.08	126.42^{a} ± 1.67
GPT, U/L	166.7^{c} ± 0.019	172.3^{ab} ± .54	177.0^{a} ± 0.16

$^{a-----g}$ Means ± standard error in the same row with different superscripts are significantly different ($P\leq 0.05$).

Table 5: Effect of dietary treatments on the economical efficiency of the experimental diet (L.E* in 2007) of growing Japanese quails

Item	Treatments		
	Control	1% yeast culture	2% yeast culture
Price of 1 kg of yeast / L.E.	6.00	6.00	6.00
Price of 1 kg of diets / L.E. (a)	2.08	2.14	2.20
Feed / gain ratio (b)	2.723	2.506	2.308
Feed cost of kg weight gain (a, b)	5.665	5.363	5.077
Market price of 1 kg live weight (c)	12.00	12.00	12.00
Net revenue [c-(a.b)], L.E.	6.445	6.637	6.923
Relative economical efficiency, %	100	102.97	107.41

* L.E. = Egyptian pound

Generally, it could be concluded that, adding yeast culture to growing Japanese quails up to 2 % improved the growth performance, metabolic responses and economic efficiency.

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Evaluation of yeast spraying as a tool for reducing fungus diseases in grapevines

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INTRODUCTION

Within the general frame of the European Community "ORWINE" STREP Project (SSSPE-CT-2006-22769), we studied the possibility to reduce fungal diseases on grapevines by spraying *Saccharomyces* yeasts on damaged grape berries before harvest in order to create a competition among microorganisms at their surface. The effectiveness of yeast spraying by different commercial *Saccharomyces cerevisiae* strains was first evaluated on two different model species of fungus diseases: *Botrytis cinerea* (invasive disease fungus) and *Aspergillus carbonarius* (undesirable fungus responsible for ochratoxin A (OTA) production). A general inhibition effect was observed with all tested yeast strains, but some of them seemed more efficient. Yeast spraying was found to be very efficient for reducing *A. carbonarius* development *in vitro*. A field experiment conducted during 2007 and 2008 vintages in a vineyard artificially contaminated by *A. carbonarius* spores reinforce this last observation.

MATERIALS AND METHODS

Microbiological strains. 17 industrial wine yeast *Saccharomyces cerevisiae* strains (Lallemand, Montreal, Canada) were tested in this study. They were named A to Q in the present study for strategic and economical reasons. All of them are available commercially as dry yeasts. Two strains of Botrytis cinerea M04/51 and M04/63, respectively isolated on Gamay and Pinot Noir vine varieties by IFV-France (Beaune, France), were used. The strain of *Aspergillus carbonarius* was isolated by IFV-France (Nîmes, France) in the vineyard of INRA Pech-Rouge (Gruissan, France) in 2004.

Culture media and growth conditions. All media were heat-sterilized (110°C, 20 min). The yeast strains were grown in a standard nutrient medium, YPD [10 g L^{-1} yeast extract (Difco, Detroit, MI), 20 g L^{-1} Bacto-peptone (Difco), and 50 g L^{-1} glucose]. The fungi strains were grown in the standard nutrient medium, PDA [200 g L^{-1} potatoes extract (Difco) and 20 g L^{-1} glucose]. Fungi spores were obtained by growing fungi strains on PDA solid medium for 7 days, and gentle rinsing the surface of the Petri dishes with saline buffer containing 0.2 % Tween 80 as a surfactant. Spores were centrifuged (3000 × g for 2 min at 4°C) and resuspended in sterile saline buffer before numbering.

Cell and spore numbering. Yeast or fungi spores suspensions were sonicated (30 s, 10 W), and number and volume were determined using an electronic particle counter (model ZB2, Beckman-Coulter, Margency, France) fitted with a 100 µm aperture probe.

Determination of grape mycoflora. Each sample was plated onto YPD agar medium containing 0.4 g L^{-1} chloramphenicol (Sigma) and 0.15 mg L^{-1} biphenyl (Sigma) for yeast determination, or onto Czapek yeast extract agar (Difco) for mold determination. The plates were incubated for 1 week at 28°C. For fine *Aspergillus carbonarius* identification, cultures were grown on malt extract agar, 25% glycerol nitrate agar, and Czapek yeast extract with

20% sucrose agar, and identified according to the methods given by Pitt and Hocking (1999) and Samson, Hockstra, Frisvad and Filtenborg (2000).

***In vitro* tests on damaged grape berries.** Grape berries were purchased in supermarkets from the south hemisphere (before 2006 harvest) or directly in the field (during 2006 harvest). Berries were artificially damaged (standardized 1 cm length- and 2 mm depth-scalpel cut) under sterile conditions after total surface disinfection by three sodium hypochlorite successive baths ($CaHCl_2O_2$, 90 g L^{-1}). Contaminations of berries with different amounts of controlled populations of the two model species (*B. cinerea* and *A. carbonarius*) were then realized as stated in the text. Spraying of *S. cerevisiae* species was furthermore simulated by immerging grape berries in yeast suspensions at different cellular concentrations and at different times after initial contamination by the two model species. Berries were then incubated for weeks at 28°C in sealed sterile boxes with constant moisture. The effectiveness of yeast spraying was assessed by the monitoring of the growth of the different microbiological populations at the surface of the grape berries. Since grape berries vary in size according to their origin, grape berries were first carefully weighted. Assuming an identical density of 1.0 for all berries, each berry surface was then calculated from the following formulae :

(1) $$S = p\ (m/4)^{2/3}$$

where S and m represent the surface (in cm^2) and the mass (in g) of the grape berry, respectively. Three grape berries were used for each treatment, and the experiments were run twice. Numeration of the contaminant species populations was assayed on the supernatant obtained by vortexing each grape berry in 5 mL saline buffer containing 0.2 % Tween 80 as a surfactant and two glass beads (7 mm diameter) as a mechanical help for 3 min. After global electronic numbering of particles and consequent proper dilution in sterile saline, supernatant was plated on selective culture media to estimate fungus and yeast surface contaminations.

Experimental design at the field scale. In order to get the corresponding blank experiments, we use the experimental design schedule described in Table 1, by defining 4 different blocks in the same vineyard (Mourvèdre variety, a variety very susceptible to fungi infections in the south border of France). Each block contains 2 rows of 76 vine stocks fitted on two adjacent rows. The average yield of this vineyard is 2.5 kg per vine stock, and about 6-7 bunches per vine stock. All blocks were harvested separately by hand at grape maturity. Three weeks before the expected technological harvest time, vine rows were sprayed with a suspension of *A. carbonarius* spores in sterile water in order to get an average contamination of each bunch by 10^5 spores (which represents about 2500 spores per berry): for this purpose, 30 L containing 10^9 *A. carbonarius* spores were sprayed uniformly on the corresponding blocks. Since *A. carbonarius* is a potential very invasive fungi, such a low contamination rate was previously shown enough to get an impact on the hygienic quality of the grapes, even in dry weather situations (Perrone et al., 2006; B. Molot, IFV France, personal communication). About three days before the expected technological harvest time, vine rows were sprayed with a suspension of *S. cerevisiae* "F" cells in order to get about 10^5 S. cerevisiae cells per berry (about to 10^7 S. cerevisiae cells per bunch). For this purpose, 30 L containing 40 g de Solugel® (Martin Vialatte Oenologie, Epernay, france) et 50 g of *S. cerevisiae* "F" cells previously rehydrated in sterile water were sprayed uniformly on the corresponding blocks. Just before harvest, the grapes of the 4 blocks were carefully inspected and photographed for estimating their overall hygienic quality. At the time of harvest, about three different lots of 100 grape berries were aseptically randomly harvested in each block, rinsed with sterile water in the presence of Tween 80 (an anionic detergent) and plated on selective culture media to estimate fungus and yeast surface contaminations. For estimating fungi contamination inside the grape berries, the previous berries were surface-disinfected with sodium hypochlorite solution (1%) for 1 min, rinsed in sterile distilled water three times rinsed two more times

with sterile water and aseptically hand-crushed in sterile small poly bags (Sigma-Aldrich, St Louis, Mi). The resulting juices were then plated on selective culture media to estimate internal invasive contamination of grape berries by fungus.

Experimental winemaking. For each harvested block, 200 kg of grape bunches were first de-stemmed and the corresponding grape berries poured into stainless steel fermentation tanks (100 L each one containing about 100 kg of grapes). Each tank was then inoculated with 10 g of rehydrated ICV-INRA K1M S. cerevisiae strain (Lallemand, Toulouse, France). Fermentations were conducted at 22°C until mid-fermentation. Then the tanks were heated to 28°C at a rate of 0.125°C h^{-1}. The final temperature of 28°C was maintained up to the end of fermentation. Fermentation kinetics were followed by automatic measurement of CO_2 release by the means of gas flowmeters. Fermenting must was manually punched on a regular basis every day until the end of the fermentation. All fermentations were completed (residual sugars < 2 g L^{-1}) in less than 300 h. Grapes were then pressed, and all liquid parts blended and poured into 50 l stainless steel vats. A lactic starter (Vitilactic H+®, Martin Vialatte Oenologie, Epernay, France) was used to initiate malolactic fermentation at the temperature of 24°C.

Ochratoxin A determination. Ochratoxin A level in the grape musts was roughly analyzed with the help of the Ochracard® kit (R-Biopharm Rhône Ltd, St-Didier au Mont d'or, France). This simple screening test for the detection of ochratoxin A in complex food matrices combined an extraction step on an immunoaffinity column, and a card system. The presence or absence of colour on the test port after sample application indicates ochratoxin A contamination above or below a chosen detection level. Analysis of ochratoxin A levels in the finished wines were assessed by the recommended OIV methodology (Norm EN 14133) based on a purification by an immuno-affinity column, followed by HPLC analysis with fluorimetric detection. These analyses were performed by Inter-Rhône analysis facilities (Orange, France).

RESULTS

Selection of yeast species. Firstly, 17 industrial *S. cerevisiae* strains were tested on Petri dishes for their potential inhibition effect on fungus growth. Extemporaneous inoculation of solid PDA growth media with different fungi (2 strains of *B. cinerea* and 1 strain of *A. carbonarius)* and each industrial *S. cerevisiae* strain was performed. Mycelium growth was observed and annotated around the yeast inoculum droplets. After 3 days of incubation a general inhibition effect was observed with all tested yeast strains, but some of them seemed more efficient (Figure 1). The F strain was peculiarly efficient for inhibiting *A. carbonarius* mycelium development, while other strains are completely inefficient (data not shown). We therefore conserved this *S. cerevisiae* F strain for all the experiments.

Effect of simultaneous yeast inoculation on infected damaged berries. In a second set of experiments, extemporaneous spreading of *S. cerevisiae* F strain was performed *in vitro* at the surface of previously artificially damaged grape berries contaminated with the different fungus species.

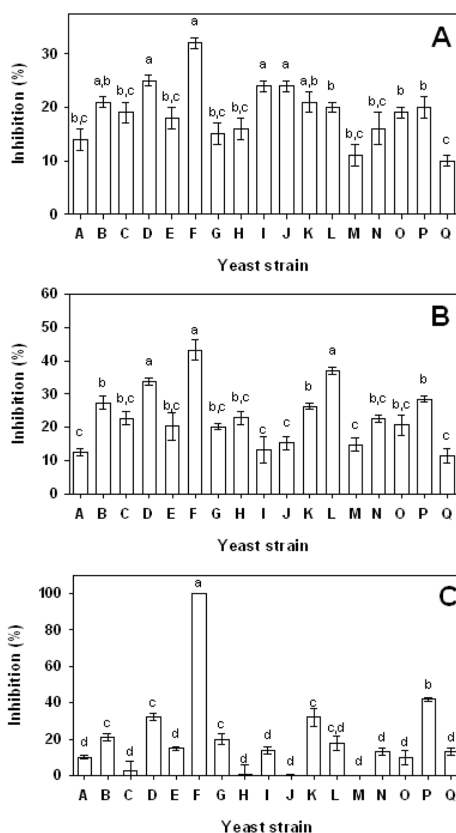

Figure 1. Test of 17 industrial *S. cerevisiae* strains for their potential inhibition effect on fungus growth (mean and standard deviation of duplicates). (A) 10^6 *B. cinerea* M04/51 and (B) M04/63 spores or (C) 10^4 *A. carbonarius* spores were first spread on PDA Petri dishes and 2.5 10^6 cells of each *S. cerevisiae* strain were extemporaneously inoculated in a 100 µL-droplet of sterile saline solution. Petri dishes were observed after 70 h incubation at 28°C. Mycelium growth was observed and annotated around the yeast inoculum droplets. The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

As shown in Figure 2, yeast spreading was very efficient for reducing both *B. cinerea* and *A. carbonarius* mycelium growth after 48 h of incubation. This was not the case for a bacterial contamination with *Gluconobacter oxydans*, where a much lower effect is observed (data not shown).

Time-scale effect of yeast inoculation on infected damaged berries. We tried finally to evaluate the time-scale of the effect of yeast spraying on the reduction of grape infection by the two tested fungi. For this purpose, we delayed the time of yeast inoculation after initial infection of damaged grape berries by the two model fungus species studied. As seen on Figure 3, yeast spraying at the surface of *B. cinerea* infected berries delayed differently the growth of the fungus depending on the strain studied. For all the fungus tested, the effect of yeast spraying is much more sensitive during the first three days after infection. For *A. carbonarius* infected berries, the effect of yeast spraying is significant for about 4 to 5 days after the initial infection by the fungus (Figure 4, P values = 0.044 and 0.042, respectively). Therefore, from an applied point of view, yeast spraying should be done about 2-5 days after initial infection by the fungi in order to get an optimal antagonistic effect.

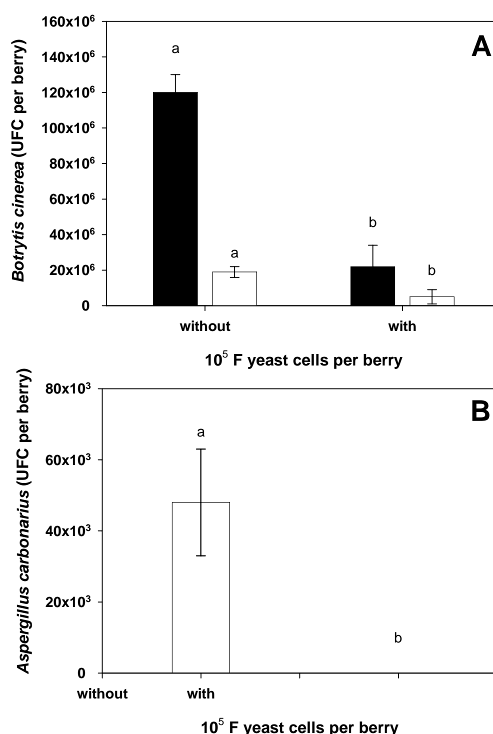

Figure 2. Effect of the inoculation of 10^5 *S. cerevisiae* F cells on the surface of wounded grape berries previously and extemporaneously inoculated in the wound with (A) 10^6 *B. cinerea* M04/51 (black boxes) and M04/63 (white boxes) spores or (B) 10^4 *A. carbonarius* spores. Fungi numbering was realized after 48 h incubation at 28°C (mean and standard deviation of two replicates of three grape berries for each situation). The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

Results at the field scale : 2007 and 2008 vintages. Three weeks before the expected technological harvest time, vine rows were sprayed with a suspension of *A. carbonarius* spores in sterile water in order to get an average contamination of each bunch by 10^5 spores (which represents about 2500 spores per berry). Careful visual inspection of the *A. carbonarius* contaminated grapes did not reveal any visible *A. carbonarius* infection one week before the expected technological harvest time. About three days before the expected technological harvest time, vine rows were sprayed with a suspension of *S. cerevisiae* F cells in order to get about 10^5 *S. cerevisiae* cells per berry (about to 10^7 *S. cerevisiae* cells per bunch) (Table 1).

Table 1: Summary of the experimental design

Block n°	**Dates**		
	3 weeks before THT	**3 days before THT**	**Technological harvest time (THT)**
A	*A. carbonarius* inoculation	-	Grape harvest
B	-	*S. cerevisiae* spraying	Grape harvest
C	*A. carbonarius* inoculation	*S. cerevisiae* spraying	Grape harvest
D	-	-	Grape harvest

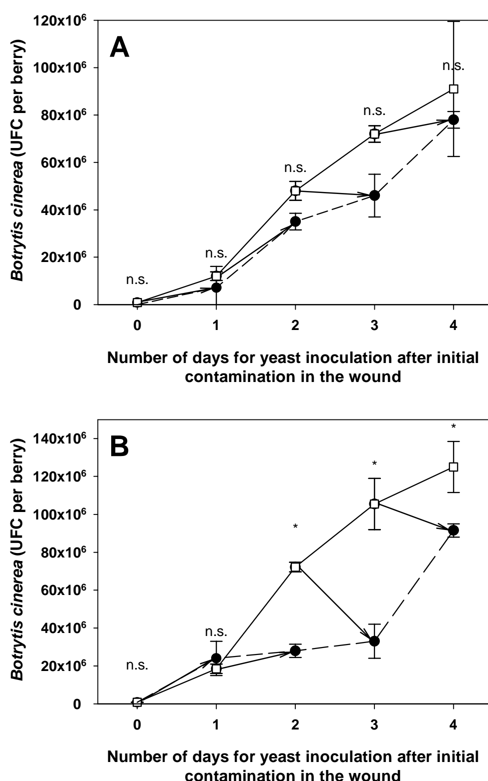

Figure 3. Effect of the delayed inoculation of 10^5 *S. cerevisiae* F cells on the surface of previously wounded disinfected grape berries inoculated with 10^6 *B. cinerea* spores ((**A**) M04/51, (**B**) M04/63) in the wound at T=0 (mean and standard deviation of two replicates of three grape berries for each situation). (□) *B. cinerea* population on non-treated berries, (●) *B. cinerea* population on yeast-treated berries. Incubation was performed at 28°C in sealed sterile boxes with constant moisture. Numeration was performed 48 h after yeast inoculation. (n.s.) non significant differences between groups at the 95% confidence level, (*) significant differences between groups at the 95% confidence level, as tested by Tukey statistical test.

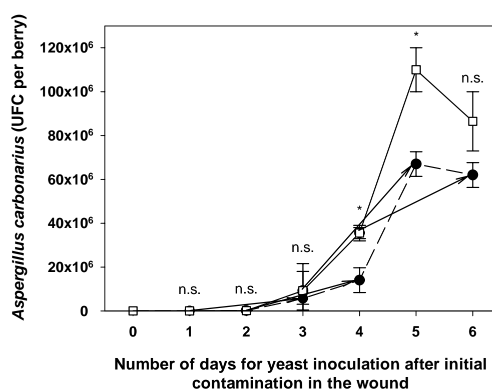

Figure 4. Effect of the delayed inoculation of 10^5 *S. cerevisiae* F cells on the surface of previously wounded disinfected grape berries inoculated with 10^4 *A. carbonarius* spores in the wound at T=0. (□) *A. carbonarius* population on non-treated berries, (●) *A. carbonarius* population on yeast-treated berries. Incubation was performed at 28°C in sealed sterile boxes with constant moisture. Numeration was performed 48 h after yeast inoculation. (n.s.) non significant differences between groups at the 95% confidence level, (*) significant differences between groups at the 95% confidence level, as tested by Tukey statistical test.

At the time of harvest, a careful inspection of all bunches allows us to find only few (less than 1% of the harvested bunches) exhibiting the symptoms of *A. carbonarius* external infection (black fungi between the berries). This observation could be attributed to the low rainfalls and the very low main humidity that were observed during grape maturation in 2007 and 2008 (data not shown). After grape harvest, external fungus contamination was assessed on 90 randomly chosen berries picked in each block (Figure 5).

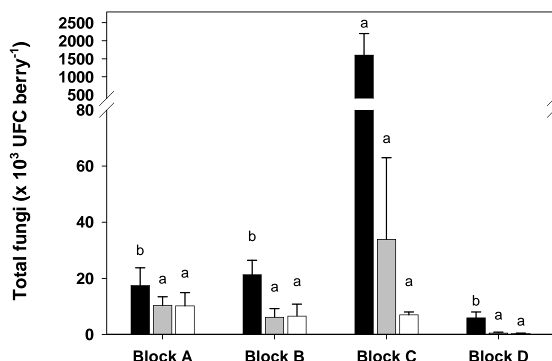

Figure 5. Distribution of yeasts and fungi at the surface of grape berries (mean and standard errors of 90 randomly chosen berries from three different lots of 100 grape berries for each block). (Black boxes) *Saccharomyces* strains, (grey boxes) non-*Saccharomyces* strains, (white boxes) filamentous fungi. The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

Fungi were detected in each block, but *A. carbonarius* was not specifically detected in infected blocks. *Saccharomyces* yeasts were significantly detected on berries harvested in the blocks where yeast spraying was realized (P value = 0.024), confirming its effectiveness. In a second experiment, we crushed aseptically the berries harvested in the different blocks and studied again the occurrence of *A. carbonarius* in the entire grape berries. As shown on Figure 6, we found that *A. carbonarius* infection occurred specifically in the artificially contaminated blocks. However, due to the high standard errors between sample duplicates, no significant differences were observed between the blocks, with respect to *A. carbonarius* surface infection. Before fermentation, a preliminary rough determination of ochratoxin A level in the corresponding grape musts with the help of the Ochracard® kit did not reveal any contamination above 2 µg L^{-1}.

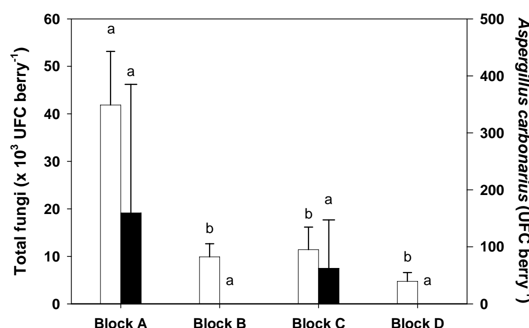

Figure 6. Distribution of *A. carbonarius* and other fungi in crushed berries (mean and standard errors of 90 randomly chosen berries from three different lots of 100 grape berries for each block). (Black boxes) *A. carbonarius*, (white boxes) other fungi. The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

Winemaking Fermentation kinetics were very similar for all blocks. Close inspection of the fermentation kinetics shows that initial maximum fermentation rates of the musts issued from grapes contaminated with *A. carbonarius* were lower than controls unless the first must punching was performed. This observation is reminiscent of a limitation of the inoculated industrial K1M *S. cerevisiae* strain growth, which can be likely attributed to nutrient deficiency (mainly lipids) in the must (Fornairon-Bonnefond, Demaretz, Rosenfeld and Salmon, 2002). This effect was corrected in the fermentation performed on musts issued from *A. carbonarius* contaminated grapes and sprayed with F *S. cerevisiae* strain. Moreover, attempts to detect sprayed F *S. cerevisiae* strain amongst the viable yeasts during all alcoholic fermentations were unsuccessful (data not shown). This result proved that remnant F *S. cerevisiae* sprayed yeasts did not interfere with the alcoholic fermentation performed by the inoculated K1M *S. cerevisiae* strain. Although wines were all inoculated with a lactic acid bacteria starter, the wines resulting from modalities contaminated with *A. carbonarius* exhibited one week- to ten days-delay before the beginning of malolactic fermentation (data not shown). At the exception of volatile acidity, the global chemical analysis of the final wines did not reveal significant differences between the different modalities (data not shown). Volatile acidity was higher in the blocks where *A. carbonarius* initial spraying was performed (Figure 7). On the contrary, the sole *S. cerevisiae* spraying seems to significantly reduce volatile acidity (Figure 7, P value = 0.036). Sensory analysis of the final wines did not allow the finding of significant differences between the 8 experimental wines (data not shown). Analysis of ochratoxin A levels in the finished wines was assessed by purification of the toxin on an immunoçaffinity column, followed by HPLC analysis with fluorimetric detection. The final ochratoxin A levels were given on Figure 8. Significant levels of ochratoxin A were detected in wines resulting from grapes harvested in blocks contaminated with *A. carbonarius* (P values = 0.035 and 0.032, respectively), although only few harvested bunches exhibit the visual symptoms of *A. carbonarius* external infection. Yeast spraying reduces significantly by a 1.5 fold factor the final level of ochratoxin A in the wine (P value = 0.043). This reduction is compatible with the levels of *A. carbonarius* infection observed in the crushed berries (Figure 6).

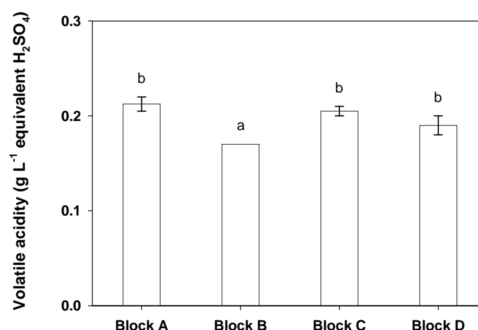

Figure 7. Volatile acidity levels in the finished wines (mean and standard errors of duplicates). The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

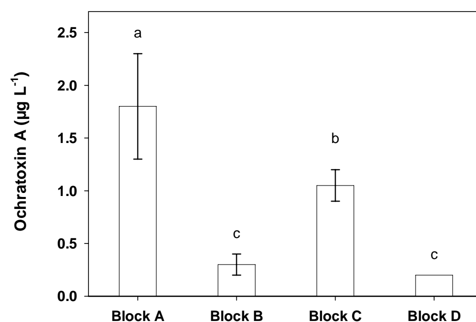

Figure 8. Ochratoxin A levels (µg L^{-1}) in the finished wines (mean and standard errors of duplicates). The same letters indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

CONCLUSIONS

The main objective of this work was the potential reduction of microbial diseases on damaged grape berries by spraying *S. cerevisiae* yeasts on the grapes by creating a competition among microorganisms at their surfaces. Such surface competitions were successfully performed to control post-harvest diseases (molds) of fruits or vegetables by pre-harvest applications of yeasts (Elad, Köhl and Fokkema, 1994; Benbow and Sugar, 1999). Natural saprophytic yeasts were generally used for this purpose (Elad, Köhl and Fokkema, 1994; Chand-Goyal and Spotts, 1996). Such natural yeasts (mainly *Cryptococcus* and *Rhodotorula* spp.) are known to colonize plant surfaces or wounds for long periods under dry conditions, utilizing available nutrients for rapid multiplication, and to be minimally impacted by pesticides (Wisniewski and Wilson, 1992). Limitation of the use of such yeasts relies on the fact that their production in mass at an industrial scale is very difficult, or even impossible (Villetaz, 1992). However, to our knowledge, nobody tried to test classical industrial S. cerevisiae strains, which are easily available in great amounts, for their ability to control fungus development. The choice of enological *S. cerevisiae* strains was dictated by the fact that most of these available strains were originally isolated from grapes or wines (Villetaz, 1992), and therefore seemed more adapted to the peculiar substrate represented by damaged grape berries. We first assess the quantification of the effect of *S. cerevisiae* on the development of undesirable bacteria or fungi at the surface of voluntarily damaged grape berries. A general inhibition effect was observed in vitro by a set of 17 industrial *S. cerevisiae* strains against *B. cinerea* and *A. carbonarius* mycelium growth. However, only few of them are really very efficient. We therefore conserved the most promising *S. cerevisiae* strain, named F.

A first set of experiments performed at the laboratory scale show that extemporaneous spreading of *S. cerevisiae* F strain at the surface of previously artificially damaged grape berries contaminated with different microbial species was very efficient for reducing fungus mycelium growth after 48 h of incubation. This was not the case for bacterial *G. oxydans* contamination, where no effect is observed. From this first part of the work, it could be roughly concluded that *S. cerevisiae* F spraying by its mass impact could lower grape infection by fungi. In a second set of experiments, we demonstrate that *S. cerevisiae* F spraying should be done about 2-5 days after initial infection by the fungi in order to get an optimal antagonistic effect. After this period, the potentiality of fungi to initiate disease remains, indicating that a competition for nutrients has taken place between protagonists

(Hocking et al., 2007). The effect of yeast spraying on *A. carbonarius* development on the grape berries was particularly significant. From all these experiments we therefore think that such yeast spraying before grape harvest could represent for the viticulturist a biological alternative for limiting the occurrence of *A. carbonarius* in the vineyard.

In subsequent field scale experiments performed during 2007 and 2008 vintages, we show that yeast spraying with the selected industrial *S. cerevisiae* F strain on an artificially *A. carbonarius* infected vineyard was able to reduce the *A. carbonarius* proliferation inside the grape berries, even if the external black mycelia form of *A. carbonarius* is not observed at the grape berry surfaces. The ability of strains of A. carbonarius to colonize and penetrate intact berries is indeed known, finding OTA in the pulp, although berry skin was considered the major source of OTA in grapes (Battilani, Pietri and Logrieco, 2004). However, the way *A. carbonarius* penetrates the fruit in undamaged grapes when not using the portal of entry that the stem provides, is still unknown (Belli et al., 2006). From the obtained results, it should the hypothesized that yeast spraying at the surface of intact grape berries reduce partially *A. carbonarius* penetration into undamaged grapes. This effect should be studied in more details. Moreover, the reduction of *A. carbonarius* proliferation was accompanied by a significant reduction of the final level of ochratoxin A in the corresponding wines. The chemical and sensory properties of the final wines were also not detrimentally affected by yeast spraying.

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Effect of Probiotic (*Saccharomyces cerevisiae*) Adding to Diets on Intestinal Microflora and Performance of Hy-Line Layers Hens

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Abstract: As experiment was conducted to evaluate the affect of adding various levels of a live yeast to laying hen diets on their laying and feeding performance, egg shell, egg components and some blood constituents, as well as the intestinal microflora make-up. This were studied to validate the mode of a live yeast action in improving laying hens performance. For this purpose 75 Hy line (W-36) white layers were sited from 70 to 79 week of age in individual cages and randomly distributed into five experimental groups of 15 layers each. The individual hen was represented as an experimental unit. The five experimental groups were fed on five graded levels of a live yeast as 0.0% (control), 0.4%, 0.8%, 1.2% and 1.6%. The main results indicated an increase in egg production percentage of layers fed with 0.4% and 0.8% a live yeast which recorded 83.4% and 80.6% respectively compared with 74% of control which was similar to the groups of layers fed 1.2% (74.9%) and 1.6% (74.6%). Average egg weight was not influenced by adding yeast into diets. Egg mass results were parallel to these of egg production where the values of 46.7, 51.0, 50.2, 48.3 and 46.1 g egg/hen/day were recorded for the group of birds fed with 0%, 0.4%, 0.8%, 1.2% and 1.6% a live yeast respectively. Egg albumen and egg yolk were affected significantly. There was a slight improvement in egg shell thickness and percentage. Feed intake values were approximately similar within the different treatments. Feed conversion ratios (g feed/g egg) of layers fed yeast levels of 0.4% (2.08) and 0.8% (2.07) were better than the control group (2.27). Blood total protein levels of birds fed 0.4% (3.82), 0.8% (3.65) and 1.2% (3.97) yeast were lower than the control (4.16), while the value of 1.6% yeast (4.16) was slightly higher than control. Blood albumen levels were parallel to those of blood protein while blood globulin values were not affected. Blood cholesterol levels of layers fed yeast-supplemented diets were lower than the control. Blood total lipids were not affected by treatments. Ileal content pH of layers fed 0.8% and 1.2% yeast levels was lower than the control. Microbiological examination of ileal content indicated an obvious reduction in bacterial total count. While Lactobacilli bacterial count was increased. There were reductions in bacterial strains of *Escherichia coli (E.coli), Klebsiella sp., Staphylococcus sp., Micrococcus sp., Campylobacter sp., and Closterdium perfringers* of layers fed various yeast levels. The results of this study suggest adding live yeast at 0.4% or 0.8% into laying hen diets can enhance the productive performance and nutrients utilization via the inhibitory effect of yeast against pathogenic bacteria. [Journal of American Science. 2010;6(11):159-169]. (ISSN: 1545-1003).

Keywords: yeast level, laying hen, egg production, ileal microflora, blood constituents.

1. Introduction

Microorganisms used as probiotics in animal mutrition: Probiotics are live microorganisms that, when administered through the digestive tract, have a positive impact on the host's health. Microorganisms used in animal feed are mainly bacterial strains belonging to different genera, e.g. *Lactobacillus, Enterococcus*, *Pediococcus* and *Bacillus*. Other probiotics are microscopic fungi, including *Saccharomyces* yeasts. Some probiotic microorganisms are normal residents in the digestive tract, while others are not (Guillot, 2009). Different mechanisms of probiotic action have been suggested,

but most are only hypothetical. The positive effect can result either from a direct nutritional effect of the probiotic, or a "health" effect, with probiotics acting as bioregulators of the intestinal microflora and reinforcing the host's natural defences (Fuller, 1977; Fuller, 2001).

Kabir (2004) indicated that the gut microflora forms with its host animal a complex ecosystem and microbial interactions ensure the stability of the ecosystem and the health of the host. In some cases the gut microflora is unbalanced and the biological defences against pathogenic agents less effective. The positive effect observed can be the result of either a direct nutritional effect, similar to the effect obtained with antibiotics, or a "health" or sanitary effect, where the probiotic act as a bioregulator of the gut microflora and reinforces the natural defences.

The different mechanisms of action suggested are: (i) nutritional effect include: (1) Reduction of metabolic

reactions that produces toxic substances (2) Stimulation of indigenous enzymes (3) Production of vitamins or antimicrobial substances.

(ii) Sanitary effect include (1) Increase in colonization resistance. (2) Stimulation of the immune response. Some experiments have demonstrated in vitro the effects of strains of *Saccharomyces cerevisiae* on the activity of anaerobic rumen microorganisms. The addition of S. cerevisiae live cells to cultures of some cellulolytic fungal species stimulated zoospores germination and cellulose degradation. The addition of yeasts stimulates also the growth of some anaerobic bacteria, including the cellulolytic and the lactic acid utilising bacteria (Chaucheyras et al., 1995; Yoon and Stern, 1996).

Kizerwtter and Binek, (2009) reported that probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both in vitro and in vivo through several different mechanisms. The mode of action of probiotics in poultry includes: (i) maintaining normal intestinal microflora by competitive exclusion and antagonism (ii) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production (iii) improving feed intake and digestion iv) stimulating the immune system (Apata 2008; Kabir, 2009).

Kabir et al. (2005) attempted to evaluate the effect of probiotics with regard to clearing bacterial infections and regulating intestinal flora by determining the total viable count (TVC) and total *lactobacillus* count (TLC) of the crop and cecum samples of probiotics and conventional fed groups at the 2nd, 4th and 6th week of age. Their result revealed competitive antagonism. The result of their study also evidenced that probiotic organisms inhibited some nonbeneficial pathogens by occupying intestinal wall space. They also demonstrated that broilers fed with probiotics had a tendency to display pronounced intestinal histological changes such as active impetus in cell mitosis and increased nuclear size of cells, than the controls. Recently, Mountzouris et al. (2007) demonstrated that probiotic species belonging to *lactobacillus, Strepococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida,* and *Saccharomyces* have a potential effect on modulation of intestinal microflora and pathogen inhibition.

A few years ago active living yeast, has been documented as probiotic feed additive for poultry, due to its improvement effect on performance characteristics. Including a live yeast into laying hen diets improved egg production percentage (Kim et al., 2002 and Shivani et al., 2003), and egg weight (Han et al., 1999; Park et al., 2001 and Park et al., 2002). Dumanovski (2000); Sharma et al. (2001); Kim et al. (2002) and Kabir (2009) reported that, adding a live yeast into laying hens diet improved feed intake and feed conversion ratio.

Inclusions of yeast into laying hen diets enhanced egg shell breaking strength (Park et al., 2002), and reduced soft or broken eggs (Park et al., 2001).

In Egypt, a very few studies have been conducted to investigate the effect of feeding yeast on performance of laying hens. Soliman (2003) studied the effect of supplementing a constant level of live yeast into laying hens diets, he observed an improvement in average egg weight, feed conversion values and nutrients utilization. The mode of beneficial action of yeast can be attributed to its antagonistic bacteria and altering gut microflora make up Line et al., 1998; Wakwak et al. (2003) and Kabir 2009) observed a sharp reduction in bacterial total count of ileum content, due to supplementing yeast into Japanese quail diets. In contrast ileal content of lactobacilli bacteria increased significantly due to adding yeast into laying hen diets (Kim et al., 2002; Hossain et al., 2005). Adding yeast to poultry diets leads to reduced bacterial counts of *E.coli and Closterdium perfringers* (Park et al., 2002; Nava et al., 2005), *Salmonella and Campylobacter* (Line et al., 1998). In this concern the research is still lacking under Egyptian conditions.

the objective of this study aimed to investigate the effect of enriching Hy line (W-36) laying hen diets with various levels of active a live yeast on their laying and feeding performance, egg shell, egg components and some blood constituents. As well as ileal bacterial make-up will be studied to validate the mode of yeast action in improving performance of laying hens.

This study provides a summary of the use of probiotic (Saccharomyces cerevisiae) for prevention of bacterial diseases in poultry as well as demonstrating the potential role of probiotics in the growth performance and immune response of poultry.

2. Materials and Methods

This study was carried out at (Layer Nutrition Research Unit), Faculty of Agriculture, Ain Shams University.

It was conducted using 75 Hy-Line (W-36) white layers which were randomly sited from 70 to 79 week of age in individual battery cages located in open sided laying house. The hens were randomly

distributed into five treatment groups of 15 layers each. The individual hen was represented as experimental unit. For nine weeks experimental period the hens were fed on a basal diet supplemented with five graded levels of active live yeast *Saccharomyces cerevisia* (produced by Starch, Yeast and Clean Co., Alex.) as 0.0% (control) 0.4%, 0.8%, 1.2% and 1.6%.

The basal diet was formulated (Table 1) to meet all nutrient requirements of laying hens according to (Hy-Line 2000) management guide. Feed was provided ad lib in an individual feeders and water was supplied through automatic nipples. Lighting hours were 17 hours per day. Egg weight in grams was recorded daily for each hen throughout the experimental period. Average egg weight, egg production percentage and average egg mass (g/hen/day) were calculated for each hen and treatment group. Feed consumption in grams per hen was recorded weekly and average feed consumption per treatment group was calculated. Feed conversion ratio was calculated as gram feed consumed per gram egg produce (g. feed/ g. egg). Body weight gain was calculated for each hen and treatment group by subtracting individual body weight of hen at 70 weeks from that at 79 weeks of age. Egg component percentages were assessed by using 12 eggs per treatment represent 6 hens as two consecutive eggs per hen. For this purpose, egg was individually weighted, broken, yolk and albumin was separated weighed and related as percentage to whole egg weight. Egg shell with membrane were cleaned, dried, weighed and related as percentage e to the whole egg.

Table (1): Composition and calculated analysis of experimental diet.

Feed Ingredient	Percentage (%)
Yellow corn	59.93
Soybean meal (48%)	24.23
Corn gluten meal	2.0
Calcium carbonate	9.16
di-calcium phosphate	1.84
Oil	2.0
Common salt	0.364
Methionine	0.076
Premix*	0.4
Total	**100**
Calculated analysis:	
ME (kcal/kg)	2806
Protein (%)	17.39
Calcium (%)	3.97
Av. Phosphorus (%)	0.465
Meth. + Cyst (%)	0.66
Lysine (%)	0.86

*: Vitamins and minerals Premix: each 1 kg supplied the following per kilogram of diet; vit. A: 12000 lu, vit. D3: 3000 lu, vit. E.: 12 mg.
vit. B12 0.02 mg, vit. B1 1 mg, Choline chloride 0.16 mg, Copper 3 mg, Iron 30 mg.
Manganese 40 mg, Zinc 45 mg and Selenium 3 mg according to NRC (1994).

Egg shell thickness (millimeter) was determined using a micrometer. Initial and final body weights of layers were recorded and average body weight gain was calculated.

Blood Analysis and Microbiological Examination:

At the end of the experiment five hens per experimental group were slaughtered, blood samples were collected and centrifuged for 15 minutes. Plasma total protein was determined according to Biuret method (Henery, 1964), albumin according to Doumas et al. (1971). Plasma globulin was calculated by subtracting albumin from total protein. Then albumin to globulin ratio was calculated. Plasma total lipid was determined according to Knight et al. (1972) and total cholesterol according to Watson (1960).

For microbial experimentation, ileal content samples were collected by pressing the outer wall of cut ileal to push its content into clean, sterile glass bottle. The pH value of ileum content were determined using pH meter. Microbiological experimentation procedure was done as follows: One gram of ileal content was adjustely weighed and transferred into test tube containing 9 ml of 0.1 sterile peptone the samples were mixed well and serial dilutions were prepared.

Cultivation and Enumeration of Bacteria:

Bacterial total count was examined with nutrient agar medium composed of (per liter) yeast extract 2.5 g trypton 5 g, glucose 1 g, agar 15 g and distilled water up to one liter (Swanson et al., 1992).

Lactobacilli bacteria was counted with M.R.S. agar medium which is composed of casein peptone 10 g meat extract 10 g, yeast extract 5 g, glucose 20 g, tween 80 1 g, K_2hpo_4 2 g, sodium acetate 5 g, diammonium citrate 2 g, $Mnso_4$ 0.2 g and distilled water up to 1 liter (Laner and Kandier, 1980)..

Coliforms bacteria were counted by using MacConkey agar medium that is composed as pancreatic digest of gelatin 17 g, pancreatic digest of casein 1.5 g, peptic of animal tissue 1.5 g, lactose 10 g, bile salts 1.5 g, sodium chloride 5 g, neutral red 0.03 g, crystal violet 0.001 g, agar 3.5 g, and distilled water up to 1 liter (Oxoid, 1992).

Campylobacter strains were grown in stationary cultures in 5 ml of Rosef broth without antibiotics for 48 hours in a microaerobic atmosphere created by using BBL gas pak plus anaerobic system envelopes without the palladium catalyst. Rosef broth contains (per liter) peptone 10g, lablemco (oxid) 8 g, yeast extract 1 g, Nacl 5 g, rezasurin solution (0.025% wt/vol) 1.6 g (Ryan and Ray, 2004).

Colstridium perfringers were grown in a stationary culture in an anaerobic atmosphere and subsequently diluted in sterile Rosef broth or sterile saline to concentrations of 10^6 to 10^8 CFU per ml, then PCR procedure was used for examination (Baumgart et al., 2007).

Klebsiella and Proteus gram negative *Entero-bacteria* were grown in MacConkey agar medium and eosin/methylene blue agar medium composed (per liter) of peptone 10 g, lactose 5 g, dipotassium phosphate 2 g, eosin Y 0.4 g, methylene blue 0.065 g, and agar 13.5 g (Oxoid, 1992).

Staphylococcus sp. and Micrococcus sp. gram positive bacteria was grown in nutrient agar medium, MacConekay agar medium and *Staphylococcus* medium (No. 110) that composed (per liter) yeast extract 2.5 g, tryptone 10 g, glateene 30 g, lactose 2 g, D/manitol 10 g, Nacl 75 g, dipotassium phosphate 5 g, agar 15 g, pH 7 ± 0.02 (Mathews et al., 1997).

Statistical Analysis:

Statistical analysis was carried out using statistical program SAS (1988). Ducan's multiple tests was used to separate means.

3. Results and Discussion

Shareef and Dabbagh (2009) reported that Saccharomyces cervisiae supplementation of broilers, to the level of 1, 1.5 and 2%, were significantly, increase the body weight gain, feed consumption and feed conversion efficiency. The beneficial effect of *Saccharomyces cerevisiae* is attributed to the fact that it is a naturally rich source of proteins, minerals and B-complex vitamins.

It is well known that yeast culture, and its cell wall extract containing 1,3-1,6 D-glucan and Mannan oligosaccharide are the important natural growth promoters for modern livestock and poultry production (Van Leeuwen et al., 2005a). The advantages of these promoters over the traditional antibiotic growth promoters are 1) no withdrawal time, 2) no residual effect, and 3) no causes of microbial mutation *(*Gibson and Roberfroid, 2008). *Saccharomyces cerevisiae* is considered as one of the live microorganisms probiotic that, when administered through the digestive tract, have a positive impact on the hosts health through its direct nutritional effect. Field reports (Banday and Risam, 2002) have suggested that probiotic supplementation improved performance of broilers. The different mechanisms of probiotic action suggested are; nutritional effect by regulation of metabolic reactions that produces toxic substances; stimulation of endogenous enzymes and by production of vitamins or antimicrobial substances. Moreover, Saccharomyces cerevisiae could act as bioregulalor of the intestinal micro flora and reinforcing the host natural defenses, through the sanitary effect by increasing the colonization resistance and stimulation of the immune response (Line et al., 1998). These effects were largely reflected by using mannan Oligosaccharide, the naturally derived extract from the cell wall of *Saccharomyces cerevisiae.* This oligosaccharide content is approxi-mately50% of the carbohydrate fraction and improved body weight gain in broiler chickens and that this effect can be attributed to the trophic effect of this product on the intestinal mucosa, because it increases villus height, particularly during the first 7 days of the chickens life (Santin et al., 2001).

Oligosaccharides used to control pathogenic scours of all kinds in livestock caused by *Salmonella,* and *E.coli* etc (Laegreid and Bauer, 2004). Mannan-oligosaccharides are thought- to block the attachment of pathogenic bacteria to the animal's intestine and colonization that may result in disease, while acting as a nutrient to other beneficial bacteria. It is also thought to stimulate the animal's immune system, thereby further reducing the risk of disease (Firon and Ofek, 1983). Oyofo et al. (1989) observed that the adherence of *Salmonella typhimurium* to enterocytes of the small intestine of chicks, in vitro, was inhibited in the presence of mannose. Later, they found that inclusion of mannose in the drinking water of chicks reduced *S. typhimurium* colonization of the cecum.

Saccharomyces cerevisiae Probiotic supplementation has been shown to reduce the cholesterol concentration were reported in egg yolk by (Abdulrahim et al., 1996) and serum in chicken (Mohan et al., 1996). Recent report suggested that feeding of chicory beta fructans an oligosaccharide, a prebiotic, reduced the serum cholesterol and abdominal fat of broiler chicken (Yusrizal, 2003). Gilliland et al. (1985) suggested that the Prebiotic supplementation could have enhanced the lactobacilli count. Similar results have been reported by others *(Mohan, 1996).*

Laying Performance:

Egg production percentage of laying hens fed 0.4% (83.4%) and 0.8% (80.6%) live yeast was higher than the control value (74%) which was approximately similar to those fed with 1.2% (74.9%), 1.6% (74.6%) yeast in their diets. The differences between egg production percentages lacked significance (Table 2).

Table (2): Effect of feeding different yeast levels on laying performance and egg components.

Item	Yeast Level				
	0.0%	**0.4%**	**0.8%**	**1.2%**	**1.6%**
Egg production	74.0	83.4	80.6	74.9	74.6
Av. Egg weight (g)	63.1	61.2	62.7	64.5	61.8
Egg mass (g egg/hen/day)	46.7	51.0	50.2	48.3	46.1
Egg component					
Egg yolk (%)	27.3	28.1	28.8	27.6	27.7
Egg albumin (%)	63.7	62.6	61.7	63.1	62.9
Egg shell (%)	9.00	9.33	9.45	9.39	9.39
Egg shell thickness (mm)	0.396^{b}	0.425^{ab}	0.426^{a}	0.416^{ab}	0.420^{ab}

a, b: Means with different superscripts are significantly different ($P<0.05$).

The improvement in egg production due to low level of yeast inclusion is in agreement with the result of Kim et al., (2002); Shivani et al. (2003); Shareef and Al-Dabbagh (2009) who observed higher percentage of egg production for hens fed yeast-supplemented diets than the control hens.

Average egg weight was not influenced significantly by adding yeast into diets. Nursoy et al.

(2004) stated that, egg weight was not affected by adding yeast into diet. The improvement in egg production reflected on egg mass (g egg/hen/day) values which increased from 46.7 (control) to 51.0 and 50.2 by adding 0.4% and 0.8% yeast level respectively, while the high levels of yeast (1.2% and 1.6%) declined egg mass value to be 48.3 and 46.1 respectively.

The increment in egg production and egg mass with 0.4% and 0.8% yeast level may be attributed to the antagonistic effect of yeast against harmful enteric microflora which may cause mal-absorption of nutrients. So that, adding yeast may enhance digestion, absorption and saving more nutrients for egg formation. Soliman (2003) attributed the best hen day egg production of hens fed dietary yeast to the decrease proliferation of pathogenic bacteria. The high inclusion of yeast level has an adverse effect on nutrient digestibility (Romashko, 1999). Thereby, laying performance was not improved due to adding of 1.2% or 1.6% live yeast into diet.

Feeding Performance and Body Weight Gain:

Feed intake values of different treated groups were approximately similar and lacked significance. *Kim et al. (2002)* stated that, feed intake values were not statistically different among yeast feeding groups and control.

Feed conversion ratios (g feed/g egg) of birds fed with 0.4% (2.08) and 0.8% (2.07) dietary yeast were better than that of control (2.22), while 1.2% (2.24) and 1.6% (2.25) yeast levels did not show any improvement compared to the control. Park et al. (2002); Soliman (2003) and Zhang et al., (2005)

observed an improvement in feed conversion ratio of laying hens fed yeast supplemented diets.

The slight improvement in feed conversion inherent with low inclusion levels of yeast (0.4% or 0.8%) may be attributed to the improvement in nutrients absorption and utilization associated with adding yeast which reduces the proliferation of enteric harmful bacteria that responsible of mal-absorption (Table 3). Bradle and Savag (1995) observed an improvement in energy utilization due to feeding dietary yeast. Soliman (2003) reported that, supplementation of yeast into laying hen diets significantly improved digestion coefficient of crude protein.

Body weight gain values of layers fed different yeast levels were not significantly higher than control (Table 3). Sharma et al. (2001) stated that, the weight gain of egg type chicken fed yeast supplemented diet was higher than those fed control diet.

Table (3): Effect of feeding various live yeast levels on feeding performance and body weight gain.

Item	Yeast Level				
	0.0%	**0.4%**	**0.8%**	**1.2%**	**1.6%**
Feed intake (g/hen/day)	104.00	105.7	105.00	108.3	103.6
Feed conversion (g feed/g egg)	2.22	2.08	2.07	2.24	2.25
Initial body weight (g)	1475	1444	1480	1478	1481
Final body-weight (g)	1497	1494	1540	1555	1552
Body weight gain (g)	22	50	60	76.8	71.6

Non-significant differences.

Egg Component:

Incorporating of live yeast into laying hen diets did not influence egg albumin or egg yolk percentages and the difference; among treatments lacked significance (Table 2). Nursoy et al. (2004) did not find any affect on egg albumin or egg yolk of laying hens fed yeast-supplemented diet.

However, egg shell percentage and egg shell thickness values were improved due to feeding various yeast levels, especially at 0.8%, when compared to the control group (Table 2).

The improvement in egg shell percentage and egg shell thickness may be attributed to the enhancement of calcium absorption and retention associated with adding yeast into the diet (Bradly and Savage, 1995). Park et al. (2001) reported that, hens fed diets with yeast produced less soft shell and broken egg than control.

Blood Constituents:

Blood total protein values of birds fed on 0.4% (3.82), 0.8% (3.65), and 1.2% yeast (3.97) were lower than the control (4.16) (Table 4). However, the level of 1.6% yeast (4.33) was slightly higher than control. Similar results were recorded for blood albumin. There was no effect on blood globulins due to adding yeast to the diet.

The results of blood protein did not agree with those obtained by Wakwak et al. (2003), who did not find any effect on blood protein or albumin due to adding yeast into growing quail diets.

The lower values of blood proteins of birds fed on 0.4%, 0.8% and 1.2% yeast than the control may be attributed to the inhibitory effect of yeast against harmful intestinal microflora because harmful enteric bacteria secretes inflammatory agents lead to increase protein synthesis in liver and accordingly increased blood content of protein. Klasing and Austic (1984) observed an increase in protein synthesis in liver of chickens infected with *Escherichia coli* bacteria. Similar explanation can be introduced for the higher blood protein value of layers fed 1.6% dietary yeast, that the high inclusion of active live yeast may induce an inflammation in the small intestine wall causing increase in blood protein level.

Blood cholesterol levels of layers fed yeast supplemented diets were lower than the control (Table 4). Victor et al. (1993) and Endo et al., (1999) found that cholesterol content was lower with inclusion of yeast into broiler chicks' diets. Blood total lipid was not affected by adding yeast into diets.

Table (4): Effect of feeding various live yeast levels on blood constituents.

Item	Yeast Level				
	0.0%	**0.4%**	**0.8%**	**1.2%**	**1.6%**

Total protein (g/dL)	4.16[a]	3.82[ab]	3.65[b]	3.97[ab]	4.33[a]
Albumin (g/dL)	2.23[a]	1.83[b]	1.80[b]	2.08[ab]	2.36[a]
Globulin (g/dL)	1.93	1.99	1.87	1.89	1.97
Alb./Glob.	1.16	0.92	0.97	0.91	0.84
Cholesterol (g/dL)	161.5[a]	149[ab]	133.7[b]	158.2[ab]	149[ab]
Total lipid (mg/ dL)	418.0	395.0	396.2	437.7	423.0

a, b: Means with different superscripts are significantly different (P<0.05).

Ileal pH and Intestinal Bacteria:

Ileal content pH was not affected by adding active yeast into laying hens diets (Table 5). However, there were a reduction in digesta pH of layers fed yeast level of 0.8% and 1.2% which recorded 6.00 and 6.31 respectively against 6.58 for control. Dawson et al. (1990) and Gibson and Roberfroid, (2008) observed a reduction in ruminal pH value of steers fed active yeast.

There was an effect yeast on bacterial total count which was sharply reduced when supplemented yeast level increased. The most reduction was recorded for the birds fed 1.6% live yeast (Table 5 and Fig. 1).

Table (5): Effect of feeding active yeast levels on pH value of ileal content and intestinal bacteria make-up.

Microbial Strains	Yeast Level				
	0.0%	**0.4%**	**0.8%**	**1.2%**	**1.6%**
Ileal content pH	6.58	6.88	6.00	6.31	6.58
Log 10 cfu./mg					
Bacterial total count	15	12.5	12.7	10.1	5.4
Escherichia coli	7.0	2.5	3.5	2.5	2.25
Lactobacilli sp.	6.0	4.25	15.1	10.0	8.5
Klebsiella sp.	1	1	N.d	1	1
Staphylococcus sp.	3	1	2	1	1
Proteus sp.	2	1	2	1	1
Micrococcus sp.	2	N.d	3	N.d	N.d
Combylobacter sp.	4	N.d	3	2	N.d
Closterdium perfringers	3	N.d	2	1	N.d

N.d: Non-dectable.

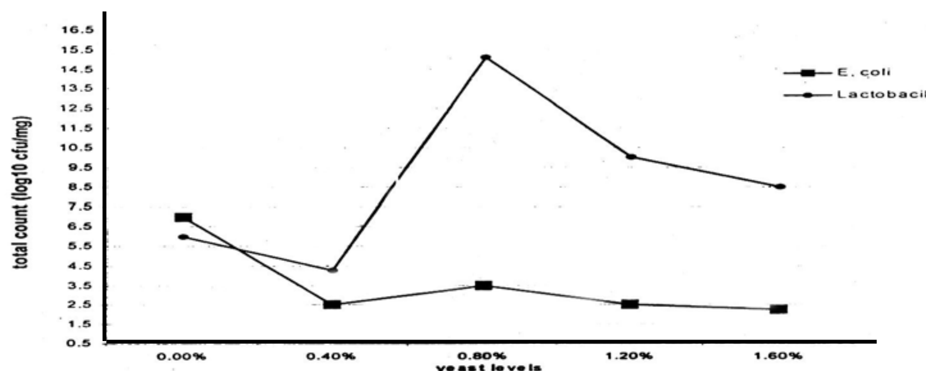

Figure (1): Effect of yeast level on total count of E.coli and lactobacilli sp..

The inhibitory effect of yeast on intestinal microflora had been established by Line et al. (1998); Wakwak et al. (2003) and Nava et al., (2005), who reported that, yeast has a reduction effect against pathogenic gut microflora.

Count of *Lactobacilli* bacteria increased due to adding active live yeast at 0.8%, 1.2% and 1.6% into laying hens diets. This result confirms those of Kim et al. (2002), who added *Pichia farinose* yeast strain into laying hens' diets and Park et al. (2002) and Kabir (2009), who included *Saccharomyces cervisiae* into broiler diets. Their results indicated an increase in viable count of ileal lactobacilli's due to adding live yeast.

The viable counts of Lactobacilli are inversely related to the pH value of ileal digesta (Table 5), where the reduction in pH values is associated with increasing Lactobacilli count. This may confirm that Lactobacilli bacterial grow well in slightly acidic media (Fuller, 2001).

Lactobacilli bacteria secrete lactic acid which reduces digesta pH so the reduction in pH value may be due to direct action of intestinal bacilli bacteria or to indirect effect of yeast on increasing intestinal bacilli bacteria. Live yeast enriched diet led to a sharply reduction in pathogenic bacterial strains of *E.coli* and *Campylobacter sp.* These strains usually cause mild to moderate gastroenteritis, diarrhea and mal-absorption of nutrients in chickens.

The current results are in agreement with those of Park et al. (2002), who stated that the counts of *closterdium perfringer* and E.coli bacteria were lower due to adding *Sacchatomyces cervisia* yeast into broiler chicks' diets. The antagonistic effect of live yeast against intestinal microflora was elucidated by Line et al. (1998) and Laegreid and Bauery (2004) who stated that, several harmful pathogenic bacteria have been shown to exhibit a binding specific for the sugar mannose. A live yeast cells contain mannose in their wall. This mannose in the cell wall may cause the yeast to act as a decoy for the attachment of pathogens. Because yeast has been demonstrated not to permanently colonize animals, the yeast and any yeast-bound pathogens pass out in the bird excretion and bacterial colonization is diminished.

Kabir et al., (2004) reported that probiotic microorganisms, once established in the gut, may produce substances with bactericidal or bacteriostatic properties (bacteriocins) such as lactoferrin, lysozyme, hydrogen peroxide as well as several organic acids. These substances have a detrimental impact on harmful bacteria, which is primarily due to a lowering of the gut pH. A decrease in PH may partially offset the low secretion of hydrochloric acid in the stomach. In addition, competition for energy and nutrients between probiotic and other bacteria may result in a suppression of pathogenic species. Numerous factors such as animal to animal variation, strain of yeast, and experimental procedures have contributed to the variation in results of yeast culture studies. However, the digestive advantages of enhanced nutrient digestibility, cecal fermentation and subsequent production parameters provide justification for nutritionists to continue to research yeast culture supplementation.

4. Conclusion:

It can be concluded that adding live yeast *Saccharomyces cervisiae* can enhance the productive performance of laying hens and nutrients utilization via the inhibitory effect of yeast against pathogenic bacteria which may cause mild enteritis and mal-absorption of nutrients.

Probiotics constitutes now an important aspect of applied biotechnological research and therefore as opposed to antibiotics and chemotherapeutic agents can be employed for growth promotion in poultry. Scientists now are triggering effort to establish the delicate symbiotic relationship of poultry with their bacteria, especially in the digestive tract, where they are very important to the well being of man and poultry (Kabir, 2009). Since probiotics do not result in the development and spread of microbial resistance, they offer immense potential to become an alternative to antibiotics. The present study reveals that probiotics could be successfully used as nutritional tools in poultry feeds for promotion of growth, modulation of intestinal microflora and pathogen inhibition, immunomodulation and promoting meat quality of poultry.

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Effects of dietary antibiotic growth promoter and *Saccharomyces cerevisiae* fermentation product on production, intestinal bacterial community, and nonspecific immunity of hybrid tilapia (*Oreochromis niloticus* female × *Oreochromis aureus* male)[1]

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ABSTRACT: To investigate the effects of a dietary antibiotic growth promoter (florfenicol) and a *Saccharomyces cerevisiae* fermentation product (DVAQUA) on growth, G:F, daily feed intake, intestinal bacterial community, and nonspecific immunity of hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂), a 16-wk feeding trial was conducted in a recirculating aquaculture system. Four feeding regimens were evaluated: control, dietary florenicol (0.02 g/kg; 16 wk), dietary DVAQUA (0.5 g/kg; 16 wk), and sequential use of florenicol (0.02 g/kg; 8 wk), and DVAQUA (0.5 g/kg; 8 wk). Each regimen had 4 replicate tanks (0.5 × 0.5 × 0.5 m) and each tank contained 12 fish (initial BW: 46.88 ± 0.38 g). Dietary florfenicol improved growth ($P = 0.089$), G:F ($P = 0.036$), and serum complement component concentrations ($P < 0.001$) of hybrid tilapia. However, the compound decreased the estimated intestinal bacterial count estimated by *rpo*B quantitative PCR ($P < 0.001$) and bacterial diversity (visual band numbers, Shannon diversity index, and Shannon equitability index based on 16S rDNA V3 denaturing gradient gel electrophoresis fingerprints) compared with the control. Although sequential use of florfenicol and DVAQUA improved growth and G:F numerically to a similar extent as dietary florfenicol, and increased intestinal bacterial count to normal quantities, the sequential use of florenicol and DVAQUA decreased intestinal bacterial diversity (visual band numbers, Shannon diversity index, and Shannon equitability index) as well as serum complement component concentrations ($P < 0.001$) compared with their respective use and the control. These findings might be negatively related to disease control and host defense, and the sequential use of florenicol and DVAQUA should be practiced with caution. Feeding DAVQUA to the fish improved nonspecific immunity and increased intestinal bacterial count and bacterial diversity, but further research, including challenge studies, should be conducted before recommendation of DVAQUA supplementation to hybrid tilapia diets.

Key words: antibiotic growth promoter, hybrid tilapia, intestinal microbiota, nonspecific immunity, *Saccharomyces cerevisiae* fermentation product

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INTRODUCTION

The use of dietary antibiotic growth promoters in aquaculture has been criticized for the potential development of antibiotic-resistant bacteria and the presence of antibiotic residues in resultant seafood products (Sapkota et al., 2008). Florfenicol has been approved for use in aquaculture in China to replace quinocetone since 2002 (MOA, 2002). However, no information is available about the effects of florfenicol on production, intestinal microbiota, and nonspecific immunity of fish, which are important factors with respect to disease control (Celli and Knodler, 2008) and host defense (Chow and Mazmanian, 2009).

The dietary *Saccharomyces cerevisiae* fermentation product (**DVAQUA**) has been reported to improve the growth and survival of shrimp (*Litopenaeus vannamaei*;

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Table 1. Ingredients and chemical compositions of the experimental diets (%; as-fed basis)[1]

Item	Control diet	Florfenicol-added diet	*Saccharomyces cerevisiae* fermentation product (DVAQUA) added diet
Ingredient			
Fish meal (60.2% CP)	4.00	4.00	4.00
Intestine casing meal (55.6% CP)	4.00	4.00	4.00
Corn gluten meal, Shandong (58.0% CP)	3.00	3.00	3.00
Soybean meal (44.4% CP)	6.00	6.00	6.00
Cottonseed meal (40.0% CP)	4.00	4.00	4.00
Rapeseed meal (38.0% CP)	12.00	12.00	12.00
Single-cell protein (73.8% CP)	4.00	4.00	4.00
Wheat middlings (16.7% CP)	26.00	26.00	26.00
Wheat flour (12.7% CP)	10.00	10.00	10.00
Bentonite	2.00	2.00	1.95
Rice bran (14.2% CP)	12.00	12.00	12.00
Soybean oil	3.00	3.00	3.00
Phospholipid oil	1.00	1.00	1.00
Vital wheat gluten (75.0% CP)	4.00	4.00	4.00
Corpuscle powder (95% CP)	2.00	2.00	2.00
DVAQUA	0	0	0.05
Florfenicol	0	0.002	0
Premix[2]	3.00	3.00	3.00
Chemical composition			
Moisture	5.82	4.94	5.32
CP	32.55	32.08	31.82
Crude lipid	5.68	5.47	5.76

[1]*Saccharomyces cerevisiae* fermentation product (DVAQUA) was supplied by Diamond V Mills Inc., Cedar Rapids, IA; florfenicol was provided by Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China; and other ingredients were obtained from Xinxin Feed Co. Ltd., Jiaxing, Zhejiang, China.

[2]According to Lv et al. (2007).

Burgent et al., 2004) and rainbow trout (*Oncorhynchus mykiss*; Barnes et al., 2006). Recently, it has been reported that dietary DVAQUA could enhance the nonspecific immunity of hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) and modulate the intestinal bacterial community, though its supplementation failed to stimulate BW gain and feed conversion efficiency (He et al., 2009). To reduce the use of dietary antibiotics and obtain satisfactory productions, some Chinese producers supplement the antibiotic growth promoter florfenicol and DVAQUA sequentially during the 16-wk feeding period based on the assumption that dietary DVAQUA can restore the intestinal bacterial community and the nonspecific immunity decreased by dietary florfenicol of the fish.

The aim of the present study was to investigate the effects of sequential use of dietary florfenicol and DVAQUA on growth, intestinal bacterial community, and nonspecific immunity of fish. Hybrid tilapia was chosen as they are a fish that is fairly easy to raise, fast-growing, and widely cultured in China (Lv et al., 2007).

MATERIALS AND METHODS

The experiment was conducted under the Guidelines for the Care and Use of Laboratory Animals (NRC, 1985).

Experimental Diets

Three isonitrogenous and isoenergetic experimental diets were formulated (Table 1). *Saccharomyces cerevisiae* fermentation product was supplied by a commercial company (DVAQUA, Diamond V Mills Inc., Cedar Rapids, IA). According to a previous study (He et al., 2009), the optimum amount of DVAQUA to feed, approximately 0.5 g/kg of diet, promoted nonspecific immunological function (serum complement component concentrations and head kidney macrophage phagocytic activity) of hybrid tilapia. Florfenicol (purity 99.8%) was supplied by another commercial company (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China). The feed was processed by an extrusion machine (Muyan Inc., Yangzhou, Jiangsu, China) at 120°C for 15 s (Li, 2001) and stored under dark and cold conditions until use. Moisture, protein, and lipid contents in the diets were determined as described before (Xie et al., 1997).

Feeding Trial

Juvenile hybrid tilapias (approximately 40 g) were obtained from a local aquaculture farm (Jiaxing, Zhejiang, China). Before the experiment, the fish were acclimatized for 2 wk in an indoor recirculating system at the Zhejiang Aquaculture Station of Feed Research

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Institute, Chinese Academy of Agricultural Sciences. After 1 d of fasting, 192 fish (46.88 ± 0.38 g) were randomly and evenly distributed into 16 tanks (0.5 × 0.5 × 0.5 m; water volume, 112.5 L) with 12 fish per tank. To evaluate the effects of florfenicol and DVAQUA on biological characteristics of cultured tilapia, 4 feeding regimens were used: control (16 wk; **CC**), dietary florfenicol (16 wk; **FF**), dietary DVAQUA (16 wk; **YY**), and dietary florfenicol (8 wk) and subsequently DVAQUA (8 wk; **FY**). Each feeding regimen had 4 replicate tanks. The fish were hand fed 1% of BW 3 times per day (0730, 1130, and 1730 h), and feed offered was adjusted weekly (He et al., 2009). Each tank was individually aerated, and about 20% of the tank water was exchanged with fully aerated tap water per day. Water temperature and water quality were monitored during the feeding period: rearing temperature, 25.4 ± 1.6°C; dissolved oxygen, >5.0 mg of O/L; pH, 7.7 ± 0.1; NH_4^+ N, < 0.50 mg of N/L; and NO_2^- N, < 0.05 mg of N/L. The photoperiod was 14 h light and 10 h dark with the light period from 0500 to 1900 h.

The following variables were determined: BW gain (**WG**, %) = 100 × [(final BW − initial BW)/initial BW]; G:F = (final BW − initial BW)/feed consumed; ADFI, g/d = (diet consumed/d)/fish number and % survival = 100 × (survival number/total number).

Denaturing Gradient Gel Electrophoresis Analysis

After 16 wk of feeding, 5 fish from each tank were randomly collected for the analysis of the gut microbiota. After 1 d of fasting, the fish were killed by a sharp blow on the head. The intestinal microbiota from each feeding regimen was sampled as described before (Zhou et al., 2009a). In the present study, pooled samples from 20 fish (5 fish/tank × 4 tanks; each with equal weight of gut wall) were used for gut microbiota evaluation. This procedure was carried out to avoid interindividual variations (Spanggaard et al., 2000; He et al., 2009), which have been demonstrated in several previous studies (Ringø et al., 2006; Zhou et al., 2009a,b). By using pooled samples, the general trend of the intestinal microbiota can be presented for each feeding regimen and can easily be compared. The baseline samples of gut microbiota (**BL**) were collected after the acclimation and 1 d of fasting. The denaturing gradient gel electrophoresis (**DGGE**) analysis of the autochthonous gut microbiota was carried out as described previously (Zhou et al., 2009a). Deoxyribonucleic acid extraction of gut microbiota, PCR reaction specific for bacterial 16S rDNA V3 regions, DGGE, and sequence analysis were carried out as described before (Liu et al., 2008). The gel images were analyzed using the public domain NIH Image program (National Institutes of Health, Bethesda, MD). Lanes were individually converted to linear plots. Intensity of PCR product bands was equivalent to peak heights and peak areas (Simpson et al., 1999). Peak areas were calculated and converted to percentage of total sample area. Representative sequences were deposited in the NCBI database (National Center for Biotechnology Information, Bethesda, MD) under accession numbers FJ746695 to 746696 and FJ746698 to 746715.

Relative abundance (%) was represented by the percentage of a specific band intensity to the total band intensity and defined as a statistically significant difference when the value was equal to or greater than 1.5 fold or less than one-half (Zhou et al., 2009b). The Shannon diversity index, $\boldsymbol{H} = -\Sigma\ RA_i \ln (RA_i)$, and Shannon equitability index, $\boldsymbol{E_H} = H/\ln (S)$, where RA_i is the proportion of the ith band and S is the total number of visual bands, were calculated (Dethlefsen et al., 2008).

Cluster analysis was performed based on the unweighted pair-group method using the arithmetic mean algorithm. In this study, similarity coefficients ($\boldsymbol{C_s}$) < 0.60 were regarded as a statistically significant difference, $0.60 \le C_s < 0.85$ as a marginal difference, and $C_s \ge 0.85$ was treated as similar.

Total Bacterial Count Estimated by RNA Polymerase β Subunit Gene Quantitative PCR

The total bacterial count of the pooled intestinal samples in each tank of a feeding regimen (n = 4) was estimated based on the predominant cultured bacteria identified by DGGE and RNA polymerase β subunit gene (***rpo*B**) quantitative PCR (Dolan et al., 2009). Briefly, several cultured bacteria were selected based on the predominant microbiota present in DGGE with an abundance index greater than 5%. In this study, *Pseudomonas* sp. B231 (gram-negative) and *Lactococcus lactis* ssp. *lactis* (gram-positive) were chosen as standards. Both bacterial species were cultured overnight in Luria-Bertani medium, and the total number of bacteria was counted using a hemocytometer as described by Alongi (1988). Thereafter, each bacterial strain was mixed equally at 0.5×10^8 cells/mL, and total genomic DNA was extracted from 1 mL of the combined mixture using cetyl trimethylammonium bromide (Griffiths et al., 2000) and lysozyme methods (Miller et al., 1999). The intestinal samples were thereafter precooled at −70°C for approximately 2 h and then freeze-dried (Martin Christ GmbH, Osterode, Germany) overnight. Then, DNA was extracted using approximately 200 mg of dried intestinal sample and purified as described previously. Serial dilutions of standards at 10^3, 10^4, 10^5, and 10^6 copies of the gene per reaction were prepared for calibration. The *rpo*B (1 copy in bacteria) was used for primer design. The primers were *rpo*B1698f (5′-AACATCGGTTTGATCAAC-3′) and *rpo*B2041r (5′-CGTTGCATGTTGGTACCCAT-3′; Dahllof et al., 2000). The reaction mixture (20 μL) was prepared according to the manufacturer's protocol: 7

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Table 2. Effects of the experimental feeding regimens on the growth, G:F, and survival of hybrid tilapia (n = 4)[1]

Item	CC	FF	YY	FY	Pooled SD	*P*-value
Initial BW, g	46.71	47.04	47.06	46.95	0.43	0.669
BW gain, %	301.48[a]	344.05[b]	319.29[a]	333.32[ab]	22.20	0.089
G:F, g/g	0.65[c]	0.75[a]	0.67[bc]	0.72[ab]	0.04	0.036
ADFI, g/d	1.73	1.74	1.74	1.73	0.01	0.665
Survival, %	95.84	97.92	89.58	97.92	7.32	0.364

[a–c]Within a row, means without a common superscript differ ($P < 0.10$).

[1]CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China): 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk).

μL of PCR-grade water, 1 μL of each primer (5 μ*M*), 10 μL of 2 × real-time PCR master mix (SYBR Green, Toyobo, Shanghai, China), and 1 μL of DNA template (50 ng/μL). The PCR conditions consisted of initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. The concentration of each standard in cfu/mL was input into the LightCycler 2.0 software using the threshold cycle value to construct a standard for absolute quantification analysis. The number of bacteria present in unknown samples (12 samples) was calculated based on the standard curves. Each sample was analyzed in at least quadruplicate.

Nonspecific Immunity Indexes

At the end of the feeding trial (16 wk), blood samples were obtained from the caudal vasculature by using a 1-mL syringe and 5-gauge needle within 2 min without use of anesthetics (Davis and Griffin, 2004). Five fish were randomly chosen from each tank (n = 20). The blood sample of each fish was transferred into an Eppendorf tube and centrifuged at 5,000 × *g* for 10 min at 4°C. The supernatant, serum, was stored at –20°C for lysozyme activity and complement component assay (He et al., 2009). After blood sampling, the head kidney was removed from the fish for macrophage phagocytic assay. Standard phagocyte isolation techniques were performed according to Secombes (1990), and the macrophage phagocytic index was determined as described previously (He et al., 2009).

Statistical Analysis

Results are presented as mean ± SD. Data were subjected to 1-way ANOVA to test the effects of different feed regimens. When significant differences were detected ($P < 0.10$ for production or 0.05 for estimated intestinal bacterial count and nonspecific immunity indexes), Fisher's LSD was used to compare mean values among feeding regimens. All statistical analyses were carried out using SPSS (Aspire Software International, Ashburn, VA).

RESULTS

Effects of the feeding regimens on WG, G:F, ADFI, and percentage survival of hybrid tilapia are shown in Table 2. No differences were observed in ADFI or percentage survival of hybrid tilapia in the experiment. Fish in FF showed greater ($P = 0.089$) WG and G:F ($P = 0.036$) than those observed in CC and YY, respectively. However, no differences were observed in WG and G:F between FF and FY.

Intestinal Bacterial Community

The C_s of the intestinal bacterial community of hybrid tilapia based on the PCR-DGGE fingerprints (Figure 1) are shown in Table 3. The bacterial gut community of CC was marginally different ($C_s = 0.77$) from that of BL. Furthermore, FF ($C_s = 0.67$) and FY ($C_s = 0.69$) showed marginal modulation of the intestinal bacterial community compared with CC. However, the gut microbiota of YY was similar ($C_s = 0.86$) compared with the CC group. The C_s value between FY and FF was large (0.90), whereas C_s between FY and YY or CC was smaller ($C_s = 0.79$ or 0.69, respectively.

Twenty representative bands were retrieved from DGGE, in which 9 of them were unculturable bacteria (Table 4). In the present study, *Actinobacteria* (3 bands), *Proteobacteria* (11 bands), and *Firmicutes* (2 bands) were the predominant bacteria in the intestine of hybrid tilapia. Compared with CC, FF reduced the intensity of 9 bands (bands 2, 7, 10, 12, 17, 18, 19, 20, and 21) but increased intensity of band 15. By feeding the fish YY, bands 13, 14, 15, and 16 showed increased intensity, but decreased intensity was noted for bands 2, 17, 19, and 20. The band pattern of FY showed some similarity to FF except that FY increased the intensity of bands 1, 13, 15, and 16.

Intestinal Bacterial Diversity

Total counts of bacteria estimated by quantitative PCR in the tilapia intestine after 16 wk of feeding were greater ($P < 0.001$) compared with those of BL (Figure 2a). Total counts of bacteria in the gut of FF tilapia

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Table 3. Pairwise similarity coefficients (C_s) matrix for intestinal bacterial community of hybrid tilapia under the experimental feeding regimens[1]

Item	BL	CC	FF	YY	FY
BL	1.00				
CC	0.77*	1.00			
FF	0.64*	0.67*	1.00		
YY	0.80*	0.86^{ns}	0.69*	1.00	
FY	0.67*	0.69*	0.90^{ns}	0.79*	1.00

[1]BL = baseline sample; CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China): 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk). Similarity coefficients (C_s): $0.60 \leq C_s < 0.85$ is regarded as a marginal difference (*), and $C_s \geq 0.85$ is treated as similar (ns).

were less ($P = 0.010$) compared with CC, YY, and FY, respectively. However, no differences were observed among the CC, YY, and FY groups. Visual band numbers based on PCR-DGGE fingerprints in the FF and FY groups were less than in the CC and YY groups (Figure 2b). Furthermore, FF and FY decreased H and E_H of the intestinal microbiota (Figure 2c and 2d). Although FY slightly increased visual band number compared with FF, the FY regimen further decreased H and E_H compared with FF.

Nonspecific Immunity Indexes

Dietary DVAQUA (YY), FF, and FY did not affect serum lysozyme activity compared with CC (Figure 3a). However, serum complement component concentrations (C3 and C4) were increased ($P < 0.001$) in groups FF and YY, whereas FY showed decreased ($P < 0.001$) complement component concentrations compared with the other feeding regimens (Figure 3b and 3c). Head kidney macrophage phagocytic index for the YY group was the largest ($P < 0.001$) and that in the FY group was the second largest ($P = 0.081$), but no difference was observed between CC and FF (Figure 3d).

DISCUSSION

The disadvantages of using culture-dependent techniques have been discussed in several previous reports; the techniques are time-consuming and inaccurate and cannot detect unculturable bacteria (Cahill, 1990; Ringø et al., 2006). Therefore, there has been a general trend toward culture-independent approaches without involving bacterial cultivation (Huber et al., 2004; Pond et al., 2006; Kim et al., 2007). The use of the genetic fingerprint method based on PCR amplification of 16S rDNA and DGGE in the present study provides a rapid survey of the intestinal microbial community in tilapia, and 45% (9 out of 20) of the representative bands successfully retrieved were unculturable bacteria.

In the present study, the samples were processed identically for each feeding regimen, including sampling after 1 d of fasting at the end of the feeding trial, and bias could therefore be excluded when making the comparison. However, one should be aware that the sam-

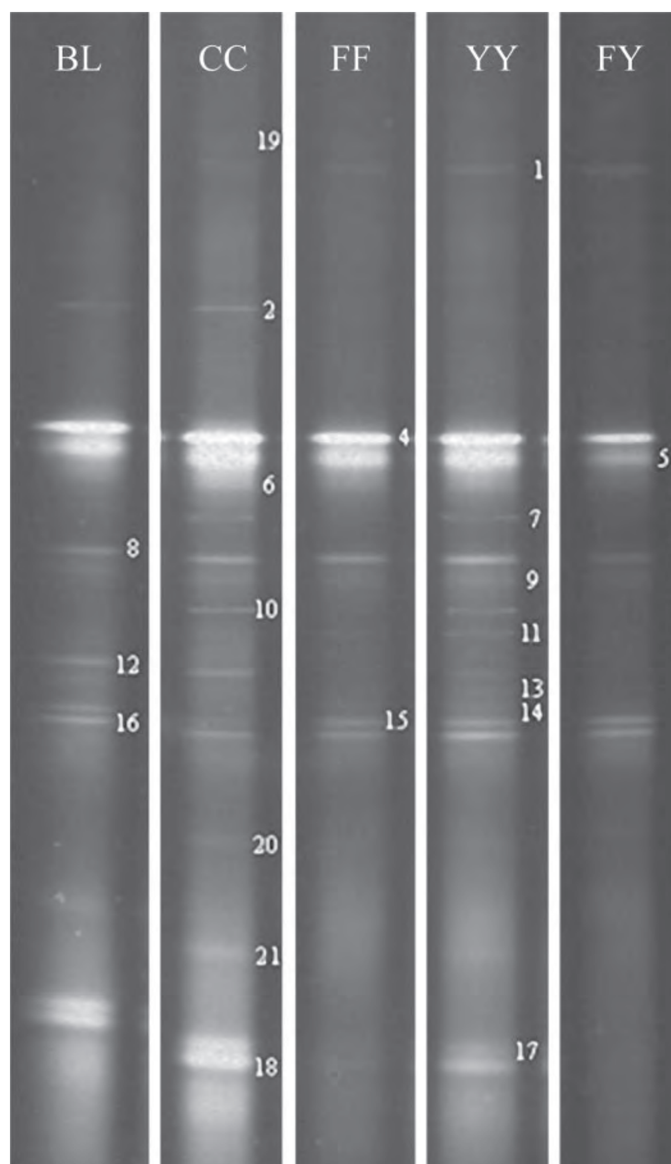

Figure 1. Denaturing gradient gel electrophoresis profile of the V3–16S rDNA regions of the gut microbiota of hybrid tilapia *Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂. BL = baseline sample; CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China); 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk); 1 to 2 and 4 to 21 = the order of the bands in the denaturing gradient gel electrophoresis profile.

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Table 4. Representative of bacteria or clones and their abundance isolated from the intestine of hybrid tilapia under the experimental feeding regimens[1]

Phylogenetic group	Band No.	Accession No.	Relative abundance, %					Closest relative (obtained from BLAST search)	Identity, %
			BL	CC	FF	YY	FY		
Actinobacteria	8	FJ746702	6.7	8.8	11.3	13.9	6.1	*Acinetobacter* sp. (FM865882.1)	100
	17	FJ746711	7.3[a]	5.7[a]	0[b]	1.9[c]	0[b]	*Leifsonia* sp. (FJ772014.1)	100
	21	FJ746715	4.4[a]	3.3[a]	0[b]	2.8[a]	0[b]	Uncultured *Actinobacterium* (AM690843.1)	100
Proteobacteria	1	FJ746695	0[a]	1.1[b]	1.9[bc]	2.5[bc]	4.6[c]	*Pseudomonas migulae* (FJ715746.1)	100
	2	FJ746696	3.3[a]	3.2[a]	0[b]	0[b]	0[b]	*Pseudomonas plecoglossicida* (EU834261.1)	100
	5	FJ746699	18.3[ab]	14.3[ab]	29.8[a]	18.1[ab]	9.5[b]	*Pseudomonas fluorescens* (FJ787327.1)	100
	9	FJ746703	2.4	3.3	2.6	3.2	1.8	*Escherichia coli* (AM157447.1)	100
	10	FJ746704	0[a]	3.7[b]	0[a]	3.0[b]	0.3[c]	Uncultured *Acinetobacter* sp. (FJ268994.1)	100
	11	FJ746705	0[a]	1.7[b]	0.8[bc]	1.2[b]	0.4[c]	*Shigella boydii* (CP001063.1)	100
	12	FJ746706	5.6[a]	4.3[ab]	0[c]	1.5[b]	0[c]	*E. coli* (FJ823386.1)	100
	13	FJ746707	0.3[ac]	0[b]	0[b]	0.2[a]	0.8[c]	Uncultured *Proteobacterium* (EF701302.1)	94
	14	FJ746708	0[a]	0[a]	0[a]	0.8[b]	0[a]	Uncultured *α-Proteobacterium* (FJ517741.1)	100
	16	FJ746710	5.7[ab]	5.2[a]	5.3[a]	9.5[bc]	9.6[c]	*Brevundimonas vesicularis* (EU862355.1)	99
	18	FJ746712	6.3[a]	15[a]	0[b]	9.5[a]	0[b]	Uncultured *Alcaligenes* sp.(FJ572672.1)	100
Firmicutes	15	FJ746709	1.7[ab]	0.4[a]	1.4[b]	2.2[bc]	4.7[c]	*Clostridium* sp. (EU862317.1)	99
	4	FJ746698	35.0	21.4	42.1	22.1	60.7	*Lactococcus lactis* (FJ824739.1)	100
Unclassified bacteria	6	FJ746700	2.9	4.6	4.4	4.1	2.0	Uncultured bacterium (EU777814.1)	100
	7	FJ746701	0[a]	3.3[b]	0[a]	3.3[b]	0[a]	Uncultured bacterium (EU537702.1)	100
	19	FJ746713	0[a]	3.0[b]	0[a]	0[a]	0[a]	Uncultured bacterium (EU539943.1)	99
	20	FJ746714	0[a]	1.6[b]	0[a]	0[a]	0[a]	Uncultured bacterium (AY707559.1)	100

[a–c]Within a row, means without a common superscript differ (see the definition of difference below).

[1]Difference = value (the relative abundance of a specific band) out of the range of less than 1.5 fold or larger than one-half of others in the same row. BL = baseline sample; CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China): 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk).

pling protocol might alter the gut microbial diversity because animals shed the lining of the intestine when it is empty (Chang and Liu, 2002). We therefore suggest that the effects of fasting on the gut microbiota of tilapia merit further investigation.

Although the intestinal microbiota of hybrid tilapia were sensitive to dietary florfenicol, resulting in a decrease of total intestinal bacterial counts and visual band number based on DGGE fingerprints, differences in the composition of intestinal bacteria as influenced by feeding tilapia florfenicol were observed. The relative abundance of *Leifsonia* sp.-like, *Pseudomonas plecoglossicida*-like, *Escherichia coli*-like, and 6 uncultured bacteria were modulated by dietary florfenicol, whereas *Clostridium* sp.-like bacteria seemed to be less affected. The reason why *Clostridium* spp. are less sensitive to dietary florfenicol has not been elucidated and should be a topic of further studies because the relationship between the insensitivity of clostridia and antimicrobial resistance is less known in fish (Akinbowale et al., 2007).

Researchers reported previously that florfenicol had no effect on the antibody production and quantity of blood leukocytes in rainbow trout (*Oncorhynchus mykiss* Walbaum), but could suppress the chemiluminescence response/phagocytic cells (granulocytes) after 5 to 6 wk (Lunden et al., 1999). In the present study, we observed that serum lysozyme activity and head kidney macrophage phagocytic index were unaffected by dietary florfenicol, whereas serum complement component concentrations were stimulated by dietary florfenicol. These results are in contrast to those reported by Lunden et al. (1999) in rainbow trout. The reason for this difference is not clear but might be due to the reduced supplementation (20 mg/kg) used in the present study compared with 3,000 mg/kg used by Lunden et al. (1999).

In a recent study with hybrid tilapia, He et al. (2009) concluded that various bacteria (uncultured *Mycobacterium* sp.-like, *Cetobacterium somerae*-like, and uncultured *Flavobacteria* bacterium-like) were selectively stimulated by dietary DVAQUA, whereas *Escherichia coli*-like, uncultured bacilli-like, and *Pseudomonas fluorescens*-like bacteria were decreased. In the present study, the gut bacteria; *Leifsonia* sp.-like, *Pseudomonas plecoglossicida*-like, and 2 uncultured bacteria were

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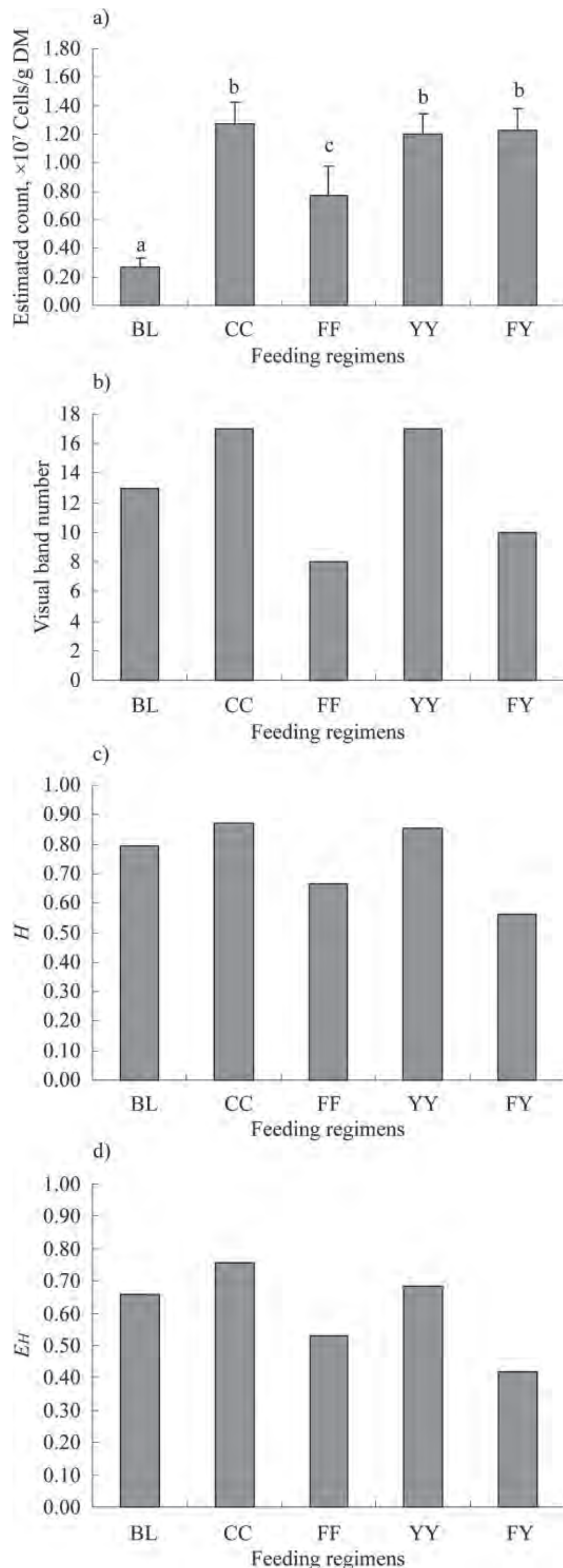

Figure 2. Effects of the experimental feeding regimens on the intestinal bacterial diversity of hybrid tilapia. Data (mean ± SD) sharing a common letter (a–c) are not different ($P < 0.05$). BL = baseline sample; CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China); 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk). a) Count; b) visual band number; c) Shannon diversity index (H); and d) Shannon equitability index (E_H).

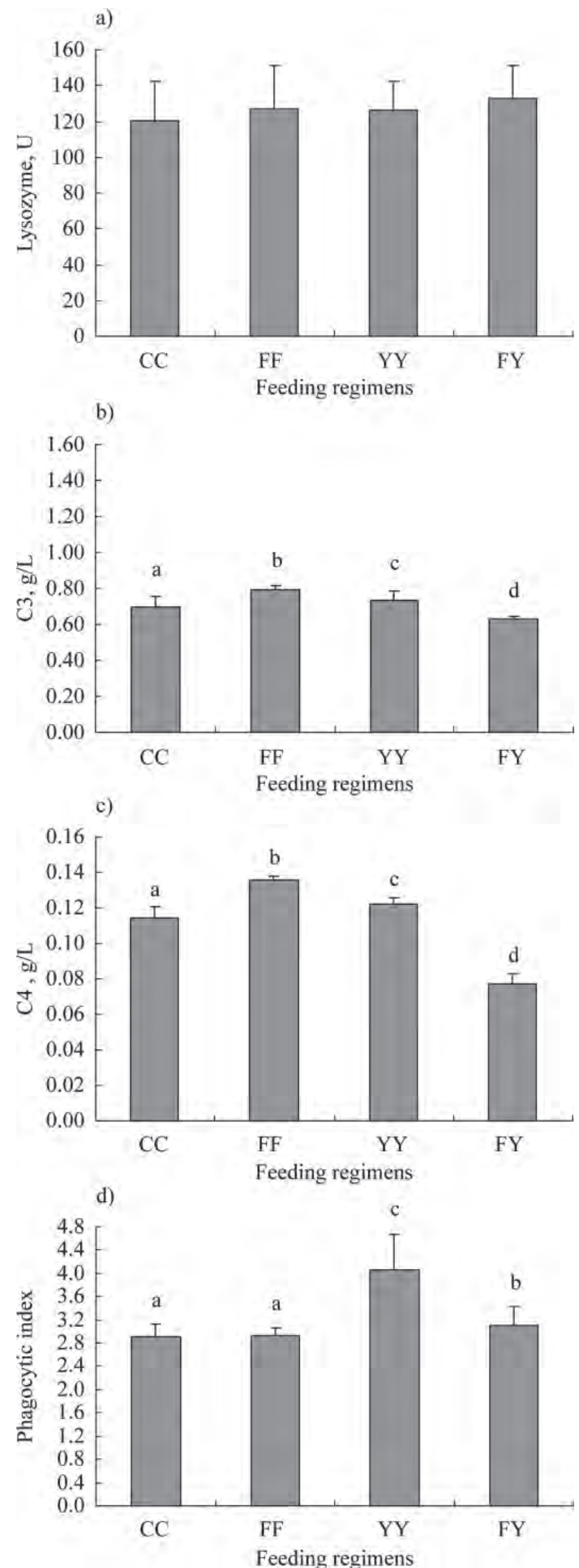

Figure 3. Effects of the experimental feeding regimens on the nonspecific immunity of hybrid tilapia. Data (mean ± SD) sharing a common letter (a–d) are not different ($P < 0.05$). CC = the control (16 wk); FF = dietary antibiotics [florfenicol (Zhejiang Hisoar Pharmaceutical Co. Ltd., Taizhou, Zhejiang, China); 16 wk]; YY = dietary *Saccharomyces cerevisiae* fermentation product [DVAQUA (Diamond V Mills Inc., Cedar Rapids, IA); 16 wk]; FY = sequential use of dietary florfenicol (8 wk) and DVAQUA (8 wk). a) Lysozyme [unit (U)/mL]; b) C3 (serum complement component C3 concentration); c) C4 (serum complement component C4 concentration); and d) phagocytic index.

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decreased by dietary DVAQUA, whereas *Clostridium* sp.-like and 2 uncultured bacteria were stimulated. As our results are different from those reported by He et al. (2009), we suggest that this might be due to different rearing conditions [i.e., the indoor recirculating aquaculture system with aerated tap water in the present study compared with the net cages in a pond used by He et al. (2009)]. On the other hand, Zhou et al. (2009a) reported that autochthonous *Clostridium* sp.-like bacteria were stimulated in juvenile hybrid tilapia fed DVAQUA in a similar recirculation aquaculture system as used in the present study.

Based on the work of He et al. (2009) that showed selective stimulation of intestinal microbiota by dietary DVAQUA, we put forward the hypothesis that feeding hybrid tilapia florfenicol for 8 wk and subsequent use of DVAQUA for the next 8 wk decrease the intestinal bacterial diversity. This hypothesis was confirmed in the present study that the sequential use of dietary florfenicol and DVAQUA could re-establish the intestinal bacterial count to a normal CC level, but failed to re-establish microbial richness (visual band number) and diversity (H and E_H) to the normal level. The increase of *Clostridium* sp.-like bacteria from the sequential use of florfenicol and DVAQUA might be due to its insensitivity to dietary florfenicol and stimulation of DVAQUA, but verification merits further investigations. Some *Clostridium* spp. (e.g., *Clostridium perfringens*), have been reported to be important pathogens that colonize the animal gastrointestinal tract and cause necrotic enteritis and subclinical disease (Barbara et al., 2008). The insensitivity of *Clostridium* sp.-like bacteria to dietary antibiotics observed in the present study could probably be ascribed to its resistance to dietary florfenicol (Akinbowale et al., 2007).

In summary, dietary florfenicol decreased intestinal bacterial count and bacterial diversity compared with the control. Sequential use of florfenicol and DVAQUA decreased intestinal bacterial diversity as well as serum complement component concentrations compared with their respective use and the control, which might be negatively related to host defense. Therefore, we recommend that the sequential use of florfenicol and DVAQUA should be practiced with caution. Feeding hybrid tilapia dietary DVAQUA improved nonspecific immunity and increased intestinal bacterial count and bacterial diversity. However, before using DVAQUA, challenge studies should be included as a standard to assess the effects on fish health.

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Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*)

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Abstract

This study evaluated the effects of three types of probiotics, two bacteria and one yeast on growth performance in Nile tilapia. Three diets were formulated containing the optimum protein level (40%) for tilapia fry: one supplemented at 0.1% with a bacterial mixture containing *Streptococcus faecium* and *Lactobacillus acidophilus*; a second supplemented at 0.1% with the yeast *Saccharomyces cerevisae*; and a third, a control diet without supplements. Two additional diets were formulated to contain 27% protein to serve as a stress factor, and were supplemented at 0.1% with either the bacterial probiotic mix or the yeast. The diets were fed for 9 weeks to tilapia fry stocked in 20-l tanks at two densities: a high density of 20 fry per tank as a stress factor; and a low density of 10 fry per tank. Results indicate that the fry fed with diets with a probiotics supplement exhibited greater growth than those fed with the control diet. Of the four probiotic treatments, the 40% protein diet supplemented with yeast produced the best growth performance and feed efficiency, suggesting that yeast is an appropriate growth-stimulating additive in tilapia cultivation.
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Keywords: Probiotics; Tilapia; *Oreochromis niloticus*; *Streptococcus*; *Lactobacillus*; *Saccharomyces*

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1. Introduction

The demand for animal protein for human consumption is currently on the rise and is largely supplied with terrestrial farm animals. Aquaculture, however, is an increasingly important option in animal protein production. This activity requires high-quality feeds with high protein content, which should contain not only necessary nutrients but also complementary additives to keep organisms healthy and favor growth. Some of the most utilized growth-promoting additives include hormones, antibiotics, ionophores and some salts (Fuller, 1992; Góngora, 1998; Klaenhammer and Kullen, 1999). Though these do promote growth, their improper use can result in adverse effects in the animal and the final consumer, as well as lead to resistance in pathogenic bacteria in the case of antibiotics.

Though probiotics are widely used in poultry and swine rearing, little has been done to incorporate them into aquaculture. Thus, this study was designed to evaluate the use of a bacteria mix containing *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae*, as probiotic supplements in diets for Nile tilapia fry.

2. Materials and methods

The experiment was conducted for 9 weeks, using 3-week old Nile tilapia (*Oreochromis niloticus*, 152.3 mg average weight) fry obtained from the Aquaculture Laboratory of CINVESTAV in Mérida, Yucatán, Mexico.

The experimental system was a closed recirculation system consisting of 40 self-cleaning 20-l plastic tanks, sedimentation tanks, a biological filter and a UV filter to prevent cross-contamination of microorganisms between treatments. The system was installed in an environment-controlled laboratory maintained at 22 °C, with a photoperiod of 12 h light and 12 h darkness. Water in the system was maintained at a temperature of 28 °C with two 2000-W bayonet-titanium heaters, and the dissolved oxygen level was controlled by adjusting water flow into each tank to 1 l min^{-1}. For water quality control, temperature and dissolved oxygen were measured daily, and weekly analyses were done of total ammonium, nitrite, nitrate and pH levels, using standard methods (APHA, 1989). The following values ($\pm$ S.D.), appropriate for tilapia cultivation, were used: temperature, 28.83 ± 0.45 °C; dissolved oxygen, 6.17 ± 1.64 mg l^{-1}; pH, 8.46 ± 0.32; ammonia, 0.07 ± 0.02 mg l^{-1}; nitrite, 0.07 ± 0.03 mg l^{-1}; nitrate, 5.93 ± 0.61 mg l^{-1}.

2.1. Experimental diets

Five isocaloric diets were formulated: three containing 40% protein, and two with 27% protein. The lower protein in the latter diets was used as a stress factor because at this growth stage, the optimum protein level for tilapia is 40% (Tacon, 1984). A commercial probiotic for terrestrial vertebrates based on *S. faecium* and *L. acidophilus* (ALL-LAC™, AllTech, Nicholasville, KY) was added to one of the 40% protein diets (ALL40) and one of the 27% protein diets (ALL27). The yeast (*S. cerevisiae*, BioSaf™, SafAgri, Minneapolis, MN) was added to separate 40% (Y40) and 27% (Y27) protein diets. Finally, a control diet was formulated with 40% protein and no supplements (CON40). To all five

diets, 0.5% chromic oxide was added as a marker for determining digestibility. Table 1 shows diet formulation and proximate composition.

Population density in the tanks was also used as a stress factor, under the assumption that overpopulation is one of the main growth-inhibiting factors in intensive aquaculture systems. To this end, 20 tanks were stocked at a density of 10 organisms per tank (one fry per 2 l) and the other 20 tanks at 20 organisms per tank (one fry per liter). All fry had similar average initial weights. The different diet formulations were assigned within the tanks in each density set so that for each protein level there were four diets within each density category. The animals were allowed to adapt to the experimental system for a week, and fed with a conventional diet, after which time the different treatments were randomly assigned to the tanks, with four replicates per treatment.

Feed was manually administered ad libitum four times a day, for 9 weeks. A daily record was kept of feed offered. Bulk weight was measured weekly to follow growth in weight and calculate survival and feeding ration. Briefly, the fish were taken from each tank using a net previously disinfected with a 1% benzalkonium chloride solution. This was then passed over fabric towels to eliminate excess moisture and the fish weighed on an electronic balance. Every third day, each tank was partially cleaned and the water partially changed. Once a week, the same day bulk weight measurement was done, the tanks were completely cleaned and a total change of water in the system carried out.

Beginning in the third week of the experiment, feces were collected by siphoning the tank 30 min after the second daily feeding to minimize leaching. Scales were removed

Table 1
Formulation and proximate composition of experimental diets

Ingredients (%)	Diets				
	CON40	ALL40[a]	Y40[b]	ALL27	Y27
Anchovy fish meal	54.23	54.23	54.23	36.60	36.60
Cod liver oil	0.00	0.00	0.00	1.85	1.85
Soybean oil	3.26	3.26	3.26	6.40	6.40
Yellow corn starch	34.50	34.40	34.40	47.04	47.04
Mineral premix[c]	1.50	1.50	1.50	1.50	1.50
Vitamin premix[d]	3.00	3.00	3.00	3.00	3.00
Carboxy methyl cellulose	3.00	3.00	3.00	3.00	3.00
Chromic oxide	0.50	0.50	0.50	0.50	0.50
Probiotic	0.00	0.10	0.10	0.10	0.10
Proximate composition (% wet weight)					
Moisture	7.30	6.63	6.48	7.27	7.43
Crude protein	39.56	39.92	37.91	25.84	26.49
Ether extract	8.02	8.04	7.47	9.53	9.69
Ash	9.55	10.14	9.77	9.17	9.32
Nitrogen-free extract	34.03	33.53	36.55	44.53	43.04
Gross energy (kJ g^{-1})	19.954	19.753	20.087	19.830	19.920

[a] ALL = Bacterial mixture.
[b] Y = Yeast.
[c] Jauncey and Ross, 1982.
[d] Tacon, 1984.

from the collected feces, the feces was oven dried at 105 °C for 24 h, and then stored in hermetic containers under refrigeration until analysis.

2.2. Chemical analyses

Proximate chemical analyses were made of diet ingredients and a sample of fish at the beginning and end of the experiment according to standard methods (A.O.A.C., 1992). Gross energy in the feed was determined by combustion in a Parr adiabatic calorimeter. To evaluate digestibility, the chromic oxide content of each diet and the collected feces were analyzed using the acid digestion method (Furukawa and Tsukahara, 1966). Protein content was also determined for the feces to assess protein digestibility.

2.3. Statistical methods

Growth performance and feed utilization efficiency parameters were statistically compared using a one-way ANOVA ($P<0.05$), and differences among means were identified using the Duncan Multiple Range Test. Analyses were carried out with the STATISCA ver. 4.3 (1993) and StatGraphics Plus ver. 2.1 (1996) computer softwares. Arcsin transformations were done when necessary.

3. Results

Of the five experimental diets and the two density categories, the 40% protein diet supplemented with yeast administered to the 10-fry density treatment (Y40/10) produced the best growth rate (Table 2). All the diets supplemented with yeast showed better results than those with the microbial mixture and control diets, though ALL27/10 and ALL27/20 showed similar responses to Y27/20. The four diets ALL40/10, ALL40/20, CON40/10 and CON40/20 had the lowest growth performance.

Table 2 shows growth and feed utilization data. Fish fed with the CON40/10 and CON40/20 diets had statistically lower survival than those fed with probiotic-supplemented diets ($P<0.05$). The highest survival was recorded with the probiotic treatments.

The addition of yeast to the diets with optimum protein content (40%) administered in the low organism density tanks (Y40/10) produced the best growth (individual weight gain, IWG; specific growth rate, SGR), with values statistically higher than the other treatments ($P<0.05$). The diets supplemented with probiotics produced an IWG and SGR significantly higher than the control diets ($P<0.05$).

The ALL40/10 treatment had the statistically highest feed conversion ratio of the probiotic-supplemented diets, though all the other probiotic-supplemented diets had feed conversion ratios significantly lower than those for the control diets ($P<0.05$). The best conversion ratio was recorded for the Y40/20, Y40/10, Y27/20 and ALL27/20 treatments. In general, fish fed with the diets supplemented with yeast showed better feeding efficiency than those fed with diets containing the bacterial mixture.

The Protein Efficiency Ratio (PER) was significantly higher in the treatments containing 27% protein supplemented with probiotics and administered to tilapia at high densities

Table 2
Growth and feeding performance of fish fed diets supplemented with probiotics

Mean values[1]	Diets										
	CON40/10	CON40/20	Y40/10	Y40/20	Y27/10	Y27/20	ALL40/10	ALL40/20	ALL27/10	ALL27/20	± S.E.M.[2]
Survival (%)	75.00^{ab}	64.81^{a}	87.50^{bc}	92.59^{c}	91.67^{bc}	96.29^{c}	91.67^{bc}	85.18^{bc}	95.83^{c}	85.18^{bc}	5.163
Initial mean weight (g)	0.156^{a}	0.152^{a}	0.154^{a}	0.150^{a}	0.156^{a}	0.149^{a}	0.151^{a}	0.148^{a}	0.153^{a}	0.156^{a}	
Final mean weight (g)	1.285^{a}	1.439^{a}	6.164^{d}	4.570^{bc}	4.831^{c}	4.026^{bc}	1.910^{a}	2.081^{a}	4.145^{bc}	3.826^{c}	0.264
SGR (% day^{-1})[4]	3.33^{a}	3.57^{a}	5.80^{d}	5.43^{c}	5.46^{cd}	5.24^{c}	4.03^{b}	4.19^{b}	5.23^{c}	5.10^{c}	0.119
FCR[6]	3.113^{e}	3.260^{e}	1.430^{abc}	1.010^{a}	1.620^{bc}	1.170^{ab}	4.130^{f}	2.220^{d}	1.620^{bc}	1.170^{ab}	0.153
PER	0.830^{ab}	0.780^{ab}	1.890^{c}	2.640^{d}	2.260^{c}	3.170^{e}	0.610^{a}	1.140^{b}	2.230^{c}	3.380^{e}	0.121
CND[7]	0.442^{a}	0.482^{a}	1.902^{c}	2.402^{d}	2.227^{cd}	3.150^{e}	0.565^{a}	1.035^{b}	2.422^{d}	3.472^{e}	0.136
ANU (%)[8]	13.58^{a}	12.44^{a}	30.35^{c}	42.70^{d}	32.95^{c}	47.30^{d}	9.62^{a}	19.27^{b}	32.95^{c}	47.51^{d}	1.843
AOMD (%)[9]	89.59^{bc}	71.30^{a}	96.18^{cd}	96.90^{cd}	90.27^{c}	92.97^{cd}	75.35^{a}	84.78^{b}	97.16^{d}	97.10^{d}	2.967
APD (%)[9]	94.28^{cd}	68.79^{a}	97.75^{cd}	98.46^{d}	91.78^{c}	96.21^{cd}	95.53^{cd}	84.78^{b}	97.16^{cd}	98.25^{d}	2.686

[3]Individual weight gain, IWG (mg day^{-1}) = 1000[(Σ weekly WG)/time (days)].

[5]Individual feed intake, IFI (mg day^{-1}) = 1000[(Σ weekly FI)/time (days)].

[1] Values with the same superscript in the same row are not statistically different ($P < 0.05$).

[2] Standard error, calculated from the mean-square error of the ANOVA.

[4] Specific Growth Ratio, SGR (% day^{-1}) = 100 ((log final b.w. − log initial b.w.)/time(days)).

[6] FCR = IFI/IWG.

[7] Carcass N deposition, CND (mg day^{-1}) = 1000[(final body weight × percent final carcass protein) − (initial weight × percent initial carcass protein)]/100/time (days)/6.25.

[8] Apparent N utilization, ANU(%) = 100(CND/N intake).

[9] Apparent organic matter and protein digestibility.

Table 3
Carcass proximate composition of fish fed diets supplemented with probiotics

Composition (%)	Initial	Diets									
		CON40/10	CON40/20	Y40/10	Y40/20	Y27/10	Y27/20	ALL40/10	ALL40/20	ALL27/10	ALL27/20
Moisture	81.09	73.36^{a}	74.26^{a}	73.40^{a}	73.09^{a}	73.46^{a}	74.10^{a}	73.85^{a}	73.19^{a}	73.70^{a}	73.90^{a}
Crude protein	11.80	15.61^{bc}	15.72^{bc}	15.83^{bc}	16.22^{c}	14.72^{ab}	14.55^{ab}	15.39^{bc}	16.39^{c}	15.50^{bc}	13.77^{a}
Crude lipid	3.06	5.64^{a}	7.76^{bc}	8.37^{c}	7.03^{b}	10.25^{d}	9.86^{d}	7.84^{bc}	7.32^{bc}	9.50^{d}	10.21^{d}
Ash	2.46	3.49^{a}	3.76^{cd}	3.56^{b}	3.54^{a}	3.72^{c}	3.81^{d}	3.73^{c}	3.55^{ab}	3.52^{a}	3.85^{d}

Values with the same superscript in the same row are not statistically different ($p > 0.05$).

(Y27/20 and ALL27/20) than in other treatments (Table 2). The lowest PER was recorded for the ALL40/10 and the control treatments. For these same fish, Apparent Nitrogen Utilization (ANU) was significantly greater in comparison with the other treatments. The lowest apparent biological value was observed in the control diets, with results significantly lower than those obtained with the diets including probiotics.

In general, Apparent Organic Matter Digestibility (AOMD) and Apparent Protein Digestibility (APD) values were variable among the treatments. The maximum value was seen in the ALL27/10 treatment, though this was not statistically different from the ALL27/20, Y40/10 and Y40/20 treatments. In contrast, digestibility results for the control diets, mainly CON40/20, were lower than those for the probiotic-supplemented diets, but APD for the low population control treatment (CON40/10) showed better results than those for CON40/20, suggesting an adverse effect of overcrowding on digestibility performance.

No statistical differences were observed in carcass moisture content between treatments (Table 3). Differences were observed in carcass protein content, with the highest value recorded in fish fed with the ALL40/20 and Y40/20 diets, which were statistically different from values produced in the ALL27/20, Y27/20 and Y27/10 treatments. Carcass protein was clearly related to dietary protein, with the lowest values in fish fed with the 27% protein diets. Carcass lipid content was also affected by dietary protein content, with the highest values in the 27% protein treatments, which were statistically different from the 40% protein treatments. The lowest overall lipid content was recorded for the CON40/10 treatment, which was statistically different from all other treatments. Statistical differences were observed also in the carcass ash content among the fish fed with the different diets, but it was not possible to establish a relation of this parameter with the protein level, fish density or with the type of probiotic used in each treatment.

4. Discussion

All the probiotic-supplemented diets resulted in growth higher than that of the control diets, suggesting that the addition of probiotics mitigated the effects of the stress factors. This resulted in better fish performance, with better growth results in the diets supplemented with the yeast. Similar results were observed by Vázquez-Juárez et al. (1993) when yeast isolated from the intestines of wild rainbow trout was introduced into the digestive tracts of domestic rainbow trout, producing a significant increase in the growth of the cultured trout. In contrast, the use of the bacterial mixture in the optimum protein diets at either density caused no significant growth increases when compared to the control and yeast treatments. These results may be explained by the greater adaptive capacity of yeasts in aquatic environments in contrast to bacteria such as *Lactobacillus* and *Streptococcus*. It is also necessary, however, to consider the possibility of interspecies differences, as suggested by Noh et al. (1994), who studied the effect of supplementing common carp feeds with different additives, including antibiotics, yeast (*S. cerevisiae*) and bacteria (*S. faecium*). They observed better growth response with probiotic-supplemented diets, but obtained the best growth with a bacterium, not a yeast. Similar results were reported by Bogut et al. (1998), who fed common carp diets supplemented with *S. faecium*, reporting that the bacterium has a better probiotic additive for carp than yeast, clearly in contrast to the present results for tilapia.

The best FCR values observed with probiotic-supplemented diets suggest that addition of probiotics improved feed utilization even under stress conditions, with yeast being the most effective of the supplements tested in the present study. Similar results have been reported for probiotics use in diets for piglets (Gil, 1998a). In practical terms, this means that probiotic use can decrease the amount of feed necessary for animal growth which could result in production cost reductions.

The PER and ANU results indicate that supplementing diets with probiotics significantly improves protein utilization in tilapia. This contributes to optimizing protein use for growth, a significant quality given that protein is the most expensive feed nutrient. The improvement in the biological value of the supplemented diets in those treatments with high population and low dietary protein demonstrated that the probiotics supplements performed more efficiently in stress situations (Ringo and Gatesoupe, 1998).

The better digestibility obtained with the supplemented diets suggests that the addition of probiotics improved diet and protein digestibility, which may in turn explain the better growth and feed efficiency seen with the supplemented diets. Similar results were obtained by De Schrijver and Ollevier (2000), who reported a positive effect on apparent protein digestion when supplementing turbot (*Scophthalmus maximus*) feeds with the bacteria *Vibrio proteolyticus*. They attribute this effect to the proteolytic activity of bacteria. The lower digestibility values for the CON40/20 treatment support this supposition. It may also indicate that high density had adverse effects on digestibility in the control diets, resulting in lower growth in the fish receiving these diets. Similar effects have been reported for terrestrial animals in which digestibility is shown to increase considerably with the use of a probiotic in the diet (Bougon et al., 1988; Rychen and Nunes, 1994; Gil, 1998b). The results of the present study suggest the same effect in aquatic organisms. To confirm this, more research is needed using other ingredients and protein sources because costs can be reduced by using cheaper proteins with higher digestibility.

It can be concluded that the addition of 0.1% probiotics in tilapia fry diets improves animal growth, and mitigates the effects of stress factors. The two bacterial strains used in the present study were effective in stimulating fish performance, though the yeast produced the best results, being the most viable option for optimizing growth and feed utilization in intensive tilapia culture. Feed utilization was highest in tilapia fry fed with the yeast-supplemented diets, meaning the nutrients were more efficiently used for growth and energy. Based on these results, use of a 0.1% supplement of yeast in tilapia fry feeds is recommended to stimulate productive performance. Further research is needed to determine the most appropriate supplement levels for optimum growth results in larger animals at a commercial scale.

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Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops*×*M. saxatilis*)

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Abstract

Two separate feeding trials were conducted to evaluate graded levels of dried brewers yeast in the diet of hybrid striped bass. A basal diet was formulated to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g. In Trial 1, three incremental levels of dried brewers yeast (1%, 2% and 4% of diet) were added to the basal diet in place of cellulose. In Trial 2, the same levels of brewers yeast were added to the basal diet, but menhaden fish meal and menhaden oil were adjusted to provide isonitrogenous and isolipidic diets. Each diet was fed to three (Trial 1) or four (Trial 2) replicate groups of juvenile hybrid striped bass twice daily at rates approximating apparent satiation for 6 or 8 weeks. After the second feeding trial, a *Streptococcus iniae* bath challenge was executed to test the effects of diet on disease resistance.

Enhanced weight gain and feed efficiency were generally observed in fish fed the diets supplemented with yeast compared to the basal diet in both trials. In the second trial, body composition of whole fish, hemocrit and serum lysozyme levels were observed to be within normal ranges and not influenced by the various dietary treatments. After 9 weeks of feeding in the second trial, exposure to *S. iniae* resulted in no mortality and reduced signs of disease in fish fed diets supplemented with 2% and 4% brewers yeast, while 20% mortality was observed in fish fed the control diet ($P = 0.1$).

In the second trial, blood neutrophil oxidative radical production, extracellular and intracellular superoxide anion production of head kidney macrophages and serum lysozyme were measured after 16 weeks of feeding each diet. Fish fed the diet with 2% brewers yeast were found to have significantly ($P < 0.01$) higher blood neutrophil oxidative radical and extracellular superoxide anion production of head kidney macrophages than control fish. However, no significant differences in intracellular superoxide anion and serum lysozyme were observed among the treatments.

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Based on the result of this study, it is concluded that brewers yeast positively influenced growth performance and feed efficiency of hybrid striped bass as well as resistance to *S. iniae* infection. In addition, results of immune response assays demonstrate that brewers yeast can be administered for relatively long periods without causing immunosuppression.
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Keywords: Hybrid striped bass; Brewers yeast; *Saccharomyces cerevisiae*; Immune response; Bacterial resistance; *Streptococcus iniae*

1. Introduction

Hybrid striped bass production is considered to be the fastest growing segment of the U.S. aquaculture industry over the past decade and is poised to become a global seafood delicacy in the 21st century (Harrell and Webster, 1997; Kohler 2000). However, one major constraint to hybrid striped bass aquaculture is suboptimal production efficiency stemming from intrinsically high sensitivity to various stressors and susceptibility to infectious agents during normal aquacultural production. Although viral diseases are not a primary threat to fish culture activity thus far, heavy economic loss may be caused by other pathogenic organisms, including bacteria, fungus and protozoan ectoparasites (Kohler, 2000).

In recent years, there have been growing concerns about the adverse effects of the bacterium *Streptococcus iniae* in the aquaculture of many economically important marine and freshwater fish species including rainbow trout (Eldar and Ghittino, 1999), Nile tilapia (Bowser et al., 1998), hybrid tilapia (Perera et al., 1997), yellowtail (Kaige et al., 1984), Japanese flounder (Nguyen et al., 2001a,b), hybrid striped bass (Sealey and Gatlin, 2002a), red drum (Eldar et al., 1999; Colorni et al., 2002), rabbitfish *Siganus* (Yuasa et al., 1999) and even wild fishes (Colorni et al., 2002). This bacterium may cause heavy losses from mortality, reduced growth and unmarketable appearance in various fish species. Unfortunately, hybrid striped bass is one of the most susceptible fish to *S. iniae* infection. *S. iniae* is also associated with acute cellulitis in humans. Although some reports suggest that the fish and human *S. iniae* might be genetically different (Dodson et al., 1999), all reported occurrences of the human disease were associated with puncture wounds or abrasions and handling of infected fish or contaminated water (Greenlees et al., 1998). Therefore, an effective preventive strategy is not only needed to limit economical loss in aquaculture, but also to protect the health of aquaculturists and fish processing workers.

In aquaculture, traditional methods for treating infective pathogens include a limited number of government-approved antibiotics and chemotherapeutics. However, the disadvantages such as marginal effectiveness and high cost are obvious (Sealey and Gatlin, 2001). These treatments also may cause the accumulation of chemicals in the environment and/or fish, thus posing potential threats to consumers and the environment. An alternative strategy, besides vaccine development, is nutritional modulation of immune responses and disease resistance of aquaculture species. Research on the subject of nutritional modulation, especially evaluation of natural extracts or synthetic compounds, which may enhance the immune responses and disease resistance of hybrid striped bass, is still in

its infancy. In the United States, because there is no therapeutic approved by the U.S. Food and Drug Administration specifically for hybrid striped bass, research on this topic is of special urgency.

Yeast by-products from the brewing industry are natural diet additives that have been shown to positively influence non-specific immune responses (Siwicki et al., 1994; Anderson et al., 1995) as well as growth (Rumsey et al., 1991; Oliva-Teles and Goncalves, 2001) of some fish species. However, yeast products have not been investigated with hybrid striped bass. In addition, doses and time of administration have been recognized to have important effects on immunostimulant function, and efficacy of oral administration of immunostimulants has been reported to decrease over time (Sakai, 1999). The present study was conducted to determine the effects of graded levels of brewers yeast *Saccharomyces cerevisiae* on growth performance, body composition and resistance to *S. iniae* infection in hybrid striped bass. In addition, the efficacy of long-term oral administration of brewers yeast was explored by comparing various immune responses.

2. Materials and methods

2.1. Experiment 1

An initial feeding trial was conducted to evaluate growth performance of hybrid striped bass fed graded levels of brewers yeast. The basal diet of Keembiyehetty and Gatlin (1997), which utilized menhaden meal as the protein source, was modified to contain 40% protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/kg (Table 1). This diet satisfied and/or exceeded all known nutrient requirements of hybrid striped bass (Gatlin, 1997) or other warmwater fishes (National Research Council, 1993). Dried brewers yeast (Brewtech®) was supplied by International Ingredient Corporation (St. Louis, MO, USA). Three incremental levels (1%, 2% and 4% of diet) were added to the control diet in place of cellulose. Procedures for diet preparation and storage were as previous described (Rawles and Gatlin, 1998).

Juvenile hybrid striped bass (*Morone saxatilis* × *M. chrysops*) were obtained from a commercial supplier (Keo Fish Farm, Keo, AR) and maintained indoors at the Texas A&M University Aquacultural Research and Teaching Facility prior to the feeding trial. Fish were then graded by size and groups of 10 fish with a total weight of 253 ± 5 g/group were stocked into 110-l aquaria. The basal diet was fed to all fish in 110-l aquaria during a 1-week conditioning period. Water flow rate were maintained at approximately 650 ml/min via a recirculating system, which maintained water quality through mechanical and biological filtration (Sealey and Gatlin, 2002a). Salinity was maintained at 2.5–3.5‰ using well water and synthetic sea salt (Fritz Industries, Dallas, TX, USA). Low-pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen (DO) levels at or near saturation. Water temperature was at 26 ± 1 °C throughout the trial and a 12-h light:12-h dark photoperiod was maintained with fluorescent lights controlled by timers.

Each diet was fed to fish in triplicate groups at 3% of body weight daily, except during the last week of the trial, in which feeding rate reduced to 2.5% of body weight. The

Table 1
Composition of experimental diets in Experiment 1

Constituent	Basal diet	Yeast supplementation (%)		
		1	2	4
Ingredient (% dry weight)				
Menhanden fish meal[a]	59.0	59.0	59.0	59.0
Dextrin[b]	22.5	22.5	22.5	22.5
Menhaden oil[a]	3.7	3.7	3.7	3.7
Mineral premix[c]	3.0	3.0	3.0	3.0
Vitamin premix[c]	4.0	4.0	4.0	4.0
Carboxymethyl cellulose[b]	2.0	2.0	2.0	2.0
Cellulose[b]	5.8	4.8	3.8	1.8
Brewers yeast[d]	0	1	2	4
Proximate analysis (% dry matter)				
Dry matter	88.5	92.6	92.4	92.5
Crude protein ($N \times 6.25$)	39.8	40.8	40.7	42.1
Crude lipid	11.2	10.4	10.8	10.4
Ash	14.2	14.0	14.5	14.4

[a] Omega Protein, Reedville, VA. Menhaden fish meal contained 67.8% protein and 10.7% lipid at a dry-weight basis.
[b] US Biochemical, Cleveland, OH.
[c] Same as Gaylord and Gatlin (2000).
[d] International Ingredient Corporation, St. Louis, MO. Brewers yeast contained 50.7% crude protein and 2% crude lipid (dry-weight basis).

feeding trial was conducted for 6 weeks. Group weights of fish in each aquarium were obtained weekly and feed amounts adjusted accordingly. At the end of both feeding trials, three representative fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222), and approximately 0.5 ml of blood was collected from the caudal vasculature using a 1-ml syringe and 27-gauge needle for hematocrit determination.

2.2. Experiment 2

The second feeding trial was conducted to further evaluate growth responses of hybrid striped bass fed graded levels of brewers yeast as well as some of their immune responses. The basal diet formulation was the same as one in Experiment 1. Three incremental levels of dried brewers yeast (1%, 2% and 4% of diet) were added to the basal diet and cellulose, menhaden meal and menhaden oil were adjusted to provide isonitrogenous and isolipidic diets (Table 2).

Prior to initiation of this feeding trial, juvenile hybrid striped bass obtained from Keo Fish Farm were subjected to a 2-week conditioning period to adjust to standardized regimes in a recirculating culture system consisting of 38-l aquaria (Gaylord and Gatlin, 2000). Water was maintained in the system at 25 ± 1 °C and was provided to each aquarium at a rate of 500 ml/min. Salinity was maintained to 1.5–2 ‰. Optimal water quality (DO $\geq$ 6 mg/l, total ammonia nitrogen $\leq$ 0.3 mg/l) was maintained by biofiltration and aeration as in Experiment 1. Groups of 15 juvenile hybrid striped bass weighing

Table 2
Composition of experimental diets in Experiment 2

Constituent	Basal diet	Yeast supplementation (%)		
		1	2	4
Ingredient (% dry weight)				
Menhanden fish meal[a]	57.9	57.2	56.4	55.0
Dextrin[b]	24.9	25.1	25.0	24.9
Menhaden oil[a]	2.4	2.4	2.5	2.7
Mineral premix[c]	3.0	3.0	3.0	3.0
Vitamin premix[c]	4.0	4.0	4.0	4.0
Carboxymethyl cellulose[b]	2.0	2.0	2.0	2.0
Cellulose[b]	5.8	5.3	5.1	4.4
Brewers yeast[d]	0	1.0	2.0	4.0
Proximate analysis (% dry matter)				
Dry matter	89.6	88.5	88.6	88.8
Crude protein ($N \times 6.25$)	40.4	40.5	40.7	40.7
Crude lipids	9.6	9.0	9.5	9.2
Ash	14.2	14.4	14.4	14.2

[a] Omega Protein, Reedville, VA. Menhaden fish meal contained 69% protein and 13.2% lipid at a dry-weight basis.
[b] US Biochemical, Cleveland, OH.
[c] Same as Gaylord and Gatlin (2000).
[d] International Ingredient Corporation, St. Louis, MO. Brewers yeast contained 50.7% crude protein and 2% crude lipid (dry-weight basis).

approximately 9.7 g/fish were stocked into individual aquaria such that initial weight averaged 142 ± 5 g/group. Each experimental diet was fed to four replicate groups of fish for 8 weeks. All groups were fed their respective diets at the same fixed rate (initially 5% of body weight per day and gradually reduced to 3%). This rate was adjusted each week to maintain a level approaching apparent satiation. Fish were fed in the morning and evening, 7 days each week. Growth and feed efficiency were monitored weekly by collectively weighing each group of fish.

At the end of this feeding trial, three representative fish from each aquarium were anesthetized with MS-222, and blood collected as previously described for Experiment 1. After a sample of whole blood was taken for hematocrit determination, serum was isolated by centrifugation ($3000 \times g$ for 5 min) and kept at -80 °C for lysozyme assay (Engstad et al., 1992). After bleeding, the fish were frozen for whole body composition analysis (Rawles and Gatlin, 1998).

An additional 30 fish previously fed each of the experimental diets for a total of 9 weeks were exposed to an estimated LD_{50} dose of *S. iniae*. A virulent isolate of *S. iniae* originally obtained from the brain of an infected tilapia (*Oreochromis* sp.) was biochemically identified and provided by the Texas Veterinary Medicine Diagnostic Laboratory. The bacterial suspension was prepared according to the method described by Sealey and Gatlin (2002b) and diluted to a concentration of 2.6×10^5 CFU/ml in fresh well water. Thirty fish from each dietary treatment, pooled separately in mesh baskets, were immersed in the bacterial suspension for 2 h. After bacterial exposure, the 30 fish from each dietary

treatment were divided into three groups of 10 and placed into 38-l aquaria in an isolated culture system that received a constant supply of well water at 25 ± 1 °C. Fish continued to be fed their respective diets to apparent satiation twice daily and mortality was monitored for 2 weeks. The brains of dead fish were streaked on modified selective agar (Nguyen and Kanai, 1999) to confirm death from *S. iniae*.

After the second feeding trial and diseases challenge, additional fish continued to be fed each experimental diet to apparent satiation for a total of 16 weeks. After this extended feeding trial period, four fish from each treatment were bled from the caudal vasculature. Neutrophil oxidative radical production was determined following the procedure described by Siwicki et al. (1994). Absorbance was converted to Nitro Blue Tetrazolium (NBT) units based on a standard curve of NBT diformazan per milliliter of blood. Serum samples were separated as described above for lysozyme assay (Engstad et al., 1992). Also, after 16 weeks, eight fish per treatment were euthenized and head kidney samples were taken for macrophage isolation and assay of extracellular and intracellular superoxide anion. This assay followed the procedure of Sealey and Gatlin (2002a). The amount of extracellular superoxide anion was calculated from the formula: nmol superoxide anion/well=(Δabsorbance after 60 min × 100)/6.3 (Pick and Mizel, 1981).

Data from both feeding trials and the bacterial challenge were subjected to analysis of variance and Duncan's multiple range test using the Statistical Analysis System (SAS, 1985). Differences in treatment means were considered significant at $P < 0.05$.

3. Results

3.1. Experiment 1

In Experiment 1, hybrid striped bass fed the diets supplemented with 1% and 2% Brewtech dried brewers yeast had up to 20% more weight gain compared to fish fed the basal diet during the course of the feeding trial (Table 3). However, fish fed the diet supplemented with 4% brewers yeast had weight gain similar to fish fed the basal diet and

Table 3
Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 6 weeks in Experiment 1[a]

Dietary brewers yeast (%)	Weight gain (% of initial weight)[b]	Feed efficiency (g gain/g feed)	Survival (%)	Hematocrit (%)
0	86	0.41	85	37.7
1	104	0.54	96.7	44.4
2	98	0.51	93.3	39.3
4	85	0.48	100	37.6
$Pr > F$[c]	0.26	0.15	0.24	0.28
Pooled se	8.51	0.038	3.97	5.23

[a] Values represent means of three replicate groups except the basal diet that had two replicate groups.
[b] Fish initially averaged 25.3 g.
[c] Significance probability associated with the F statistic.

Table 4
Performance of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 8 weeks in Experiment 2[a]

Dietary brewers yeast (%)	Weight gain (% of initial weight)[b]	Feed efficiency (g gain/g feed)	Survival (%)	Hematocrit (%)	Lysozyme (10^3 units/l)
0	396	0.91	98.3	44.6	939
1	418	0.96	98.3	49.4	811
2	388	0.93	98.3	44.1	811
4	413	0.93	93.3	45.8	712
$Pr>F$[c]	0.10	0.19	0.24	0.36	0.50
Pooled se	8.14	0.013	3.97	2.08	165

[a] Values represent means of four replicate groups.
[b] Fish initially averaged 9.7 g.
[c] Significance probability associated with the F statistic.

responses of fish fed the various diets were not significantly different. One replicate group of fish fed the control diet also was excluded from analysis because of mortality experienced during weighing due to toxicity from net disinfectant. Survival and hematocrits of fish in all other replicates were high and not affected by diet (Table 3).

3.2. Experiment 2

In Experiment 2, weight gain, feed efficiency and survival of fish fed all experimental diets were excellent (Table 4). In this experiment, however, dietary effects on weight gain tended to be significant at $P<0.1$, and fish fed the diets with 1% and 4% brewers yeast had the highest gain. Hematocrits of fish fed the various diets were not significantly affected by diet (Table 4). Serum lysozyme was highly variable in hybrid striped bass in Experiment 2 (Table 4), with no significant effects of dietary yeast supplementation observed. Whole body composition of hybrid striped bass was not significantly affected by the dietary supplementation of brewers yeast in Experiment 2 (Table 5).

Table 5
Body composition of hybrid striped bass fed diets containing various amounts of dried brewers yeast for 8 weeks in Experiment 2[a]

Dietary brewers yeast (%)	Moisture (%)	% Fresh weight		
		Protein	Lipid	Ash
0	68.9	17.1	8.4	4.1
1	69.2	17.2	7.8	4.3
2	68.1	17.6	7.8	4.5
4	69.4	17.1	8.3	4.2
$Pr>F$[b]	0.512	0.057	0.516	0.250
Pooled se	0.14	0.37	0.16	0.064

[a] Values represent means of composite samples of three fish from each of four replicate groups except the basal diet and 2% brewers yeast supplementation group that had three replicate groups.
[b] Significance probability associated with the F statistic.

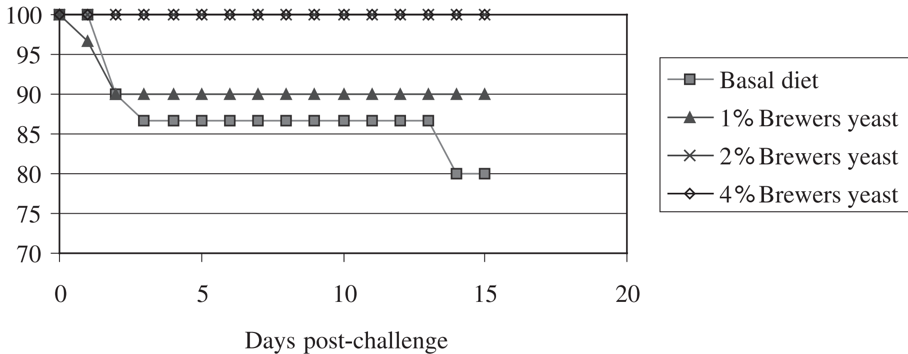

Fig. 1. Percent cumulative survival of hybrid striped bass fed incremental levels of brewers yeast (1%, 2% and 4%) for 9 weeks and subsequently exposed to *S. iniae* bath ($P = 0.1$). Symbols represent means of three replicate tanks per treatment. Pooled S.E. was 5.77.

3.3. *S. iniae* challenge

The controlled exposure of hybrid striped bass to a virulent strain of *S. iniae* at the end of the feeding trial in Experiment 2 resulted in limited mortality after the challenge (Fig. 1). Fish fed diets with 2% and 4% Brewtech brewers yeast did not experience mortality, while 20% and 10% mortality was observed in fish fed the control diet and 1% brewers yeast supplemented diet, respectively, although differences in survival were not statistically significant.

3.4. Immune responses after long-term administration

After 16 weeks of feeding the diet in Experiment 2, neutrophil oxidative radical production, serum lysozyme, intracellular and extracellular superoxide anion of head

Table 6
Blood neutrophil oxidative radical production (NBT test), serum lysozyme, intracellular superoxide anion production of head kidney macrophages and extracellular superoxide anion production of hybrid striped bass after long-term (16-week) oral administration of graded levels of brewers yeast in Experiment 2[a]

Dietary brewers yeast (%)	NBT test (mg ml^{-1})	Lysozyme (unit ml^{-1})	Intracellular superoxide anion (O.D. at 620 nm)	Extracellular superoxide anion (nmol O_2^-)
0	1.99^{c}	1246.7	0.897	2.679^{c}
1	2.59^{ab}	1073.3	1.039	3.694^{b}
2	3.09^{a}	965	1.293	4.511^{ab}
4	2.31^{bc}	1155	1.091	4.86^{a}
$Pr>F$[b]	0.004	0.19	0.466	0.006
Pooled se	0.083	0.013	0.062	0.123

[a] Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

[b] Significance probability associated with the *F* statistic.

kidney phagocytic cells were tested (Table 6). Significant differences ($P < 0.01$) in neutrophil oxidative and extracellular superoxide anion of head kidney phagocytic cells were observed among fish fed control diet and yeast-supplemented diets.

4. Discussion

Single cell proteins, including yeast and bacteria, have been viewed as promising substitutes for fishmeal in fish diets. Researchers have evaluated the nutritional value of brewers yeast *S. cerevisiae* in lake trout (Rumsey et al., 1990), rainbow trout (Rumsey et al., 1991) and sea bass (Oliva-Teles and Goncalves, 2001) by comparing growth performance, feed efficiency, liver uricase and nitrogen retention. Based on these studies, brewers yeast could replace up to 25–50% of fish meal protein without adversely affecting growth of these species. In the present study, brewers yeast was evaluated for its potential as an immunostimulant at relatively low inclusion levels.

In Experiment 1, the fish were not in optimal condition as reflected in chronic, low-level mortality (as high as 15%). Dietary supplementation of brewers yeast, especially at 1%, tended to improve the growth and health of fish in that experiment. In Experiment 2, weight gain, feed efficiency and survival of fish fed all diets were excellent. The fishes' high state of health and optimal environmental conditions may have limited potential expression of dietary effects on fish performance.

Hemocrits of fish fed the various diets in Experiments 1 and 2 were variable, but within normal ranges (Hrubec et al., 2001; Sealey and Gatlin, 2002c) and highest for fish fed 1% brewers yeast. Differences in fish size may have accounted for some of the differences in hematocrit values observed between experiments. Serum lysozyme, which is one measure of non-specific immunity, was highly variable in hybrid striped bass in both experiments. The average lysozyme levels in the present study were within the range reported by Sealey and Gatlin (2002c).

Although Streptococcicosis is attracting more attention, information on effect of nutrition on resistance to this disease is limited. Matsuyama et al. (1992) observed intraperitoneal injection of β-1,3-glucan derived from *Schizophyllum commune* and *Sclerotium glucanicum* could significantly enhance the percent survival of yellowtail after *Streptococcus* sp. challenge. However, for the present, few strategies other than vaccination have been proven to enhance resistance to *S. iniae* infection of any fish species. There is some evidence that antibiotic treatment has been ineffective (Klesius et al., 2000). After exposure to *S. iniae* in Experiment 2, hybrid striped bass fed diets with 2% and 4% brewers yeast did not experience mortality, while 20% mortality was observed in fish fed the control diet. Constant disease signs were observed including dermal hemorrhages around the mouth, base of fins and anus, erratic swimming, dark skin pigmentation and slow acceptance or refusal of feed, which are similar to those of tilapia described by Perera et al. (1994, 1997) and Evans et al. (2000). However, noticeable better feeding and less dark skin pigmentation were generally observed in fish fed diets with 2% and 4% yeast supplementation.

Some reports (Matsuo and Miyazano, 1993; Sakai, 1999) have indicated that long-term oral administration of immunostimulants to fish may induce immunosuppression. To

determine this phenomenon in hybrid striped bass, after 16 weeks of feeding, non-specific immune responses including blood neutrophil oxidative radical production, serum lysozyme, extracelluar and intracellular superoxide anion of head kidney macrophages were tested. Significant differences ($P<0.01$) in blood neutrophil oxidative radical production and extracellular superoxide anion of head kidney phagocytic cells were observed among fish fed the basal diet and yeast-supplemented diets. These results confirm early reports (Siwicki et al., 1994; Anderson et al., 1995) on effects of dietary brewers yeast on immune responses including neutrophil oxidative radical production and serum immunoglobulin level in rainbow trout. No significant differences of serum lysozyme and intracellular superoxide anion of phagocytic cells were observed among fish. The intracellular superoxide anion and extracellular superoxide anion of head kidney phagocytic cells of fish fed the basal diet were observed to be similar to that of Sealey and Gatlin (2002b,c).

Brewers yeast is a source of nucleic acids and polysaccharides including glucans. β-1,3-glucans have been recognized to effectively enhance immune functions of many aquaculture species including African catfish (Yoshida et al., 1995), Atlantic salmon (Engstad et al., 1992), rainbow trout (Jorgensen et al., 1993; Siwicki et al., 1994) and shrimp *Penaeus monodon* (Thanardkit et al., 2002). β-1,3-Glucan is generally viewed as the factor in brewers yeast with a known immunological mechanism (Gannam and Schrock, 2001). Sakai et al. (2001) reported that the nucleotides from brewers yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp as well as resistance to *Aeromonas hydrophila*. Burrells et al. (2001) also reported that dietary nucleotides, extracted from brewers yeast, could enhance resistance to various pathogenic infections in Atlantic salmon. However, the extent to which RNA in brewers yeast contributes to the beneficial influences of dietary brewers yeast on immune responses and resistance to *S. iniae* infection of hybrid striped bass is not clear.

It was concluded that brewers yeast positively influenced growth performance and feed efficiency of hybrid striped bass as well as resistance to *S. iniae* infection. In addition, results of immune response assays demonstrate that brewers yeast can be administered for relatively long periods without causing immunosuppression.

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NOTE

Yeast as a Biofertilizer Alters Plant Growth and Morphology

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Susanne Schmidt, and Chanyarat Paungfoo-Lonhienne*

ABSTRACT

It has been previously demonstrated that dicotyledonous plants perform rhizophagy, a process in which live microbial cells are engulfed by root cells and digested to acquire the nutrients from the microbes. Here we tested the hypothesis that rhizophagy is a mechanism of nutrient acquisition that is not restricted to dicotyledonous plants. We report that the monocotyledonous species sugarcane (*Saccharum officinarum* x *spontaneum*), grown in controlled axenic conditions, incorporated yeast cells into root cells. This suggests that rhizophagy is an evolutionarily conserved trait that predates the divergence of dicot and monocot species. To explore the potential relevance and practical application of rhizophagy, we investigated brewers' yeast (*Saccharomyces cerevisiae*), a waste product of the brewing industry, for its role as biofertilizer. The addition of live or dead yeast to fertilized soil substantially increased the nitrogen (N) and phosphorus (P) content of roots and shoots of tomato (*Solanum lycopersicum*) and young sugarcane plants. Yeast addition to soil also increased the root-to-shoot ratio in both species and induced species-specific morphological changes that included increased tillering in sugarcane and greater shoot biomass in tomato plants. These findings support the notion that brewers' yeast is a cost-effective biofertilizer that improves not only plant nutrition but also plant vigor during the early growth phase. It remains to be established which yeast-derived substances trigger the observed plant growth effects, and how rhizophagy contributes to plant nutrient acquisition.

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Abbreviations: GFP, green fluorescent protein, ^{GFP}yeast, yeast expressing green fluorescent protein; MS, Murashige and Skoog; YPD, yeast-extract peptone dextrose.

There is an urgent need to improve agricultural practices to ensure that food production is balanced with environmental sustainability. Globally, many high-production crop systems are characterized by low nutrient-use efficiency, with only ~30 to 50% of the applied N and P fertilizers acquired by crops. Consequently, up to 10-fold more fertilizer is applied than is recovered by crops (Robinson et al., 2011; Tilman et al., 2002; Zhang et al., 2012). Overfertilization of crop plants causes off-site pollution of hydrosphere, atmosphere, and pedosphere, results in a loss of biota, and threatens the integrity of ecosystems and global biogeochemical cycles (Gruber and Galloway, 2008; Rockström et al., 2009). To promote long-term soil fertility, next-generation crop systems will increasingly rely on nutrient-carrying materials to supplement or replace synthetic N and natural deposit–derived fertilizers.

While synthetic and mineral fertilizers rely on limited natural resources petroleum, phosphate rock, and potassium salts, organic

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materials are available as by-products of various industries. In the United States alone, ~1 billion tonnes of organic and inorganic agricultural recyclable by-products are generated annually (Edwards and Someshwar, 2000). It is not surprising then that there is much interest in improving plant nutrient supply with recycled materials (Conyers and Moody, 2009).

There is also much interest in harnessing the benefits of plant-growth-promoting microorganisms for sustainable crop production (reviewed by Vessey, 2003). Microorganisms can promote plant growth and health through a range of mechanisms, including supplying plants with biologically fixed nitrogen, phytohormones, volatiles, defense compounds, and enzymes (Bajwa et al., 1985; Kuklinsky-Sobral et al., 2004; Lugtenberg and Kamilova, 2009; Reinhold-Hurek and Hurek, 1998; Ryu et al., 2003; Timmusk and Wagner, 1999). Further, we showed that whole bacteria and yeast cells are taken up and used as nutrient sources by dicotyledonous species *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) (Paungfoo-Lonhienne et al., 2010b). We termed the ability of roots to incorporate and digest microbes 'rhizophagy' to reflect the apparent active uptake mechanism of this process (Paungfoo-Lonhienne et al., 2013). While the mechanisms involved in rhizophagy remain to be characterized, it appears to be a trait that is not restricted to taxa with mycorrhizal or diazotrophic symbioses because we also observed it in nonmycorrhizal *Arabidopsis* (Paungfoo-Lonhienne et al., 2010b). It is currently not known whether rhizophagy contributes significantly to plant nutrient acquisition. Here we tested the hypothesis that rhizophagy is a general trait of angiosperms and examined in controlled conditions if a monocotyledonous species can acquire yeast cells.

Following from this, we then evaluated possible practical applications of supplying microbes to plants. Disposal of yeast waste generated by the brewing and fermentation industries is problematic and costly and requires technologies for recycling (Blonskaja and Zub, 2009; Kobya and Delipinar, 2008; Zak, 2005). Formulations based on organic wastes, including brewers' yeast, are considered fertilizers in organic and conventional agriculture (e.g., www.faqs.org/patents/app/20090173122, accessed 27 Dec. 2013), and numerous organic and microbial-based fertilizers are available to primary producers. However, it is unclear how these materials affect plant growth. Our second aim was therefore to explore how yeast addition affects growth and nutrient acquisition of sugarcane and tomato plants in soil culture.

MATERIALS AND METHODS

Saccharomyces cerevisiae Expressing Green Fluorescent Protein and Brewery Waste Yeast

The preparation of yeast (*Saccharomyces cerevisiae*) expressing green fluorescent protein (GFP) (^{GFP}yeast) is detailed in (Paungfoo-Lonhienne et al., 2010b). Briefly, a single colony of ^{GFP}yeast clone *TDH3* (YGR192C) (Invitrogen) expressing glyceraldehyde-3-phosphate dehydrogenase fused to GFP was used to inoculate in 1 L of yeast-extract peptone dextrose (YPD) liquid media, and the culture was grown for 48 h at 28°C. Cells were harvested and used immediately.

Brewers' yeast was cultured in 20 L YPD for 1 wk at room temperature. The supernatant was discarded and the remaining yeast suspensions of 2 L were used for the experiments. 800 mL of yeast suspension were centrifuged (3000 rpm, 2 min) and washed twice with deionized water. The pellet was dissolved in 800 mL water (0.12 g pellet/mL water) and divided into two parts. Half of the dissolved material was applied as "living yeast" to soil. The second half was boiled for 15 min in a microwave and applied to soil as "dead yeast." Water derived from the final wash of living yeast cells was used as the "water control treatment."

Plant Growth Conditions

Micropropagated commercial sugarcane (*Saccharum* sp., Q322) seedlings produced from apical meristem were used in this study. The tissue was grown on petri dishes containing Murashige and Skoog (MS, Murashige and Skoog, 1962) medium solidified by 3.2 g L^{-1} of phytagel (Sigma) at 28°C, light intensity at 400 μmol m^{-2} sec^{-1} with a 16-h day and 8-h night regime. The fully developed seedlings were obtained after 3 mo with several subcultivations. Tomato seeds (variety 'Grosse Liese') were surface-sterilized with 70% ethanol for 1 min followed by rinsing with sterile water. The tomato seeds were sterilized with 5.25% NaClO plus 0.1% Tween 20 for 30 min and rinsed five times with sterilized water. The seeds were germinated in darkness for 2 d on 0.5X MS agar plates. Tomato seedlings were grown for 1 wk in a growth room under the same conditions described for sugarcane above.

Uniform sugarcane and tomato seedlings were transplanted to 4-L plastic pots containing University of California (UC) potting mix C containing fertilizer II with readily available nitrogen plus a moderate reserve of nitrogen, pH 6.5 (Matkin and Chandler, 1957). Nutrients in the fertilizer II included (per pot): blood and bone (N–P–K: 8–5–1), 6 g; potassium nitrate, 1 g; potassium sulphate, 0.5 g; and superphosphate, 6 g. All treatments received additional 16 g (per pot) complete fertilizer (N–P–K: 15–9-12 with micronutrient blend), a standard application as instructed by the company (Osmocote Exact, Scotts). Longevity of Osmocote is 3 to 4 mo, which covered the growth period of sugarcane and tomato in the experiment. In total, the amount of N, P, and K from the two sources of fertilizers corresponded to 3.02, 3.34, and 2.59 g per pot, respectively.

Pots were supplied with 6 g wet weight of either living or dead brewers' yeast as described above. Controls received water from the final wash of living yeast cells (50 mL per pot). Each pot contained one seedling, and each treatment consisted of eight pots (replications). Plants were watered daily with tap water and grown for 10 wk (sugarcane) or 4 wk (tomato) in the glasshouse. At harvest, plant seedlings were separated into roots and shoots, and roots were washed free of soil, rinsed with tap water, and rinsed three times in 0.5 mM $CaCl_2$ to remove contamination from surfaces. Plants were dried at 60°C for 5 d, weighed, homogenized, and analyzed for elemental content.

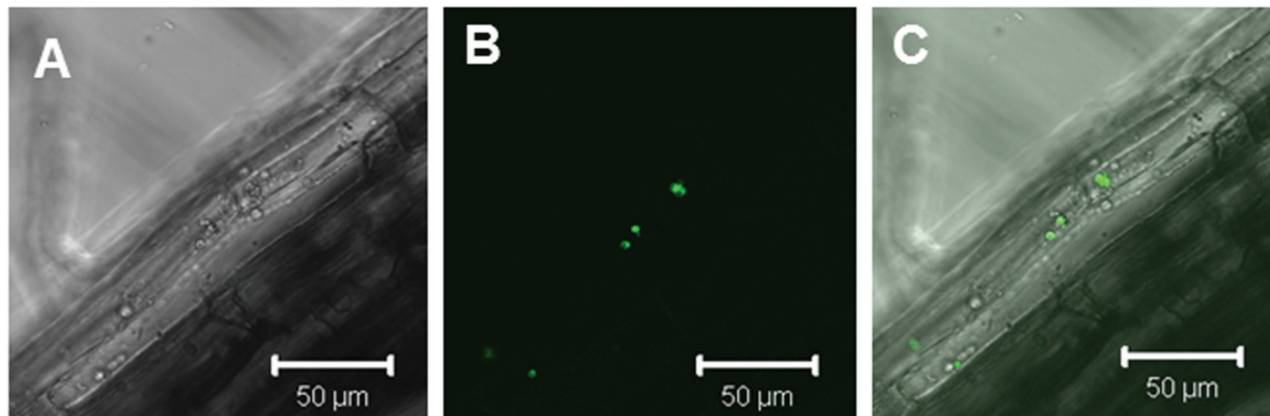

Figure 1. Uptake of yeast (*Saccharomyces cerevisiae*) expressing green fluorescent protein (GFP) (^{GFP}yeast) by axenic sugarcane (*Saccharum officinarum* x *spontaneum*) roots monitored by confocal laser scanning microscopy. Plants were grown on 0.5X Murashige and Skoog medium for 2 wk before adding ^{GFP}yeast and incubated overnight. A–C show the presence of yeast cells inside plant cells. A is the bright field image, B is the fluorescent image, and C is the combined image of A and B. A file of this image showing cytoplasmic streaming of yeast inside sugarcane root cells is presented in the supplementary movie.

Nitrogen concentrations were determined by combustion using a LECO TruSpec CHN analyzer, and phosphorus was analyzed with a Varian Vista Pro ICPOES instrument on samples digested with 5:1 nitric:perchloric acid.

Uptake of Yeast by Sugarcane Roots

To assess uptake of yeast by sugarcane, axenic sugarcane seedlings (see above) were grown vertically on 0.5X MS plates (see above) for 2 wk before incubating with 1 mL of ^{GFP}yeast solution overnight (cell density of 2 A_{600} units). At harvest, roots were washed with deionized water and analyzed by confocal laser scanning microscopy (CLSM). A confocal laser scanning microscope (CLSM, Zeiss LSM510 META, Carl Zeiss) was used with 10X dry, 20X water immersion objectives, and 40X and 60X oil immersion objectives. Green fluorescent protein was visualized by excitation with an argon laser at 488 nm and detection with a 505- to 530-nm band-path filter.

Statistical Analysis

Statistical analyses were performed using ANOVA, Tukey's Multiple Comparison post hoc test (GraphPad Prism4, GraphPad Software, Inc.).

RESULTS AND DISCUSSION

The prevalence of microbes in soil suggests that plants that can access microbes as a source of nutrients have a selective advantage. Currently, only dicotyledonous plants are known to employ rhizophagy; however, we hypothesized that rhizophagy is a much more common mechanism of nutrient acquisition among plant species. Therefore, we examined if an agriculturally important monocotyledonous species (sugarcane) can incorporate microbes into their roots. Sugarcane plants were supplied with yeast cells that constitutively express green fluorescent protein (^{GFP}yeast) to visualize yeast cells with confocal microscopy. We observed that root cells incorporated yeast cells (Fig. 1), and movement of yeast cells with cytoplasmic streaming confirmed their localization inside living root cells (Supplementary Movie 1). These results are in accordance with previous findings in tomato and *Arabidopsis* plants (Paungfoo-Lonhienne et al., 2010b). Furthermore, a gradual loss in GFP fluorescence was observed following uptake of yeast cells by roots, indicating that yeast cells are broken down in root cells and that their cell contents, including GFP, were degraded and presumably absorbed by root cells. These observations suggest that, in the controlled conditions of the experiment, sugarcane plants supplied with living yeast cells can acquire nutrients directly from yeast cells.

To test the effect of yeast addition to soil, we grew sugarcane and tomato plants in soil amended with fertilizer and dead or live yeast. Both plant species are important agricultural crops and evolutionarily diverged at least 140 million years ago (Chaw et al., 2004). Tomato plants grown in soil in the presence of living yeast increased root biomass by 56% compared with controls cultivated without yeast supplementation. Similarly, sugarcane plants supplied with living yeast produced 47% more root biomass (Fig. 2). Although shoot biomass was unaltered in sugarcane, the addition of yeast significantly ($P < 0.05$) enhanced tiller numbers by at least 23% (Fig. 3).

The enhanced root:shoot ratio in sugarcane and tomato plants supplemented with yeast is consistent with increases in root:shoot ratios observed in plants supplied with organic sources of N and P, including amides, amino acids, small peptides, and DNA (Cambui et al., 2011; Matsubayashi and Sakagami, 1996; Paungfoo-Lonhienne et al., 2010a; Soper et al., 2011; Walch-Liu et al., 2006; Yamakawa et al., 1998). Previous studies have found that organic compounds from various sources, including microbes, can enhance root and plant growth (reviewed by Paungfoo-Lonhienne et al., 2012). Therefore, it is feasible that the growth-enhancing effect of yeast supplementation that we observed does not require the yeast to be alive.

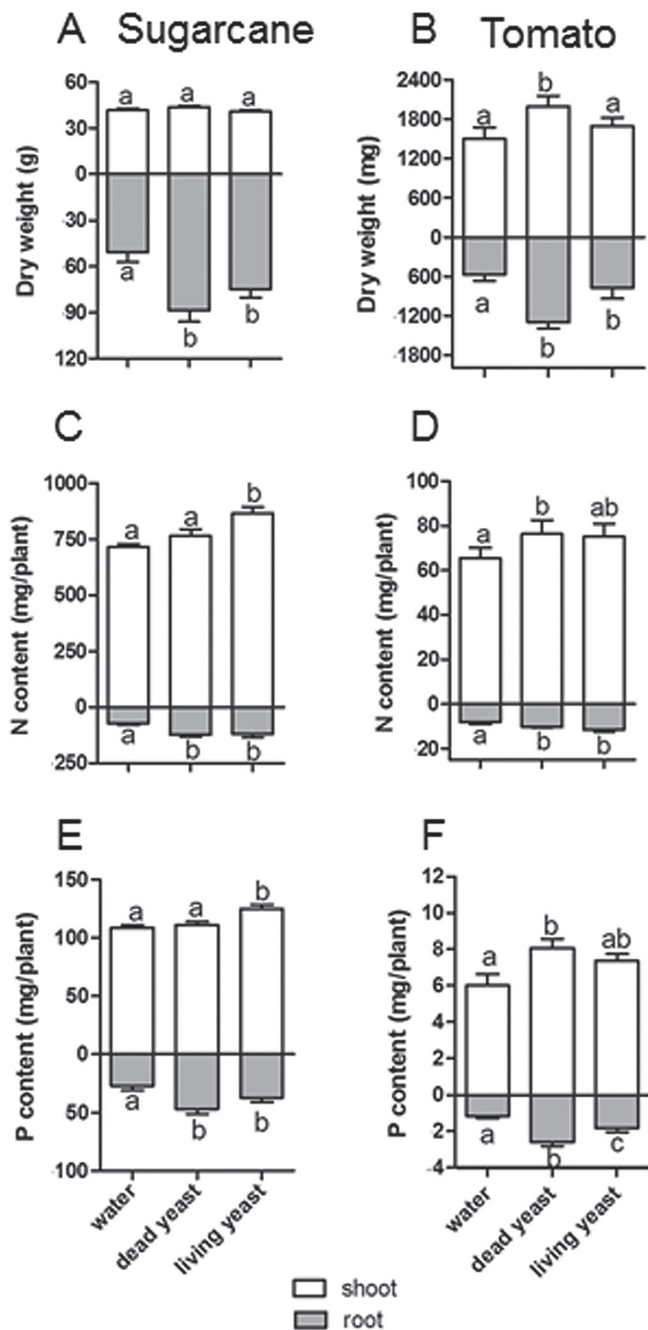

Figure 2. Plant dry weight and N and P content of sugarcane (*Saccharum officinarum* x *spontaneum*) (A,C,E) and tomato (*Solanum lycopersicum*) (B,D,F) grown in fertilized soil amended with dead or living brewers' yeast (*Saccharomyces cerevisiae*). Control plants received water from the final wash of living yeast cells. Sugarcane was grown for 10 wk and tomato for 4 wk. Compared with control treatments, biomass of sugarcane and tomato roots was significantly higher in yeast treatments, and dry weight of tomato shoots was higher in the dead yeast treatment. Root N and P content of sugarcane and tomato were higher than controls in both yeast treatments, while N and P contents of sugarcane shoots were higher in living yeast treatment and tomato shoots higher in dead yeast treatment. Bars represent averages and SEM of eight plants. Different letters indicate significant differences at $P < 0.05$, (ANOVA, Tukey's Multiple Comparison post hoc test).

To test this possibility, we supplied the plants with heat-killed yeast and examined the effects on plant growth. Sugarcane and tomato plants supplied with dead yeast cells displayed a similar increase in root biomass as plants supplied with living yeast (Fig. 2). These results indicate that the root-growth-promoting property of yeast occurs with living and dead cells. Interestingly, tomato plants provided with dead yeast produced more biomass than plants supplied with living yeast. A likely explanation is that nutrient uptake from heat-killed yeast is more efficient than from living yeast, possibly because the dead yeast cells release their contents into the soil, which can then be acquired through various nutrient transporters in the root cells.

Beyond substituting synthetic fertilizer as shown in our experiment, beneficial microbes can improve plant nutrition and vigor (Vale Barreto Figueiredo et al., 2011). Beneficial microbes stimulate plant vigor and growth via direct and indirect mechanisms that include solubilization of essential elements from soil and the production of phytohormones, volatiles, and defense compounds (Bajwa et al., 1985; Kuklinsky-Sobral et al., 2004; Lugtenberg and Kamilova, 2009; Reinhold-Hurek and Hurek, 1998; Ryu et al., 2003; Timmusk and Wagner, 1999). Thus, the increase in root:shoot ratio observed in yeast-supplied plants may be the result of the plant using organic forms of yeast-derived N and P as well as plant growth enhancing substances.

N and P control plant growth and development, and their availability is closely linked with productivity and yield. Sugarcane and tomato plants supplied with dead or living yeast displayed a significant increase in N and P content in roots (Fig. 2). Similarly, N and P content increased in shoots of plants supplied with dead yeast (tomato) and living yeast (tomato and sugarcane) (Fig. 2), indicating that plants acquire N and P from yeast in either state. Beneficial soil bacteria retain soil organic N and other nutrients in the plant–soil system and enhance release of the nutrients (Hayat et al., 2010). Our experiment shows that microbial supplementation is a means of enhancing plant growth, health, and, in the case of sugarcane, tillering. Whether rhizophagy contributes to the observed benefits conveyed by yeast is unclear. The results from this study indicate that while plants can perform rhizophagy, it is likely to be an energetically expensive mechanism and therefore unlikely to be a major mechanism of nutrient acquisition when soil nutrients are readily available. Future research is required to determine the ecological relevance of rhizophagy in the context of the availability of soil nutrients.

While yeast waste generated by the brewing industry may represent an ecologically sustainable means of stimulating crop growth, techniques have to be devised to achieve consistent benefits in field conditions. Plant-growth-promoting microorganisms are increasingly applied as biofertilizer, yet causal understanding is lacking

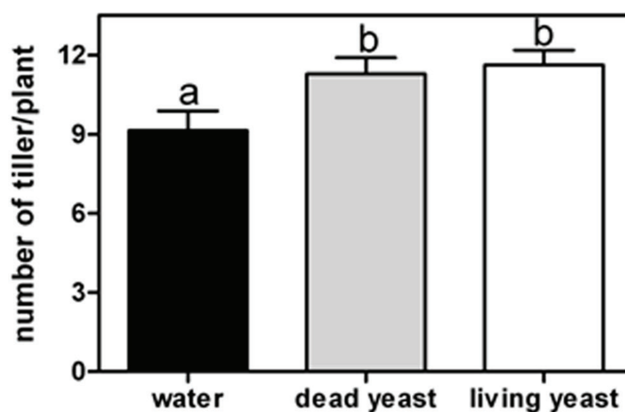

Figure 3. Performance of sugarcane (*Saccharum officinarum* x *spontaneum*) grown with brewers' yeast (*Saccharomyces cerevisiae*). Sugarcane was grown on fertilized soil amended with dead or living brewers' yeast for 10 wk. Control plants received water from final wash of living yeast cells. Compared with control treatment plants, tiller numbers increased plants in both yeast treatments. Bars represent average and SEM of eight plants. Different letters indicate significant differences at $P < 0.05$ (ANOVA, Tukey's Multiple Comparison post hoc test).

and has been hampered by the variability and inconsistency of results obtained in laboratory, greenhouse, and field studies (Malusá et al., 2012). Different plant species or cultivars may produce different types of root exudates, which may support the activity of the microbial inoculum or serve as substrate for the formation of biologically active substances by the inoculum (Figueiredo et al., 2011). Further research is required to establish whether yeast wastes can produce consistent benefits across plant taxa and environmental conditions.

In summary, supplying plants with brewers' yeast enhanced the N and P status of sugarcane and tomato plants and altered their growth and biomass partitioning. These findings indicate that yeast waste is a useful biofertilizer with potential to improve crop growth and nutrient supply. Future research will likely generate a better understanding of how microbial supplementation alters plant morphology and how it can induce specific changes that are beneficial for crop systems.

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Oral administration of yeast, *Saccharomyces cerevisiae,* enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.)

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Abstract

The effects of including lyophilised whole yeast, *Saccharomyces cerevisiae*, in the diet on the seabream innate immune response were investigated. Gilthead seabream (*Sparus aurata* L.) specimens were fed four different diets for 4 weeks: a commercial diet as control and the same diet supplemented with 1, 5 or 10 g/kg yeast. After 1, 2 and 4 weeks, serum complement titres, as a humoral parameter, and phagocytic, respiratory burst, myeloperoxidase and natural cytotoxic activities of head-kidney leucocytes, as cellular parameters, were evaluated. The results showed that yeast supplements enhanced all the latter responses, but not the humoral response. This enhancement was dose-dependent except for the cytotoxic activity that was only stimulated by the lower dose of yeast assayed. As yeast cell walls are able to enhance the seabream cellular innate immune response, these results support the possible use of whole yeast as natural inmunostimulants in common fish diets.
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Keywords: Yeast; *Saccharomyces cerevisiae*; Diet; Immunomodulation; Innate immune system; Leucocytes; Gilthead seabream (*Sparus aurata* L.)

1. Introduction

Natural immunostimulants are valuable for activating the fish immune system and protecting fish against adverse conditions (Anderson, 1992; Sakai, 1999). Among them, yeast *Saccharomyces cerevisiae* has been found to be a good enhancer of the trout immune system (Siwicki et al., 1994). Yeast cell walls are constructed almost entirely of two classes of polysaccharides, mannose polymers covalently linked to peptides (or mannoprotein) and glucose polymers (or glucans). The glucans and mannoproteins occur in roughly equal amounts in the wall. A third sugar polymer of *N*-acetylglucosamine, chitin, is also an important component of fungal walls, but is present only in minor amounts (about 1%) in yeasts (Cabib and Roberts, 1982). β-Glucans and chitin have been described as powerful immunostimulants in fish and mammals, while the use of yeast mannoproteins as immunostimulants have only been studied in mammals (Vecchiarelli, 2000). β-Glucans have generally been demonstrated to activate fish macrophages in vitro and to enhance the non-specific defence of fish to

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strengthen resistance to infection and to show adjuvant properties in fish vaccines in vivo (reviewed by Robertsen et al., 1994; Robertsen, 1999). The chitin polymer has been less studied for its role as immunostimulant but it clearly enhances the fish cellular non-specific defence and resistance to infection (reviewed by Sakai, 1999; Esteban et al., 2001).

In in vivo experiments, the most widely used method for administering sugars derived from the yeast cell wall is by intraperitoneal injection. This method is efficient and rapid because it elicits an enhanced non-specific immune response through two main pathways: the production of acute-phase proteins (APPs) and the direct activation of macrophages (Robertsen, 1999). However, this method is not practical in farm situations, where oral administration is the most suitable procedure for administering immunostimulants (Siwicki et al., 1994). This technique has been little studied in the laboratory. Some reports have demonstrated that oral administration of fungal carbohydrates enhances the fish innate immune response and protection against infection (Nikl et al., 1993; Siwicki et al., 1994; Yoshida et al., 1995; Efthimiou, 1996; Esteban et al., 2001), although such enhancement is temporary and dependent on inclusion levels and feeding period. Any information available concerning oral administration of sugars derived from the yeast cell wall in common fish diets is scarce and the mechanisms involved in the process of immunostimulation remains obscure (Robertsen, 1999).

All the above-mentioned studies have involved the use of purified yeast carbohydrates. However, the use of yeast cell walls or even whole yeast in fish farms would be of interest because different yeasts have been used successfully in fish feeds as a protein source, substituting expensive fish meal protein (Rumsey et al., 1990–1992). On the other hand, the use of substances which include different immunostimulants (β-glucans, mannoproteins and chitin in this case) could produce a more general immune response.

In this study, we examined the effects of including whole yeast cells (*S. cerevisiae*) in common fish diets upon the seabream (*Sparus aurata* L.) innate immune system, paying special attention to the dose of immunostimulant and the timing necessary to elicit an enhanced immune response.

2. Materials and methods

2.1. Fish

Sixty specimens (166 ± 16 g weight) of the hermaphroditic protandrous marine teleost gilthead seabream (*S. aurata* L.) obtained from Culmarex S.A. (Murcia, Spain) were kept in running (flow rate 1500 l/h) seawater (salinity 22‰) aquaria, at 20 ± 1 °C and with a natural photoperiod. Fish were allowed to acclimatise for 30 days before the experiment.

2.2. Feeding

Lyophilised whole yeast *S. cerevisiae* (a commercial strain) was received from the 'Instituto de Agroquímica y Tecnología de los Alimentos' (Valencia, Spain). Four experimental diets containing 0 (control), 1, 5 or 10 g yeast/kg feed were prepared in the laboratory from a commercial pellet diet (ProAqua Nutrición S.A., Palencia, Spain).

The specimens were divided randomly into four groups and each resulting group was fed one of the four different diets at a rate of 10 g dry diet kg^{-1} biomass (~1%) per day for 1, 2 or 4 weeks. The biomass of fish in each aquarium was measured after each sampling and the daily ration was adjusted accordingly.

2.3. Sample collection

Five fish of each group were randomly sampled after 1, 2 and 4 weeks of treatment. After being kept 24 h without feeding, the fish were anaesthetised with benzocaine (4% in acetone) (Sigma), weighed and measured. Blood and head-kidney samples were obtained from each specimen and different immunological parameters determined as described below.

Blood samples were collected from the caudal vein with a 27-gauge needle and a 1 ml syringe. Blood samples were allowed to clot at room temperature for 4 h. Following centrifugation, the serum was removed and frozen at −80 °C until use.

Head-kidney leucocytes were isolated as described previously (Esteban et al., 1998). Briefly, the head-kidney was dissected out by a ventral incision, cut into small fragments and transferred to 8 ml sRPMI-1640

medium: RPMI-1640 medium supplemented with 10 IU/ml heparin (Sigma), 100 IU/ml penicillin (Biochrom), 100 μg/ml streptomycin (Biochrom), 2% foetal calf serum (Gibco) and 0.35% sodium chloride (Sigma) to adjust medium osmolarity to gilthead seabream plasma osmolarity (353.33 mOs). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm^2). Head-kidney cell suspensions were layered over a 34–51% Percoll density gradient (Pharmacia) and centrifuged at $400 \times g$ for 30 min at 4 °C. After centrifugation, the band of leucocytes above the 34–51% interface were collected with a Pasteur pipette, washed twice, counted and adjusted to 10^7 cells/ml in sRPMI. Cell viability was greater than 95%, as determined by the trypan blue exclusion test.

2.4. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets (Ortuño et al., 1998). SRBC were washed in phenol red-free Hank's buffer (HBSS) containing Mg^{2+} and EGTA and resuspended at 3% (v/v) in HBSS. Aliquots of 100 μl of test serum as complement source, diluted in HBSS, was added to 100 μl of SRBC in a flat-bottomed 96-well plate to give final serum concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.1565 and 0.078%. After incubation for 1 h at 22 °C, the samples were centrifuged at $400 \times g$ for 5 min at 4 °C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm in a fluorimeter (BMG, Fluoro Star Galaxy). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. Control samples using heat-inactivated serum were also added in each assay.

The degree of haemolysis (Y) (percentage of haemolytic activity with respect to the maximum) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1-Y)$ against the volume of serum added (ml) on a $\log_{10}$–$\log_{10}$ scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units/ml was obtained for each experimental group.

2.5. Phagocytic activity

The phagocytic activity of gilthead seabream head-kidney leucocytes was studied by flow cytometry according to Esteban et al. (1998). *Vibrio anguillarum* strain R82 (serotype 01) (Toranzo and Barja, 1990), which was used as test particle, was grown and labelled with fluorescein isothiocyanate (FITC) (Sigma) according to Esteban et al. (1998). Ten microlitres of FITC-labelled bacteria, adjusted to 10^9 cells/ml in PBS, were added to each sample consisting of a 100 μl head-kidney leucocyte suspension, previously adjusted to 10^7 cells/ml in sRPMI-1640. The samples were then centrifuged ($400 \times g$, 5 min, 22 °C), resuspended and incubated at 22 °C for 45 min. At the end of the incubation time, the samples were placed on ice to stop phagocytosis and 500 μl ice-cold PBS was added to each sample. The fluorescence of the extracellular bacteria (i.e. free bacteria and bacteria adhered to phagocytes but not ingested) was quenched by adding 10 μl ice-cold trypan blue (0.4% in PBS) per sample. Standard samples of FITC-labelled *V. anguillarum* cells or head-kidney leucocytes were included in each phagocytosis assay. Samples incubated at 4 °C were used as negative controls.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10 000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), or green fluorescence (FL1) and red fluorescence (FL2) dot plots, while fluorescence histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the total cell population. The relative number of ingested bacteria per cell (phagocytic capacity) was assessed from the mean fluorescence intensity of the cells.

2.6. Respiratory burst activity

The respiratory burst activity of gilthead seabream head-kidney leucocytes was studied by a chemiluminescence method, according to Bayne and Levy

(1991). Stock solutions of 1 mg/ml phorbol myristate acetate (PMA) (Sigma) in ethanol and 10^{-2} M of luminol (Sigma) in dimethyl sulphoxide (DMSO) (Sigma) were prepared and stored at −20 and 4 °C, respectively. From these, the reactant solution containing PMA and luminol at final concentrations of 1 μg/ml and 10^{-4} M, respectively, in HBSS with calcium and magnesium was made. Fifty microlitres of cell suspension (10^7 cells/ml) and 50 μl of the solution containing PMA and luminol were added to each well of a flat-bottomed 96-well micrititre plate. The plate was then shaken and immediately read in a fluorimeter. Chemiluminescence measurements were made in 30 cycles of 2 min each. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. The backgrounds of the luminescence were calculated using a reactant solution containing luminol but not PMA.

2.7. Leucocyte myeloperoxidase content

Total myeloperoxidase content of head-kidney leucocytes was measured according to Quade and Roth (1997). Aliquots of 25 μl containing 1 million head-kidney leucocytes in Ca^{2+}- and Mg^{2+}-free HBSS were dispensed into flat-bottomed 96-well plates and incubated for 15 min with 125 μl of cetyltrimethylammonium bromide (CTAB) (Sigma) (0.02% in distilled water) and stirring at 40 rpm. Afterwards, aliquots of 25 μl of 10 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB) (Sigma) and 25 μl of 5 mM H_2O_2 (both substrates of MPO) were added. TMB and H_2O_2 solutions were prepared fresh daily. The colour-change reaction was stopped after 2 min by adding 25 μl of 4 M H_2SO_4. Plates were centrifuged ($400 \times g$, 10 min) and 150 μl of the supernatants present in each well were transferred to new 96-well plates. The optical density was read at 450 nm in a fluorimeter. Standard samples without leucocytes were also analysed.

2.8. Natural cytotoxic activity

Tumour cells from the L-1210 (mouse lymphoma, ATCC CCL-219 line) were cultured in RPMI-1640 culture medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Gibco) and incubated at 37 °C, 85% relative humidity and 5% CO_2 atmosphere and maintained in exponential growth. Tumour cells were labelled by incubating with 10 μg/ml 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) (Sigma) for 3 h in darkness. After labelling, free DiO was removed by washing three times in PBS and the staining uniformity was examined.

The natural cytotoxic activity of gilthead seabream head-kidney leucocytes was studied by flow cytometry (Cuesta et al., 1999). Fifty microlitres of DiO-labelled tumour cells (targets), adjusted to 10^6 cells/ml in supplemented RPMI-1640, were placed in 5 ml Falcon tubes, to which 250 μl of head-kidney leucocyte suspension (effectors) were added to obtain a final effector:target (E:T) ratio of 50:1 before being incubated for 2 h at 22 °C. Cytotoxic assays incubated for 0 min were used as control. At the end of the incubation time, 30 μl of propidium iodide (PI) (400 μg/ml) were added to each sample.

The samples were analysed in an FACScan. The cytotoxic activity of gilthead seabream leucocytes was related with the percentage of dead target cells showing green and red fluorescence. Standard samples of DiO-labelled target cells or head-kidney leucocytes were included in each cytotoxic assay. Cytotoxic assays incubated for 0 min were used as controls to determine initial target viability. The viability of targets maintained at 22 °C in sRPMI-1640 culture medium for 2 h was also monitored. The cytotoxic activity, expressed as the percentage of dead target cells after the cytotoxic assays, was calculated by the following formula: $\text{cytotoxic activity}\,(\%) = 100\,(\%\text{sample} - \%\text{control})/\,(100 - \%\text{control})$.

2.9. Statistical analysis

All assays were performed in triplicate and the mean + S.E. for each experimental group ($n = 5$) was calculated. The data from the flow cytometric assays were studied by using the statistical option of the Lysis Software Package (Becton Dickinson). Data were analysed statistically by one-way analysis of variance (ANOVA) to observe any difference due to time or treatment. Bonferroni's test was used to determine differences between groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Natural haemolytic complement activity

The dietary intake of yeast had no significant effect on the natural haemolytic complement activity of seabream at any time during the experiment. All the values estimated ranged from 200 to 400 ACH_{50} units/ ml serum.

3.2. Phagocytic activity

Both phagocytic ability (percentage of phagocytic cells) and capacity (number of bacteria ingested per cell) of head-kidney leucocytes were significantly enhanced by the 5 or 10 g/kg yeast-supplemented diets at week 4, although no significant differences were found between both doses (Fig. 1). No statistically significant differences in the phagocytic parameters

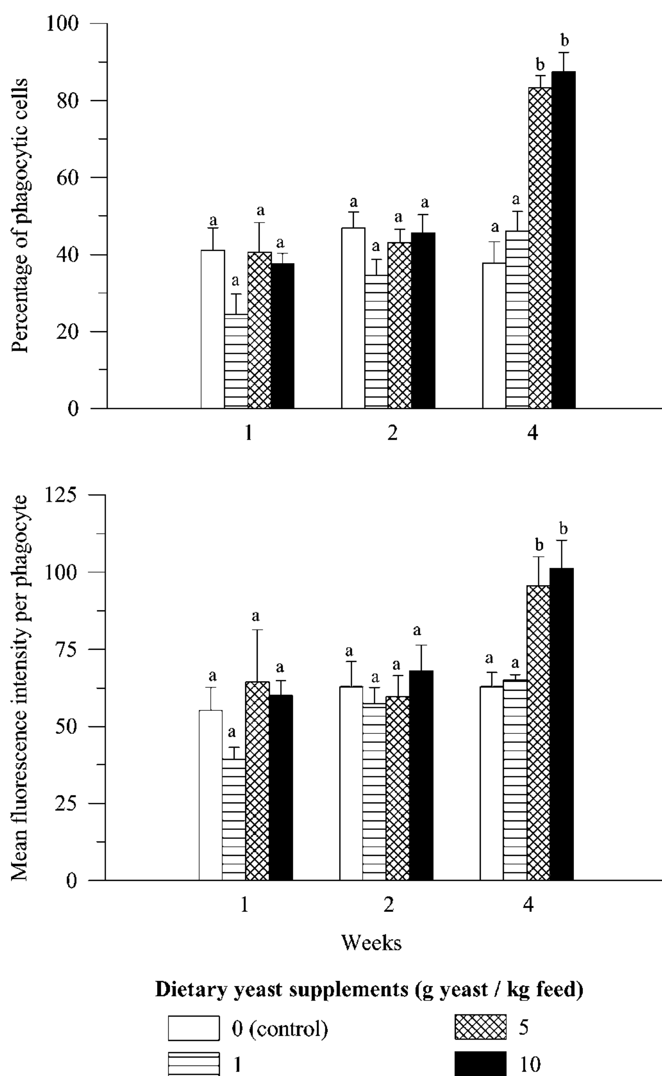

Fig. 1. Phagocytosis of FITC-labelled *V. anguillarum* by head-kidney leucocytes from seabream experimental specimens: (top) phagocytic ability; (bottom) phagocytic capacity. Data are represented as mean + S.E. Statistical differences ($P < 0.05$) between groups are indicated by different letters a and b. No significant differences appear between groups marked with the same letter.

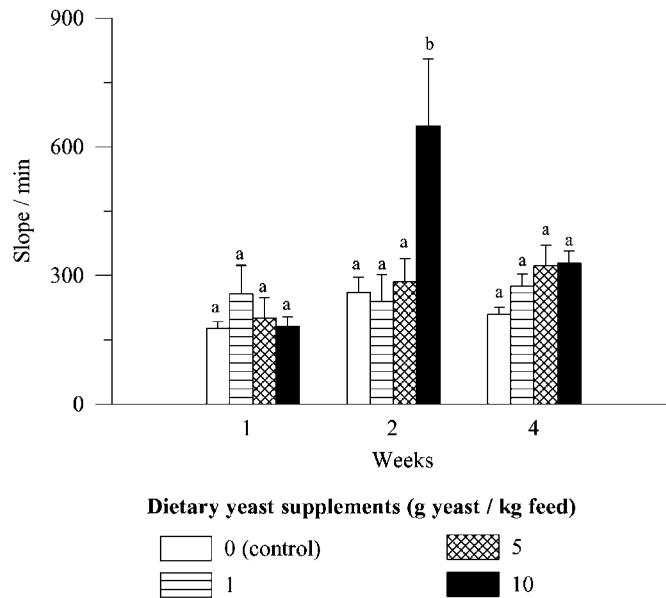

Fig. 2. Respiratory burst activity of head-kidney leucocytes from seabream experimental specimens. Data are represented as mean + S.E. Statistical differences ($P < 0.05$) between groups are indicated by different letters a and b. No significant differences appear between groups marked with the same letter.

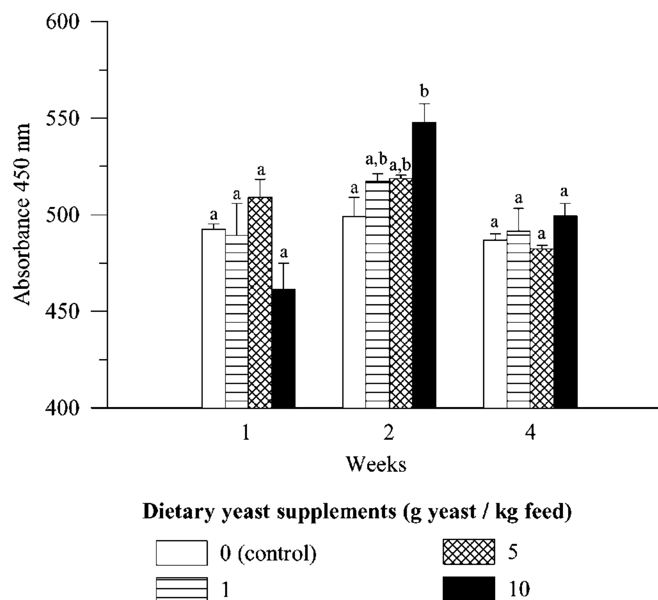

Fig. 3. Total myeloperoxidase content of head-kidney leucocytes from seabream experimental specimens. Data are represented as mean + S.E. Statistical differences ($P < 0.05$) between groups are indicated by different letters a and b. No significant differences appear between groups marked with the same letter.

compared with the fish fed the control diet were observed after feeding the specimens with the supplemented diets for 1 or 2 weeks.

3.3. Respiratory burst activity

The respiratory burst activity of head-kidney leucocytes, measured as the maximum slope of its kinetic, from the fish fed 10 g/kg yeast-supplemented diet was higher than that of the fish fed any other diet after 2 weeks of feeding (Fig. 2). No statistically significant differences were observed in the respiratory burst of head-kidney leucocytes from fish fed the yeast-supplemented diets compared to that measured in fish fed the control diet after 1 or 4 weeks of feeding.

3.4. Leucocyte myeloperoxidase content

Total activity of leucocyte intracellular myeloperoxidase was only found significantly higher in fish fed the 10 g/kg yeast-supplemented diet than in fish fed the control diet after 2 weeks of feeding. At this time, fish fed the 1 or 5 g/kg yeast-supplemented diet showed values of myeloperoxidase activity ranging between those of fish fed the control diet and those of the fish fed the 10 g/kg yeast-supplemented diet (Fig. 3). No statistically significant differences were found in this parameter at other times.

3.5. Natural cytotoxic activity

This activity was higher in fish fed any yeast-supplemented diet than in fish fed the control diet after 2 weeks of feeding, although not to a statistically significant extent, probably due to high individual variations. After 4 weeks, fish fed the 1 g/kg yeast-supplemented diet showed a significantly enhanced cytotoxic activity compared to fish fed the control or the 5 g/kg yeast-supplemented diet (Fig. 4). No statistically significant differences were observed in this activity after 1 week of feeding.

4. Discussion

The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development (Anderson, 1992; Siwicki et al., 1994; Sakai, 1999). Natural immunostimulants are biocompatible, biodegradable and safe for the environment

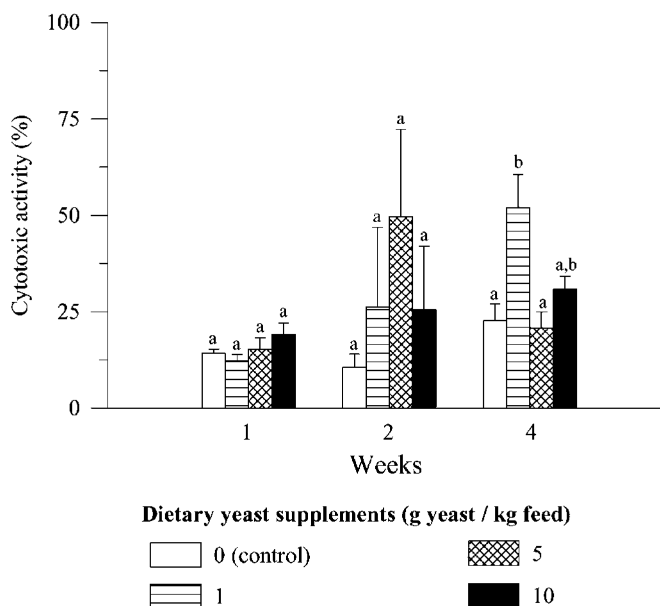

Fig. 4. Natural cytotoxic activity of head-kidney leucocytes from seabream experimental specimens. Data are represented as mean + S.E. Statistical differences ($P < 0.05$) between groups are indicated by different letters a and b. No significant differences appear between groups marked with the same letter.

and human health. Moreover, they possess an added nutritional value. In this way, yeasts have been tested in fish diets as a possible alternative to commonly used animal protein (Rumsey et al., 1990–1992). Other advantages of using yeast cells are their fast growth, low cost, high stability and the fact that they are not common constituents of fish feed. This makes it easier to work with them at known concentrations, compared with using soluble substances such as vitamins, which exist as micronutrients in feed and which are very sensitive to different factors (e.g. light, humidity or temperature) (Esteban et al., 2001). In spite of all these advantages, there is little information on the use of whole yeast in fish diets and concerning the hypothesis that the in vivo administration of whole yeast could enhance the fish immune system. Such a possibility would be of great interest for aquacultural research and the fish-farming industry.

The doses of lyophilised yeast used and the treatment times differed from those found in previous studies. For example, Siwicki et al. (1994) used a dose of 27 g *S. cerevisiae*/kg feed administrated to fish for 1 week. In this study, for reason of economy and efficiency, we looked for the minimum dose that would induce immunostimulation in seabream in the shortest time possible. Since time- and/or dose-dependent effect was to be expected, we tested several administration periods and doses, parameters which are fundamental in any immunomodulatory strategy (Sakai, 1999). As the data globally show, the optimal dose was 10 g/kg. This induced positive effects on natural immunity after 2 weeks of feeding, effects which increased after 4 weeks. Longer administration times would have been necessary to know if the immunostimulation produced was maintained. However, a deeper evaluation of the results shows that each activity reached a peak at a different time and even with a different dose of yeast. Indeed, phagocytosis increased at week 4 while respiratory burst did so after 2 weeks. Cytotoxic activity increased not with the highest but with the lowest dose of yeast (1 g/kg instead of 10 g/kg). Such differential effects on different immune parameters are common in this kind of studies concerning the modulation of fish non-specific host defences (Fletcher, 1986). The fish innate immune system lacks memory, and so the duration of its response is always shorter than that of the specific system (Anderson, 1992). In addition, innate defence is compound of several humoral and cellular factors that may present differential specificity to a given immunomodulatory substance (Secombes, 1996; Yano, 1996). Due to these peculiar characteristics, each activity shows punctual peaks that disappear with the time and that may not coincide exactly in magnitude or time with the peaks of other activities (Ortuño et al., 1999).

The natural haemolytic complement activity observed in seabream serum was similar to the values previously obtained in this fish species (Sunyer and Tort, 1995; Ortuño et al., 2001). In this study, high dietary levels of *S. cerevisiae* did not provoke any effect on the complement system. It is known that the administration of β-glucans by injection strongly activates the alternative complement pathway (Engstad et al., 1992) and serum lysozyme activity (Engstad et al., 1992; Santarém et al., 1997; Paulsen et al., 2001) both in fish and mammals. In contrast to these experiments, feeding with glucan resulted in no increase in complement or lysozyme activity (Verlhac et al., 1998), which agrees with the results of this study. This different effect of the same immunostimulant on humoral defence depending on the administration method is probably related with the induction of an inflammatory reaction and its consequent synthesis of APPs, because the oral administration does not appear to induce APPs while the injection does (Robertsen, 1999). It is not surprising therefore that the administration of yeast by injection should enhance the humoral defence. However, further experiments in this line are needed.

Yeast supplementation improved the seabream cellular innate immune response, as has been described previously for rainbow trout, where oxidative radical release, myeloperoxidase activity and phagocytic indexes were increased by the intake of *S. cerevisiae* (Siwicki et al., 1994). The same activities were increased by yeast ingestion in the present experiments, where the natural cytotoxic activity was also enhanced, which is the first time that this parameter has been seen to be increased by yeast intake in fish. *S. cerevisiae* has been shown to have antiviral properties when fed to mice (Fattal-German and Bizzini, 1992). The activation mechanisms involved are known to be related to the carbohydrates derived from the yeast cell wall. β-Glucans added to feed stimulates the phagocytic function, respiratory

burst and increases protection after challenge with pathogenic bacteria in some fish species (reviewed by Robertsen, 1999). Moreover, the existence of a β-glucan receptor on the macrophage cell surface has been demonstrated in Atlantic salmon (Engstad and Robertsen, 1994). The intake of chitin also increases the head-kidney leucocyte immune response, including phagocytosis, respiratory burst and natural cytotoxic activity (Esteban et al., 2001). Not only sugars but also nucleic acids, especially yeast RNA, could act as immune activators, since the growing evidence that nucleic acids from yeast sources, previously considered nutritionally non-essential for growth and reproduction, are essential for immune reactivity in mammals (Kulkarni et al., 1987; Rudolph et al., 1990; Cerra et al., 1991). No similar studies have been developed in fish.

To conclude, the present results provide evidences that whole yeast, *S. cerevisiae*, added to a common fish diet activates the seabream innate immune system, at a cellular level. Optimal doses and administration times have been established in an attempt to provide a useful approach for protecting cultured fish against infectious diseases.

Acknowledgements

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Immunostimulatory Effects of a Yeast (*Saccharomyces cerevisiae*) Cell Wall Feed Supplement on Rohu (*Labeo rohita*), an Indian Major Carp

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Key words: Indian major carp, nitroblue tetrazolium (NBT) assay, macrophage functional assay, lymphoproliferation assay, nitrite production assay

Abstract

The immunostimulatory role of an orally administered yeast cell wall preparation from *Saccharomyces cerevisiae* (Nutriferm™) was assessed in the Indian major carp, rohu (*Labeo rohita*). Fish were fed a diet containing the preparation for 15 days and then returned to the control diet for 20 days. Non-specific immunity was assessed at the end of the experimental feeding period (day 0) and on days 10 and 20. *In vitro* oxidative radical production, phagocytosis of leukocytes, nitrite production, and proliferation of lymphocytes were determined. All four parameters remained stable in control fish but, in fish fed the supplemented diet, reached a peak on day 10 and remained higher than in the control until day 20, indicating that ingestion of the diet containing the yeast cell wall preparation for 15 days had significant immunostimulatory effects.

Introduction

Immunostimulants play a significant role in aquaculture health management strategies. Immunostimulants include biological and synthetic compounds that activate white blood cells (leukocytes) and enhance non-specific cellular and humoral defense mechanisms in animals. Such agents are used to rectify impaired immune function and stabilize the improved status.

Fish treated with immunostimulants usually have enhanced phagocytic cell activity, pathogen killing ability, and killing mechanisms involving reactive oxygen species and reactive nitrogen species in macrophages. Lymphocytes are also activated by immunostimulants.

The route of administration affects the function of immunostimulants. Oral adminis-

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tration is the most practical and preferred method for delivery of immunostimulants because it allows mass administration regardless of fish size and results in enhancement of leukocyte function and protection against infectious diseases. Injection, on the other hand, is labor intensive and time consuming.

The immunostimulatory effects of polysaccharides and β-glucans have been widely studied in fish. Yeast, which contains polysaccharides and β-glucans, is a good enhancer of the immune system. Intake of a yeast (*Saccharomyces cerevisiae*) supplement improved the cellular innate immune response in trout (Siwicki et al., 1994) and gilthead seabream (Ortuno et al., 2002) by increasing the oxidative radical release and phagocytic indices. However, reports on the immunostimulatory effects of yeast cell wall extracts on freshwater fishes of the Indian subcontinent are meager.

As Indian carps are the backbone of freshwater aquaculture in India, the objective of the present work was to study the effects of a dietary administered yeast cell wall extract on the cellular immune responses of the Indian major carp, rohu (*Labeo rohita)*.

Materials and Methods

Experimental fish. Rohu (*Labeo rohita*) was used as a representative of the Indian major carps. Fish were purchased from a local market and maintained at the wet laboratory of the Department of Fishery Pathology and Microbiology at the West Bengal University of Animal and Fishery Sciences. Rohu (45-60 g) were maintained in circular 500-l fiberglass tanks with 24-h aeration. About 75% of the water was exchanged and waste feed and fecal materials were removed daily. Basic physico-chemical water parameters were measured every 15 days to maintain optimal levels of dissolved oxygen (5.65±0.72 mg/l), pH (8.24±0.82), nitrite (0.015±0.009 mg/l), and ammonia (0.109±0.024 mg/l). The water temperature was 25-30°C. The fish were acclimatized and fed a pelleted diet for three weeks prior to the experiment. The diet was prepared in our laboratory and fed at 1% of the body weight of the fish.

Immunostimulant. Nutriferm ™ (AB Mauri) was used as the immunostimulant. It is composed of a purified cell wall fraction gained from a pure culture of the yeast, *Saccharomyces cerevisiae*. Soluble parts were removed by digestion and the remaining cell wall fraction was purified and dried.

Feed preparation. The control diet contained 40% fish meal, 35% ground nut oil cake, 12% wheat flour, 10% rice polish, 2% vitamins and minerals, and water. The experimental diet contained the above plus 5 g of yeast cell wall per kg feed. The feed was pelletized with a hand pelletizer in our laboratory then dried at room temperature and stored at 4°C.

Experimental design. Eighty fish were stocked in eight tanks (4 control and 4 experimental) at 10 fish per tank. Before the start of the experiment, four control fish (one from each tank) were randomly chosen and cellular immune parameters were measured as below. After acclimatization, the four experimental tanks received the yeast-supplemented diet once a day for 15 days and then the control diet for another 20 days; the control tanks received the control diet throughout. Cellular activity was measured on days 0 (the day after cessation of the experimental diet), 10, and 20 in four randomly chosen fish from each treatment (one from each tank).

Oxidative radical production by leukocytes. Oxidative radical production of leukocytes was assayed by the nitroblue tetrazolium (NBT) reduction test as described by Siwicki et al. (1985). Equal volumes (0.1 ml) of blood and filtered NBT solution (0.2% in phosphate buffered saline [PBS], pH 7.2 original stock) was mixed and incubated for 30 min at room temperature. Then 0.05 ml of the mixture was added to 1 ml N, N dimethylformamide. The suspension was centrifuged for 5 min at 3000 x *g* and the optical density of the supernatant was assessed at 540 nm in a spectrophotometer (Siwicki et al., 1998).

Phagocytosis. Phagocytosis was examined as described by Yoshida et al. (1993). The number of cells was adjusted to 1 x 10^7 cells/ml in RPMI-1640 medium containing 10% fetal calf serum. One ml of cell suspen-

sion was allowed to adhere to a cover slip for 1 h at room temperature and cells that did not adhere were washed off with PBS. Cells from a young culture of *Staphylococcus* sp. (10^8 cfu/ml PBS), obtained from the Department of Fishery Pathology and Microbiology, were added to the cover slip and the slips were incubated 2 h at 28°C. The cells on the cover slip were fixed with 95% methyl alcohol and stained with Giemsa. The number of phagocytic cells per 200 cells was counted and phagocytic activity (PA) was determined according to Findlay and Mundaym (2000) as PA = 100(no. phagocytosing cells/total no. of phagocytes).

Isolation of head kidney leukocytes. Fish were anesthetized by MS-222 and the head kidney was aseptically removed, cut into small pieces, minced to obtain cells, and pushed through a stainless steel mesh with PBS (pH 7.4) containing 100 µg/ml streptomycin, 200 µg/ml gentamycin, and 100 IU/ml penicillin. The cell suspension was layered onto Histopaque® 1077 (Sigma, USA) at a ratio of 1:3 and centrifuged at 4°C for 30 min at 400 x *g* (Chung and Secombes, 1988). Following centrifugation, the white blood cell interface layer was collected using a micropipette, transferred to a clean sterile test tube, and washed thrice with PBS. Viable cells were counted by the trypan blue exclusion method using a Neubauer counting chamber (Maji et al., 2006).

In vitro production of reactive nitrogen. The production of reactive nitrogen intermediates by leukocytes was assayed following the method described by Tafalla and Novoa (2000). This method is based on the Griess reaction that quantifies the nitrite content of macrophage supernatants, as nitric oxide is an unstable molecule and degrades to nitrite and nitrate (Green et al., 1982). The number of head kidney leukocytes was adjusted to 2 x 10^6 viable cells/ml by diluting with RPMI-1640 growth medium containing 2 mM L-glutamine, 24 mM Hepes buffer, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% V/V fetal calf serum. Cell suspensions (100 µl/well) were dispensed in a 96-well flat bottom tissue culture plate (Nune, Denmark). The final volume of the wells was increased to 200 µl by adding LPS (Sigma, USA) at a concentration of 10 µg/ml and the plate was incubated at 28°C for 96 h in 5% CO_2 atmosphere.

After incubation, 50 µl aliquots of supernatants from the wells were removed, added to100 µl of Griess reagent containing 1% sulfanilamide, 0.1% N-1 napthylethylene diamine dihydrochloride, and 2.5% phosphoric acid, and incubated at room temperature for 10 min. The optical density of the solution was measured with a microplate reader (ECIL, India) at 570 nm and quantified by comparison to $NaNO_2$ (Sigma, USA) as the standard (Joardar et al., 2003).

Lymphocyte proliferation. The number of head kidney leukocytes was adjusted to 5 x 10^5cells/ml in RPMI-1640 and 100 µl cell suspensions were seeded into a 96-well tissue culture plate (Nune, Denmark). Each control (n = 4) and treated (n = 4) fish cell suspension was repeated in triplicate. The final volume of the wells was made up to 200 µl with Concanavalin A (Con-A) at a concentration of 10 µg/ml and the plate was incubated at 28°C for 48 h in a 5% CO_2 atmosphere.

The colorimetric 3-[4,5-dimethlythiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay described by Daly et al. (1995) was used to determine the proliferation of head kidney leukocytes. After 48 h culture, 20 µl of MTT (5 mg/ml PBS) were added to each well of the leukocyte culture and incubated at 28°C for 4 h. Formazan production was determined by the method of Plumb et al. (1989). The formazan crystals were dissolved by adding 150 µl of DMSO (Sigma, USA) to each well, followed by 25 µl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The contents of the wells were mixed thoroughly with a micropipette and incubated at room temperature for 10 min. Formazan development was read at 595 nm using a microplate reader (ECIL, India) and a stimulation index (SI) was calculated as SI = (mean optical density of leukocyte wells with test mitogen at time x/mean optical density of negative control wells at time x) - 1.

Statistical analysis. Results are expressed as means ± standard error (SE) and analyzed

by one-way analysis of variance (ANOVA) to test the significance of differences between the control and experimental groups (Neumann et al., 1995).

Results

Oxidative radical production of leukocytes (Fig. 1), phagocytosis (Fig. 2), production of reactive nitrogen (Fig. 3), and lymphocyte proliferation (Fig. 4) were higher in treated fish on day 0 than in the control. In treated fish, all indicators peaked on day 10 and, except for nitrite production, remained elevated until day 20. Values in the control fish on days 0, 10, and 20 were similar to pre-experiment values and, therefore, are not given in the figures.

Discussion

The *in vitro* production of reactive oxygen radicals increased from day 0, peaked on day 10, and gradually decreased to day 20, similar to some earlier reports on other species. Rainbow trout fed beta 1,3/1,6-yeast glucan for one week had a higher oxidative burst than control fish fed no glucan (Siwicki et al., 1994). The number of glass-adherent NBT-positive cells in catfish treated with oligosaccharide peaked at 12 days, decreased slightly by 30 days, dropped to the baseline level by 45 days, and was greater than but did not significantly differ from the control (Yoshida et al., 1995). In turbot fed beta 1,3/1,6-yeast glucan for five weeks, the oxidative burst of head-kidney macrophages was significantly enhanced but the enhancement was no longer observed two weeks after the fish returned to the control diet (Ogier de Baulny et al., 1996). Also in turbot, the influence of glucans on respiratory burst activity of leukocytes and production of O_2^- in all groups was higher on day 7 than on days 14 and 21 (Santarem et al., 1997). In gilthead seabream, the respiratory burst activity of head kidney leukocytes, measured as the maximum slope of its kinetic, in fish fed a diet supplemented by 10 g yeast per kg diet was higher than that of fish fed any other diet after seven weeks of feeding (Ortuno et al., 2002). On the other hand, Verlhac et al. (1998) obtained no statistically significant differences in oxidative burst of head kidney phagocytes in rainbow trout fed glucan.

Phagocytosis is an important element of the defense of fish against invading microorganisms (MacArthur and Fletcher, 1985; Olivier et al., 1986). Destruction of ingested microorganisms might be due to degranulation and metabolic activation when toxic intermediates of oxygen are produced. In our

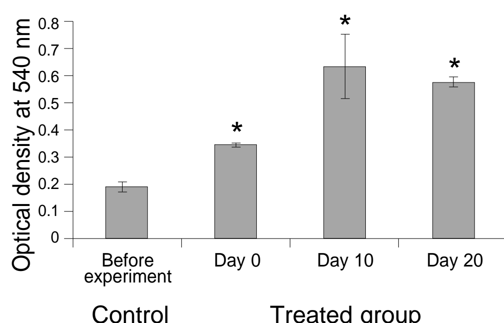

Fig. 1. Superoxide anion production by leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation, determined by nitroblue tetrazolium (NBT) reduction assay; means of four observations±SE, asterisk indicates significant difference (p<0.01) from the control. Control did not vary throughout experiment.

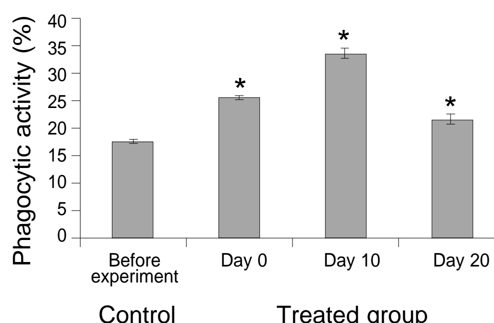

Fig. 2. *In vitro* phagocytic activity of head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation; means of four observations±SE, asterisk indicates significant difference (p<0.01) from the control. Control did not vary throughout experiment.

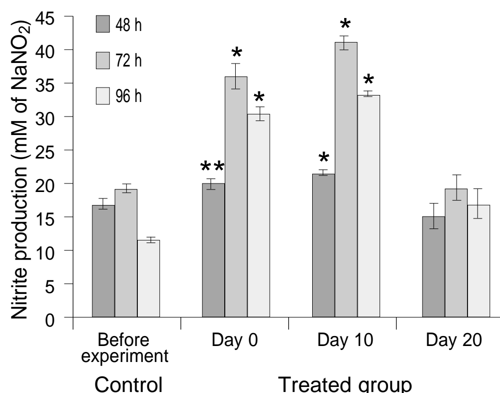

Fig. 3. *In vitro* nitrite (NO^{-}_2) production by head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation after 48, 72, and 96 h of incubation; means of four observations±SE, single asterisk indicates significant difference of $p<0.01$ from control, double asterisk indicates significant difference of $p<0.05$. Control did not vary throughout experiment.

experiment, phagocytic activity increased from day 0, peaked on day 10, and gradually decreased to day 20. The phagocyte activation mechanism might be related to the *S. cerevisiae* cell wall supplementation of the feed, as was observed by earlier workers. However, the dose and time of administration, as with other animal models, varies from experiment to experiment. Yano et al. (1989), Chen and Ainsworth (1992), and Jorgensen et al. (1993) reported that glucan raises phagocytosis. In an earlier study, oral administration of 5 or 10 g yeast preparation from *S. cerevisiae* per kg feed enhanced the phagocytic function of gilthead seabream in week 4 although there was no significant difference between doses (Ortuno et al., 2002). No statistically significant differences in phagocytic parameters were observed in fish fed supplemented diets for 1 or 2 weeks compared to fish fed a control diet (Ortuno et al., 2002). In rainbow trout fed beta-1,3/1,6-glucan, phagocytic activity was higher than in control fish at 1 week (Siwicki et al., 1994). However, Verlhac et al. (1998) reported no effect of glucan on the tested macrophage activities.

Reactive nitrogen intermediates are important molecules in regulating immune functions and have a direct antimicrobial effect (Liew et al., 1990; Schoor and Plumb, 1994). Wang et al. (1994) first demonstrated that fish macrophages synthesize nitric oxide. A macrophage activating factor secreted by mitogen stimulated goldfish kidney leukocytes induced nitric oxide production in both primary cultures and cell lines of goldfish macrophages (Wang et al., 1995). In the present study, nitrite production of the head kidney leukocytes was assessed *in vitro*. In all treated fish, nitrite production increased and peaked on day 10. However, Novoa et al. (2003) found that nitric oxide production in turbot was not affected *in vitro* or *in vivo* by any nisin treatment and Sakai et al. (1999) found no evidence of activation of reactive nitrogen species in macrophages of fish treated with various immunostimulants.

Measurement of lymphoproliferation activity is required to evaluate stimulation of specific and non-specific mitogens. In the present study, yeast cell wall supplementation significantly enhanced the proliferative response of head kidney leukocytes induced by the mitogen Con A. Treated fish possessed *in vitro* lymphoproliferative ability up to day 20, with a peak on day 10. Verlhac et al. (1998) found a significant enhancing effect of dietary glucan on the response of peripheral blood lymphocytes of rainbow trout induced by Con A just after the experimental feeding (week 0) and four weeks later but no significant variations in the effect of dietary vitamin C on Con A-induced proliferation of peripheral blood lymphocytes and no response to LPS.

In conclusion, pelleted feed supplemented with the yeast cell wall preparation, Nutriferm™, enhanced *in vitro* production of reactive oxygen and nitrogen, phagocytic activity, and lymphocyte proliferation in the Indian major carp, rohu. Bricknell and Dalmo (2005) reported that administration of an immunostimulant may up-regulate the immune system to a heightened level until the immunostimulant is withdrawn, although continuous administration may cause adverse effects such as tolerance or immunosuppression. Conversely,

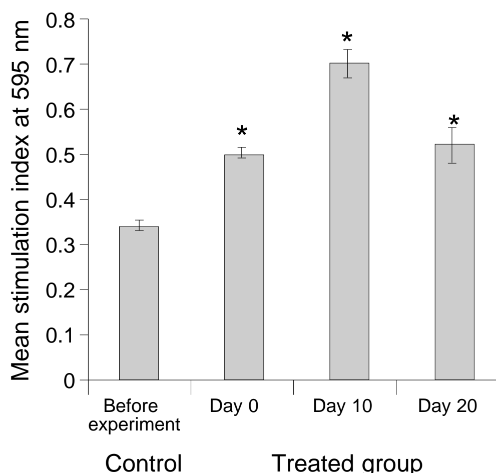

Fig. 4. *In vitro* lymphocyte proliferation assay of head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation; means of four observations±SE, asterisk indicates significant difference ($p<0.01$) from the control. Control did not vary throughout experiment.

pulse administration of immunostimulants may cause oscillations in the immune response from rest to enhanced and back to rest. As Nutriferm™ administered orally for 15 days resulted in a considerable immunomodulatary effect on the rohu, this preparation together with a pulse feeding strategy might be useful as a disease control strategy for Indian carps. A pulse-feeding strategy should be tested with 20-30 day intervals.

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[Article]

Evaluation of Dairy–Yeast Prebiotic Supplementation in the Diet of Juvenile Goldfish in the Presence or Absence of Phytoplankton and Zooplankton

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Abstract.—Prebiotics recently have been shown to increase immune responses and disease resistance in certain fish species; therefore, the current study was conducted to evaluate the commercially available dairy–yeast prebiotic, GroBiotic-A, for use with juvenile goldfish *Carassius auratus*. The study consisted of two 10-week feeding trials in which juvenile goldfish were fed practical diets that were either unsupplemented or supplemented with the dairy–yeast prebiotic at 2% by dry weight. Juvenile fish were sorted by size and stocked into 12 units within each of two culture systems: one indoor system supplied with recirculated well water and one system located outdoors with a continuous flow of pond water to provide a source of phytoplankton and zooplankton. Both diets were fed to fish in six units within each system at the same fixed percentage of body weight twice daily. Culture system (i.e., presence or absence of phytoplankton and zooplankton) was the primary factor influencing ($P < 0.0001$) percent weight gain, feed efficiency, and survival of goldfish during the feeding trials. No dietary effect was detected, although there was a significant ($P < 0.05$) interaction between culture system and diet, with supplementation of the dairy–yeast prebiotic tending to improve weight gain and feed efficiency of fish in the presence of phytoplankton/zooplankton. During a controlled disease challenge with an intraperitoneally administered dose of *Aeromonas* hydrophila that was equivalent to a predetermined LD50 (dose lethal to 50% of test fish), average survival values ranged between 67% and 83% for fish that previously had access to phytoplankton/zooplankton compared with 17–33% for fish that had no access to phytoplankton/zooplankton. The dairy–yeast prebiotic, however, did not enhance resistance of goldfish to the bacterial pathogen and did not greatly alter microbiota of the anterior or posterior gastrointestinal tract based on denaturing gradient gel electrophoresis analysis. In conclusion, the dairy–yeast prebiotic did not improve feed efficiency in goldfish or resistance to a bacterial pathogen as previously observed in golden shiners *Notemigonus crysoleucas* and hybrid bass (white bass *Morone chrysops* × striped bass *M. saxatilis*).

The goldfish *Carassius auratus* is a prominent bait and ornamental fish produced en masse by aquaculture. In the southeastern United States, goldfish are typically raised in large outdoor ponds, where fish are exposed to seasonal photoperiods and temperature cycles; supplemental fertilization of ponds is provided to encourage growth of phytoplankton and zooplankton as primary food resources (Lochmann and Phillips 2002). In comparison, goldfish raised in indoor culture systems experience constant environmental conditions and generally lack access to supplemental nutrients via phytoplankton or zooplankton. During various production phases, especially during harvest from ponds and prior to shipment to retail locations, fish are subjected to different stressors, such as netting, crowding, air exposure, transportation, and potentially different water quality conditions (e.g., temperature and dissolved oxygen level; Barton 2002). These stressors typically make the fish more susceptible to various disease outbreaks, which can result in large numbers of losses. Members of the genus *Aeromonas* are common pathogens of cyprinid species and are the agents of a disease known by several names, such as red-sore disease, hemorrhagic septicemia, and ulcer disease (Swann and White 1989; Guz and Kozinska 2004; Yildiz et al. 2005). Symptoms typically include ulcers in the fish's integument, widespread septicemia, pale gills, abnormal swimming behavior, bloating, and sudden death (Swann and White 1989; Guz and Kozinska 2004). *Aeromonas* spp. are considered to be opportunistic pathogens, and various common stressors can therefore lead to outbreaks (Swann and White 1989; Guz and Kozinska 2004). Traditionally, medications have been used in response to disease outbreaks; however, expense and limited effectiveness have prompted interest in developing strategies to increase fish resistance to disease and thus reduce the incidence of disease.

Gastrointestinal (GI) tract microbiota play a significant role in the health and nutrition of humans and

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terrestrial animals. Recently, the addition of prebiotics to diets has been shown to provide some protection against various diseases (Bailey et al. 1991; Grieshop et al. 2004; Arslanoglu et al. 2007; Sink et al. 2007; Macfarlane et al. 2008). Prebiotics were originally described as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon" (Gibson and Roberfroid 1995). These beneficial bacterial have typically been identified as *Bifidobacteria* spp. and *Lactobacilli* spp. (Gibson et al. 1999; Bouhnik et al. 2004). Prebiotics affect communities of microbiota already present in the gut; however, if the targeted beneficial bacteria are not present in the gut, prebiotics will not be able to provide any intended effects. Favorable results of prebiotics have been reported in humans (Manning and Gibson 2004; Rastall 2004), swine (Tzortzis et al. 2005), poultry (Patterson and Burkholder 2003; Chung and Day 2004; Chen et al. 2005), canines (Howard et al. 2000; Beynen et al. 2002), felines (Sparkes et al. 1998), and some fishes (Li and Gatlin 2004; Mahious et al. 2006; Sink and Lochmann 2008). While prebiotics research in fishes is still limited, a growing body of literature has reported various beneficial effects of prebiotic compounds, including increased growth, immunity, and disease resistance in several fish species (Tovar et al. 2002; Li and Gatlin 2003, 2004, 2005; Burr et al. 2005, 2008a, 2008b; Mahious et al. 2006; El-Dakar et al. 2007; Gatlin et al. 2007; Sealey et al. 2007; Sink et al. 2007; Abdel-Tawwab et al. 2008; Sink and Lochmann 2008) and in humans and terrestrial animals (Gibson and Roberfroid 1995; Howard et al. 2000; Beynen et al. 2002; Patterson and Burkholder 2003; Smiricky-Tjardes et al. 2003; Bouhnik et al. 2004; Chung and Day 2004). Further research is needed to gain greater understanding of the role of prebiotics in the diets of various fish species produced in aquaculture. Therefore, the present study was conducted with goldfish.

Methods

Experimental trials and systems.—The study consisted of two 10-week feeding trials conducted simultaneously in which juvenile goldfish were cultivated in two different culture systems to allow the presence or absence of phytoplankton and zooplankton. In each feeding trial, juvenile fish were obtained from a local retailer, sorted by size into reasonably similar groups, and stocked into 12 units within each of the two culture systems. For each feeding trial, the total initial body weight of fish in each container was similar among all groups in each culture system. The fish were allowed 1–3 weeks for conditioning in each experimental setup before the trials began. Goldfish in one feeding trial were placed into a culture system without phytoplankton or zooplankton; this indoor system consisted of 38-L aquaria supplied with recirculated well water maintained at 26 ± 1°C throughout the trial. A low-pressure blower provided supplemental aeration. Water quality conditions were monitored throughout the length of the experiment. A HACH DR/2000 direct reading spectrophotometer was used to measure alkalinity, nitrite, and total ammonia nitrogen using the appropriate HACH reagents. Total hardness was measured via EDTA titration, and dissolved oxygen, temperature, and salinity were assessed with a YSI Model 85–25 meter. Water quality conditions were maintained at acceptable levels for warmwater fish (Stickney 2005) by biological and mechanical filtration, and water exchanges were performed as needed. A diurnal photoperiod cycle of 12 h light :12 h dark was provided via fluorescent lighting.

In the other feeding trial, an outdoor system provided phytoplankton and zooplankton to 19-L plastic containers accommodated within a 1,200-L fiberglass tank, which received a continuous supply of pond water. Water was pumped from a 0.10-ha pond with dense aquatic vegetation dominated by filamentous algae, and that pond received a continuous supply of water from a 5-ha reservoir. No mechanical filtration was provided, while biological filtration consisted of a natural nitrogen cycle provided by the pond biota. A low-pressure blower provided supplemental aeration through an air stone within the fiberglass tank. Water conditions were monitored throughout the length of the experiment and remained at acceptable levels for warmwater fish (Stickney 2005). Throughout the experiment, daytime water temperatures remained relatively constant at 29.5 ± 0.5°C due to the large volume of water that supplied the fiberglass tank. A naturally occurring diurnal photoperiod cycle gradually decreased from 13.00 to 11.25 h light over the course of the trial.

Diets.—The experimental diets were composed of practical ingredients and consisted of a basal diet without supplementation and a test diet supplemented with a commercially available dairy–yeast prebiotic, GroBiotic-A, at 2% by dry weight (Table 1). GroBiotic-A is a mixture of partially autolyzed brewer's yeast *Saccharomyces cerevisiae*, dairy components, and fermentation products. Both diets were manufactured by a commercial feed mill (ARKAT Feeds, Dumas, Arkansas).

Each of the experimental diets was randomly assigned to six units within each culture system and fed to fish at a fixed percentage of body weight divided

TABLE 1.—Composition (% as fed except where indicated) of extruded basal and dairy–yeast prebiotic-supplemented diets fed to juvenile goldfish raised in the presence or absence of phytoplankton and zooplankton for 10 weeks.

Ingredient	Basal diet	Prebiotic-supplemented diet
Menhaden Select fish meal	2.0	2.0
Poultry by-product meal (feed grade)	4.0	4.0
Soybean meal	50.0	50.0
Wheat shorts	30.0	30.0
Wheat bran	9.95	7.95
Vitamin premix[a]	0.04	0.04
Mineral premix[b]	0.01	0.01
Poultry fat	4.0	4.0
Prebiotic[c]	0.0	2.0
Crude protein[d]	33.7	34.0
Total lipid[d]	7.6	7.9
Ash[d]	6.4	6.5
Energy : protein ratio (kJ/g protein)[e]	36.03	35.66

[a] Vitamin premix is U.S. Fish and Wildlife Service (USFWS) Number 30 for salmonids.
[b] Mineral premix is USFWS Number 1 for salmonids.
[c] GroBiotic-A was donated by International Feed Ingredient Corp., St. Louis, Missouri.
[d] Percent, dry basis.
[e] Estimated energy content of the diets was based on values of 16.7, 16.7, and 37.7 kJ/g for carbohydrate, protein, and lipid, respectively.

into 2 feedings/d for a total of 10 weeks. In the indoor aquarium trial, 20 fish initially averaging 1.38 g were randomly selected and stocked into each aquarium at the beginning of the conditioning period and were fed each diet initially at 3% of body weight per day for the first 3 weeks; the ration was then increased to 4% of body weight at the start of the experiment. In the outdoor trial, 10 fish initially averaging 1.74 g were stocked into each container at the beginning of the 1-week conditioning period and were fed 4% of body weight per day at the start of the experiment. Goldfish raised in the indoor experimental aquarium system were weighed once per week, whereas goldfish cultured in the outdoor system were weighed once every other week. After each weighing, the quantity of diet provided to fish in each container was adjusted according to the biomass present and established percentage of body weight per day, which was the same for each diet and culture system.

The weighing process consisted of netting the goldfish and then group-weighing by placing the fish into a tared container of water on an electronic balance; this procedure minimized handling stress. The experimental aquaria and associated equipment were located at the Texas A&M University System's Aquacultural Research and Teaching Facility in Burleson County.

Sample collection and analyses.—During the course of the feeding trials, percent weight gain, feed efficiency, and percent survival were measured as response criteria. Weight gain was expressed as a percentage of initial weight, and feed efficiency was calculated based on the total wet body weight gain of fish and the total dry feed given to fish in each group. Survival was expressed as a percentage of initial number stocked.

Gut microbiota analysis.—At the end of the 10-week feeding period, three goldfish were selected at random from each tank in the indoor aquarium system and were dissected to obtain digesta from anterior and posterior sections of their GI tracts, which were collected and stored at −80°C until further processing could begin. Digesta samples from the two GI tract sections of individual fish in each tank were processed for genomic DNA extraction as previously described (Burr et al. 2008b). The extracted DNA was subjected to polymerase chain reaction (PCR) using the method of Hume et al. (2003) with bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S ribosomal DNA. Denaturing gradient gel electrophoresis (DGGE) was then performed on the isolated DNA fragments to compare microbial communities of the gut. At the time of DGGE analysis, the replicate samples processed from all fish per tank were pooled to simplify the resulting DGGE dendrogram. The DGGE analysis was performed using the method described by Hume et al. (2003) as modified from Muyzer et al. (1993); materials and fragment analysis pattern relatedness were as described by Burr et al. (2008b). The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (version 1.6; Bio-Rad Laboratories, Hercules, California). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages for clustering (Hume et al. 2003). Relatedness of 80% or less was required to declare microbial communities as being distinct.

Disease challenge.—Remaining goldfish from each replicate container within each culture system were pooled by dietary treatment and then were randomly distributed into triplicate groups of 12 goldfish for inoculation with *Aeromonas* hydrophila. The bacterial sample was obtained from the Texas Veterinary Medical Diagnostic Laboratory (College Station) and was maintained in tryptic soy broth. The *Aeromonas* hydrophila sample was passed through goldfish in order to increase its virulence, and pretrial testing with serial dilutions of the bacteria indicated that 8.5×10^6 colony-forming units/mL was the effective dose lethal to 50% of test fish (LD50). Fish were intraperitoneally (IP) injected with 0.5 mL of the LD50, while three separate groups of 12 control fish each were injected with 0.5 mL of phosphate-buffered saline solution to ensure that no mortalities occurred due to injection method. After injection, each group of goldfish was

TABLE 2—Mean (±SE) performance, disease trial survival rates, and nitroblue tetrazolium (NBT) test results of juvenile goldfish fed basal and dairy–yeast prebiotic-supplemented diets in the presence or absence of phytoplankton and zooplankton for 10 weeks (FER = feed efficiency ratio). Values in the same column with different letters are significantly different ($P < 0.05$).

Treatment	Weight gain (% of initial weight)[a]	FER (g gain/g dry feed)[a]	Survival (%)[a]	Survival after *Aeromonas* challenge (%)[b]	NBT[c] (mg/mL)
Indoor system					
Basal diet	287 ± 7.1 z	0.43 ± 0.01 z	90 ± 2.9 y	33 ± 12.73 y	3.8 ± 0.05 y
Dairy–yeast prebiotic diet	249 ± 12.0 z	0.42 ± 0.02 z	95 ± 1.4 y	17 ± 0.00 y	3.6 ± 0.06 y
Outdoor system					
Basal diet	95 ± 2.7 y	0.26 ± 0.01 y	100 ± 0.00 z	67 ± 0.00 z	4.0 ± 0.17 z
Dairy–yeast prebiotic diet	142 ± 13.2 y	0.33 ± 0.02 y	100 ± 0.00 z	83 ± 8.33 z	4.0 ± 0.15 z
$P < F$[d]					
Culture system	<0.0001	<0.0001	<0.0001	0.0002	0.0179
Diet	0.6491	0.1493	0.1489	1.0000	0.5119
Culture system × diet	0.0003	0.0452	0.1489	0.0599	0.7095

[a] Means of sextuple groups.
[b] Means of triplicate groups.
[c] Means of duplicate groups from the outdoor system and triplicate groups from the indoor system.
[d] Significance probability associated with the F-statistic.

placed in a 38-L aquarium receiving a continuous flow of well water at 24.0 ± 0.5°C; fish were monitored every 12 h, and mortalities were recorded for 168 h after injection.

Nonspecific immunity.—Representative fish from each replicate container (three from the indoor system and two from the outdoor system) were exsanguinated via venipuncture of the caudal vasculature with heparinized needles approximately 12 h after their final feeding. Whole blood was analyzed for neutrophil oxidative radical production (nitroblue tetrazolium test; Siwicki et al. 1994; Li and Gatlin 2003, 2005) as a measure of nonspecific immunity.

Statistical analysis.—Factorial analysis of variance (ANOVA) in a 2 × 2 arrangement was used to analyze data from the two feeding trials, and Duncan's multiple-range test was used to separate treatment means when appropriate. The main factors were diet (basal versus test) and culture system (presence or absence of natural productivity; SAS Institute 1996). Responses obtained only for fish in the indoor system were analyzed by ANOVA according to a completely random design. Statistical differences were considered significant at P-values of 0.05 or less.

Results

Fish Responses in the Feeding Trials

After 10 weeks of receiving the experimental diets, fish in the indoor system without access to phytoplankton or zooplankton had a greater feed efficiency ratio (FER; $P < 0.0001$) and enhanced weight gain ($P < 0.0001$; expressed as a percentage of initial weight) in comparison with fish that were raised in the outdoor system and given access to phytoplankton and zooplankton (Table 2). There was no difference in weight gain or FER values between fish fed the two diets in either system, although there were significant ($P < 0.05$) interactions, with the dairy–yeast prebiotic tending to improve fish performance in the outdoor culture system (Table 2). Survival during the trial was higher for fish raised in the outdoor system in the presence of phytoplankton and zooplankton than for fish raised indoors ($P < 0.0001$), regardless of the dietary treatment ($P = 0.1489$; Table 2).

Gut Microbiota Analysis

Results of DGGE of the 16S rDNA amplicons from goldfish gut microbial communities are shown in Figure 1. Analysis of the dendrogram showed high relatedness (92%) among the GI microbial communities regardless of dietary treatment, presence–absence of phytoplankton and zooplankton during rearing, or GI tract section examined (anterior or posterior).

Disease Challenge and Nonspecific Immune Response

Fish reared in the presence of phytoplankton and zooplankton, regardless of dietary treatment, survived the *Aeromonas* challenge better ($P = 0.0002$) than fish reared in the absence of phytoplankton or zooplankton (Table 2). Average survival ranged between 67% and 83% for fish with access to phytoplankton and zooplankton, whereas survival was between 17% and 33% for fish without access to phytoplankton or zooplankton (Table 2). Dietary treatment had no effect on disease trial survivability ($P = 1.0000$), although the interaction between culture system and diet approached significance ($P = 0.0599$), with fish fed the dairy–yeast prebiotic tending to have greater survival when reared

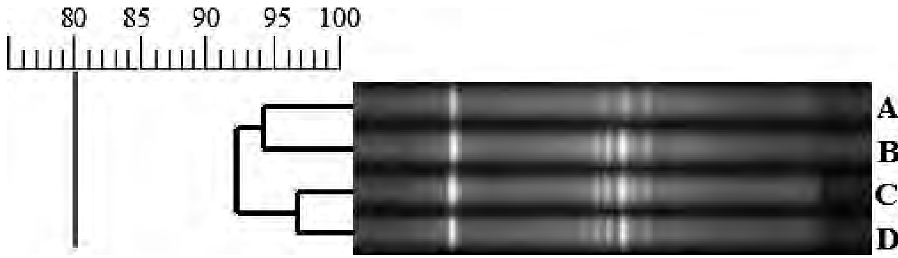

FIGURE 1.—Denaturing gradient gel electrophoresis of 16S ribosomal DNA amplicons of microbial communities from sections of the gastrointestinal (GI) tracts of goldfish cultured in an indoor aquarium system: (A) anterior GI sections of fish fed a basal diet; (B) posterior GI sections of fish fed the basal diet; (C) anterior GI sections of fish fed a dairy–yeast prebiotic-supplemented diet; and (D) posterior GI sections of fish fed the prebiotic-supplemented diet.

in the presence of phytoplankton and zooplankton (Table 2). Neutrophil oxidative radical production also was greater ($P = 0.0179$) in goldfish reared in the presence of phytoplankton and zooplankton, with no dietary effect detected (Table 2).

Discussion

The use of prebiotics in piscine diets has gained interest in recent years. GroBiotic-A, which contains partially autolyzed brewer's yeast, dairy components, and fermentation products, is a prebiotic that has been most extensively studied and has produced various beneficial effects—including increased weight gain, feed efficiency, and resistance to various pathogens—in several fish species.

Weight gain of goldfish in the current study was not influenced by the supplementation of the dairy–yeast prebiotic, although there was a tendency for improvement in the outdoor culture system. In a recent study with golden shiners *Notemigonus crysoleucas*, Lochmann et al. (2009) reported that the same dairy–yeast prebiotic slightly improved weight gain and feed efficiency of fish in an indoor aquarium trial but not in outdoor pools where fish had access to natural productivity. In the present study, somewhat surprisingly, the weight gain of goldfish located in the indoor system was greater than that of fish in the outdoor system with access to phytoplankton and zooplankton. This was most likely due to the difference in the fish culture systems, which resulted in differential access to the prepared diets. In the indoor system, diet that was not immediately eaten was available on the bottom of the aquaria for an extended period of time, allowing fish to continue foraging after the initial feeding. In contrast, the outdoor containers had mesh bottoms; uneaten diet slowly sank through the container and then through the grated bottom, making further foraging impossible. The amount of phytoplankton and zooplankton available to goldfish in the culture system was not measured at the time of the trial. However, during a subsequent trial in the same seasonal period 1 year later, zooplankton in the system averaged 207 organisms/L and was composed primarily of copepods and rotifers. Thus, goldfish had access to zooplankton as an additional food source, although the level was approximately one-third the amount found in the water of the supply pond (583 organisms/L); this difference was possibly due to destruction of organisms in the water pump. The level of zooplankton available to goldfish in the present study was considerably lower than that reported for fertilized ponds used for aquaculture production of koi (domesticated common carp *Cyprinus carpio*) and goldfish (Jha et al. 2006a, 2006b).

Unlike the results of the current study, in which dietary supplementation with the dairy–yeast prebiotic had limited effect on goldfish FER values, Li and Gatlin (2004, 2005) reported enhanced feed efficiency in hybrid bass (white bass *Morone chrysops* × striped bass *M. saxatilis*) that were fed diets supplemented with GroBiotic-A. Weight gain of hybrid bass fed GroBiotic-A in those studies also generally tended to be greater than that in fish fed the basal diet, although the differences were not always statistically significant. After 3 weeks of receiving diets supplemented with GroBiotic-A or partially autolyzed brewer's yeast, rainbow trout *Oncorhynchus mykiss* exhibited enhanced FER, but no differences in weight gain were observed (Sealey et al. 2007). The improved FER may be due to further enhanced nutrient digestibility, as reported in different fish species that were fed diets supplemented with prebiotics or live yeast (Tovar et al. 2002; Waché et al. 2006; Abdel-Tawwab et al. 2008). A recent study of red drum *Sciaenops ocellatus* (Burr et al. 2008a) also reported enhanced protein and energy digestibility coefficients for a soybean-meal-based diet supplemented with GroBiotic-A at 1% dry weight compared with the basal diet.

Feeding the dairy–yeast prebiotic to goldfish in the present study did not result in differences in the

microbiota of the GI tract according to DGGE analysis of the 16S rDNA amplicons. This analysis requires relatedness to be 80% or lower for microbial communities to be considered distinctive; values greater than 80% therefore indicate similar bacterial species. The DGGE analysis of the anterior and posterior sections of goldfish GI tracts indicated that relatedness among gut microbiota was 92% overall (Figure 1), which is in contrast to results of a recent in vitro study (Burr et al. 2008a) in which incubation of red drum intestinal contents with GroBiotic-A produced a distinct microbial population. A change in microbial composition of GI tract microbiota also was recently reported (Burr 2007) for hybrid bass fed a soybean-meal-based diet supplemented with GroBiotic-A at 1% of dry weight.

Goldfish reared in the presence of phytoplankton and zooplankton in the present study expressed far greater survival during the disease challenge with IP injection of *Aeromonas* hydrophila, regardless of dietary treatment. The IP administration of phosphate-buffered saline to the control goldfish did not kill any of the fish, thus confirming that *Aeromonas* hydrophila was the cause of mortality in goldfish exposed to the pathogen. Mortality continued until 96 h after IP injection, and then no further mortality was recorded through 168 h postinjection. Due to *Aeromonas* spp. naturally occurring in freshwater pond systems (Swann and White 1989), it is believed that goldfish in the present study reared in the outdoor system were naturally exposed to the bacterium and thus were able to build some immunological resistance to the disease. As a result, goldfish from the outdoor system had higher survival after the disease challenge (83% survival) compared with those from the indoor system. Goldfish from the outdoor system also had higher blood neutrophil oxidative radical production, indicating elevated nonspecific immunity.

Li and Gatlin (2004, 2005) demonstrated statistically improved survival of hybrid bass fed a diet supplemented with dairy–yeast prebiotic when exposed to *Streptococcus iniae* and *Mycobacterium* sp. via immersion. GroBiotic-A also has been reported to improve survival of rainbow trout after experimental exposure to infectious hematopoietic necrosis virus (Sealey et al. 2007). Similarly, golden shiners fed GroBiotic-A experienced reduced mortality when experimentally exposed to the agent of columnaris disease, *Flavobacterium columnare*, which is a major pathogen in golden shiner aquaculture (Sink and Lochmann 2008). However, based on the results of the current study with goldfish, this dairy–yeast prebiotic did not improve resistance to *Aeromonas* hydrophila. However, the presence of phytoplankton and zooplankton conferred benefits to goldfish in terms of survival after *Aeromonas* hydrophila challenge.

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Establishing a risk-assessment process for release of genetically modified wine yeast into the environment

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Abstract: The use and release of genetically modified organisms (GMOs) is an issue of intense public concern and, in the case of food and beverages, products containing GMOs or products thereof carry the risk of consumer rejection. The recent commercialization of 2 GM wine yeasts in the United States and Canada has made research and development of risk assessments for GM microorganisms a priority. The purpose of this study was to take a first step in establishing a risk-assessment process for future use and potential release of GM wine yeasts into the environment. The behaviour and spread of a GM wine yeast was monitored in saturated sand columns, saturated sand flow cells, and conventional flow cells. A widely used commercial *Saccharomyces cerevisiae* wine yeast, VIN13, a VIN13 transgenic strain (LKA1, which carries the *LKA1* α-amylase gene of *Lipomyces kononenkoae*), a soil bacterium (*Dyadobacter fermentens*), and a nonwine soil-borne yeast (*Cryptococcus laurentii*) were compared in laboratory-scale microcosm systems designed to monitor microbial mobility behaviour, survival, and attachment to surfaces. It was found that LKA1 cells survived in saturated sand columns, but showed little mobility in the porous matrix, suggesting that the cells attached with high efficiency to sand. There was no significant difference between the mobility patterns of LKA1 and VIN13. All 3 yeasts (VIN13, LKA1, and *C. laurentii*) were shown to form stable biofilms; the 2 *S. cerevisiae* strains either had no difference in biofilm density or the LKA1 biofilm was less dense than that of VIN13. When co-inoculated with *C. laurentii*, LKA1 had no negative influence on the breakthrough of the *Cryptococcus* yeast in a sand column or on its ability to form biofilms. In addition, LKA1 did not successfully integrate into a stable mixed-biofilm community, nor did it disrupt the biofilm community. Overall, it was concluded that the LKA1 transgenic yeast had the same reproductive success as VIN13 in these 3 microcosms and had no selective advantage over the untransformed parental strain.

Key words: risk assessment, genetically modified organism (GMO), *Saccharomyces cerevisiae*, yeast, *LKA1*, biofilm.

Résumé : L'utilisation et la commercialisation d'organismes génétiquement modifiés (OGM) est l'objet d'une grande préoccupation publique et, dans le cas des aliments et boissons, les produits contenant des OGM ou leurs sous-produits risquent d'être rejetés par le consommateur. La commercialisation récente aux États-Unis et au Canada de 2 lignées de levures de vin GM a fait de la recherche et développement en évaluation du risque des microorganismes GM une priorité. Le but de cette étude était de franchir une première étape en établissant un processus d'évaluation du risque de l'utilisation future et de la commercialisation de levures de vin GM pour l'environnement. Le comportement et la dispersion de la levure de vin GM ont été suivis dans des colonnes de sable saturées, des cellules à écoulement de sable saturées et dans des cellules à écoulement conventionnelles. *Saccharomyces cerevisiae* VIN13, une levure de vin commerciale largement utilisée, une lignée VIN13 transgénique (LKA1, qui comporte le gène de l'α-amylase *LKA1* de *Lipomyces kononenkoae*), une bactérie du sol (*Dyadobacter fermentes*) et une levure du sol non œnologique (*Cryptococcus laurentii)* ont été comparées dans ces microcosmes expérimentaux conçus pour suivre la mobilité microbienne, la survie et l'attachement à des surfaces. Les cellules LKA1 survivaient dans les colonnes de sables saturées mais étaient peu mobiles dans la matrice poreuse, suggérant que les cellules s'attachaient au sable avec haute efficacité. Il n'y avait pas de différences significatives entre les patrons de mobilité de LKA1 et ceux de VIN13. Les 3 souches de levures (VIN13, LKA1 et *C. laurentii*) formaient des biofilms stables; la densité des biofilms formés par les 2 souches de *S. cerevisiae* était la même, ou sinon, le biofilm formé par LKA1 était moins dense que celui de VIN13. Lorsqu'elle était inoculée avec *C. laurentii*, LKA1 n'avait pas d'in-

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fluence négative sur l'écoulement de *Cryptococcus* dans la colonne de sable, ou sur sa capacité de former des biofilms. De plus, LKA1 ne s'intégrait pas efficacement dans une communauté mixte stable au sein d'un biofilm, et ne perturbait pas la communauté du biofilm. En somme, nous pouvons conclure que la réussite de reproduction de la levure transgénique LKA1 est la même que celle de VIN13 dans ces trois microcosmes, et que LKA1 ne possède pas d'avantages sélectifs par rapport à la souche parentale non transformée.

Mots-clés : évaluation du risque, organisme génétiquement modifié (OGM), *Saccharomyces cerevisiae*, *LKA1*, biofilms.

[Traduit par la Rédaction]

Introduction

The yeast *Saccharomyces cerevisiae* has a long and distinguished history as a fermentation agent. Because of its efficiency in producing alcohol and gas, *S. cerevisiae* is, without doubt, the most important commercial microorganism with generally regarded as safe (GRAS) status (Pretorius et al. 2003). By brewing beer, making wine, and leavening bread dough, our longest serving domesticated organism made possible the world's earliest known biotechnological processes. Since the emergence of genetic engineering in the 1970s, *S. cerevisiae* has played a leading role in shifting the frontiers of molecular biology, increasing our understanding of fundamental cellular and molecular processes, and enabling the application of modern biotechnological tools to research on, and development of, industrial microorganisms. The importance of *S. cerevisiae* has extended beyond traditional fermentation. Today, the products of yeast biotechnologies impinge on many commercially important sectors, including food, beverages, biofuels, chemicals, industrial enzymes, pharmaceuticals, and agriculture.

Two genetically modified (GM) wine yeasts were recently cleared and given GRAS status by the American Food and Drug Administration, Health Canada, and Environment Canada. Unlike conventional wine yeasts, one of these commercialized GM wine strains has the capacity to conduct malolactic fermentation, thereby reducing the risk of biogenic amine formation by certain bacteria during malolactic fermentation (Husnik et al. 2006). The second GM wine yeast secretes much less urea, which limits the production of ethyl carbamate (Coulon et al. 2006). Whether consumers in key export markets accept a wine made with GM yeasts will clearly determine the commercial success of this technology and, indeed, its short-term future.

The use of GM organisms (GMOs) in commercial winemaking is controversial for the same reasons as those presented in often-heated debates whenever this topic is considered. Many members of the world wine community are watching closely to see what eventuates from developments in the United States and Canada. In most wine-producing countries, including Australia and South Africa, GMOs are prohibited in commercial winemaking, and it will take widespread consumer acceptance and legal approval by regulatory bodies to change this. Whatever the final outcome, extensive use of GM yeasts could soon become a reality in some wine-producing countries. Therefore, there is an urgent need to assess and address the perceived health and environmental risks associated with the use of GMOs in the human food chain (Bauer et al. 2004).

Generally, an appropriate risk-assessment procedure is an essential prerequisite before considering the use and release of any GMO into the environment. Almost all adopted risk-assessment strategies are based on a common set of principles and guidelines; Paoletti et al. (2008) presented a review on the different strategies followed by the European Union, the United States, Canada, and Australia. One approach to GMO risk assessment is to compare the relative fitness of the GM strain with that of the parent it was derived from. Any differences then become the focus of the safety assessment. Such comparisons require experimental systems and in situ studies that simulate the natural environments in which the GM strains are most likely to be found. In a typical winemaking environment, the yeast lees is disposed of in waste water systems and the grape skins are returned to the vineyard.

Microorganisms have been shown to associate with and become established on surfaces in aqueous and terrestrial environments. Biofilm formation is acknowledged as a universal microbial strategy for survival, and has been implicated in many natural, engineered, and medical systems and processes. Biofilms are, therefore, realistic model systems with which to study microbial behavior, including comparisons between transformed and parental strains.

Soil is an important part of the biosphere, and the movement, biofilm formation, and growth of GM yeast in this habitat will largely affect its survival prospects. In soil, microorganisms occur either as suspended cells in pore water or as biofilms on soil surfaces. Growth conditions in the soil differ substantially from those that are typically applied in the laboratory during the cultivation of microorganisms; the latter often involving pure cultures and employing rich growth media that do not closely match what is found in nature. Furthermore, research on biofilm formation has focused primarily on bacteria, while yeast-biofilm research is mostly performed on clinically relevant strains of the *Candida* genus. Many fungi involved in biofilm formation are not amenable to genetic studies, and this is one of the reasons that relatively little is known about fungal biofilms (Baillie and Douglas 1999; O'Toole et al. 2000). While searching for a model system to study fungal biofilms, Reynolds and Fink (2001) found that *S. cerevisiae* can initiate biofilm formation on a number of plastic surfaces. It was suggested that active metabolism is a requirement for adherence, since growth in a lowered glucose concentration enhanced the adherence of yeast to plastic surfaces, whereas the total absence of glucose led to a reduced adherence. The expression of *FLO11* in *Saccharomyces* may play a similar role in adherence to the glycopeptidolipids expressed on the cell surface of the nonflagellated bacterium *Mycobacterium smegmatis*. These glycopeptidolipids are thought to be required for biofilm for-

mation, as well as sliding motility behaviour, because they increase surface hydrophobicity (Recht et al. 2000).

Despite the widespread use of *S. cerevisiae* in industry and science, relatively few studies have addressed biofilm formation by this organism. In spite of many efforts to find *S. cerevisiae* in nature, it is seldom found on healthy, undamaged grapes, and is rarely isolated from vineyard soils (Martini 1993). There is, however, a school of thought that supports the idea that the primary source of *S. cerevisiae* is indeed the vineyard, with its occurrence differing entirely with each plant and grape cluster (Török et al. 1996). *Saccharomyces cerevisiae* strains isolated from vineyards are mostly found on damaged grapes, where competition has led to the development of extremely efficient fermentation behaviour (Landry et al. 2006). Others are of the opinion that *S. cerevisiae* does not have a natural habitat, but is primarily a domesticated species that has been selected as part of the winemaking process over thousands of years. Previously, *S. cerevisiae* has rarely been isolated from areas that are not closely associated with humans (Naumov and Naumova 1991; Naumov 1996; Naumov et al. 1998). Recently, Sampaio and Gonçalves (2008) reported the isolation of 4 *Saccharomyces* species from tree bark, particularly certain oak species. The sequencing of other *S. cerevisiae* strains that were isolated from oak trees has demonstrated that wild *S. cerevisiae* is significantly genetically differentiated from domesticated strains (Fay and Benavides 2005; Aa et al. 2006). However, the historical relationship of wild *S. cerevisiae* with humans might never be clearly known (Replansky et al. 2008). The dissemination of naturally occurring and commercial *Saccharomyces* yeasts has been studied by several researchers over the past decade (Pretorius et al. 1999; Mortimer and Polsinelli 1999; Khan et al. 2000; Van der Westhuizen et al. 2000*a*, 2000*b*; Jolly et al. 2003; Schuller et al. 2005; Valero et al. 2005). The conclusion from some of these studies suggests that, although commercial wine strains could be enriched from vineyards, fears of commercial yeasts ultimately dominating the natural microflora have been exaggerated (Van der Westhuizen et al. 2000*a*, 2000*b*; Valero et al. 2005). However, there have been no published data on GM yeast persistence in natural environments or their impact on the soil microflora.

In this study, the behaviour of a commercial *S. cerevisiae* wine yeast strain, VIN13, and a GM variant of it, LKA1 (VIN13 transformed with the *LKA1* α-amylase encoding gene from *Lipomyces kononenkoae*) were studied under different scenarios, along with a typical soil bacterium (*Dyadobacter fermentens*) and soil yeast (*Cryptococcus laurentii*). To evaluate the persistence and movement of VIN13 and LKA1 through soil, sand columns were prepared, and breakthrough curves and survival rates were determined over several months. It is known that microorganisms adhere to surfaces and grow as sessile biofilm communities in response to environmental stress conditions (e.g., oligotrophic conditions). Therefore, continuous-flow cells were used in combination with epifluorescence and scanning electron microscopy (SEM) to determine whether the strains formed biofilms on sand and glass surfaces, verifying the results obtained in the soil columns. The results from this study demonstrate that the approach followed, especially the use of biofilms as an experimental system, is a suitable methodology for comparing the environmental behavior of GM yeasts with their parental strains, which should form part of the risk-assessment process established for GM wine yeasts.

Materials and methods

Microbial strains and culture conditions

A commercial wine yeast, *S. cerevisiae* VIN13, and a GM VIN13 strain (designated LKA1) transformed with an α-amylase encoding marker gene from the yeast *L. kononenkoae* were used as examples of industrially relevant wine yeast strains. *Cryptococcus laurentii* UOFS Y-1880 was used as an example of a widely occurring environmental yeast. This strain was originally identified using morphological and physiological characteristics, as well as sequence analyses of the D1/D2 region of the large subunit rDNA (Botes et al. 2005). *Cryptococcus laurentii* UOFS Y-1880 is being maintained in the Yeast Culture Collection of the University of the Free State, South Africa. To establish a mixed-biofilm community, the yeasts *Schizosaccharomyces pombe* ATCC 24843, *Saccharomycodes ludwigii* CBS 2625, and *Kloeckera apiculata* ATCC 66165, and the bacterial isolates CT01, CT04, CT05, and CT08 were included in this study. Sequencing of the 16S ribosomal DNA of bacterial isolates CT01, CT04, and CT08 (in both directions to completion) identified them as *Microbacterium nematophilum*, *D. fermentens*, and *Pseudomonas* sp. AEBL3, respectively. No closely related phylogenetic affiliation could be found for CT05. The bacterial strains were isolated from biofilms, where they coexisted in an oligotrophic environment, and were selected because they could be co-cultured as a stable community. The yeast strains were selected because of their frequent association with vineyards and wine cellars.

The *LKA1* gene was expressed in *S. cerevisiae* VIN13 under the control of the yeast phosphoglycerate kinase I (*PGK1*) promoter and terminator, and was integrated into the genome of VIN13 via the sulfometuron methyl resistance marker gene (*SMR1*). *LKA1* was originally cloned from the yeast *L. kononenkoae* (Steyn et al. 1995), and encodes a raw starch-degrading α-amylase that liberates reducing groups from glucose polymers containing both α-1,4 and α-1,6 bonds. VIN13 transformants containing the *LKA1* gene were identified as colonies that produce clear zones of raw starch degradation on Phadebas agar plates. The Phadebas agar medium contained 6.7 g yeast nitrogen base with amino acids (Difco; Detroit, Michigan), 1 g glucose, 20 Phadebas tablets (Phadia AB; Portage, Michigan), and 20 g agar per litre of distilled water.

Monitoring of yeast behaviour in a porous matrix

Saturated sand columns under conditions of continuous flow

Polyethylene columns (23 mm in diameter and 140 mm long) were filled with sieved (1180 μm) autoclaved homogeneous commercial sand. The columns were presterilized with 3.5% (*v*/*v*) sodium hypochlorite for 0.5 h, and then rinsed for 4 h with sterile Ringer's solution (Merck; Whitehouse Station, New Jersey). The total pore volume (PV) was determined for each experiment. Streptomycin sulfate (0.5 g/L)

was added to the soil columns as needed to control bacterial contamination.

The inlet of each column was connected with silicon tubing (internal diameter of 1.6 mm) to a Watson-Marlow 502S peristaltic pump and a reservoir containing sterile Ringer's solution. The Ringer's solution was pumped through the column at 2.3 mL/h. One PV of the culture suspended in Ringer's solution was subsequently pumped through the columns at the same flow, after which flow with Ringer's solution was resumed. A fraction collector (Model 2110, Bio-Rad; Hercules, California) was used to collect effluent samples every hour.

Preparation of cultures

Isolates were cultivated overnight at 30 °C; strains VIN13 and LKA1 were cultivated in 50 mL full-strength YM medium (containing 0.3% yeast extract, 1% glucose, 0.3% maltose, 0.5% peptone from casein), and *C. laurentii* and *D. fermentens* were cultivated in 50 mL 10% YM medium. Cultures were centrifuged for 5 min at 5000 r/min and washed 3 times with Ringer's solution. Cells were then resuspended in 150 mL Ringer's solution and the cell suspensions were introduced into the columns as described earlier. In addition to pure culture suspensions, co-cultures of *C. laurentii* and the *Saccharomyces* strains were simultaneously introduced into columns, using equal volumes of cultures. The initial cell concentration was determined in each case by serial dilution and enumeration of viable cell numbers on YM agar plates.

Sampling methods

The total volume of selected samples (2.7 mL each) obtained from the fraction collector was centrifuged for 5 min at 5000 r/min, resuspended in 200 μL Ringer's solution, and plated in duplicate onto YM agar plates containing streptomycin sulphate (0.5 g/L). Tryptone soy agar (TSA) plates (Biolab; Ipswich, Masschusetts) without streptomycin sulfate were used in the experiments with *D. fermentens*. At the end of each experiment, the sand columns were dissected in 1 cm sections, and the sand from each section was aseptically removed to determine the penetration distance of the test organism. Each sand sample (10 g) was added to 90 mL of Ringer's solution in an Erlenmeyer flask, shaken by hand for 2 min, and mixed by vortex (Vortex Genie 2, Scientific Industries; Bohemia, New York) for 1 min at maximum speed. In separate experiments, the time required for efficient removal of cells from the sand grains showed that a minimum of 1 min of vortexing is required. In most instances, there is little improvement of dispersion efficiency with longer exposure, with reduction in viability typically occurring for vortex times >5–7 min. Samples were serially diluted, and 100 μL was plated in duplicate onto YM agar plates containing streptomycin sulphate (0.5 g/L) for yeast, and on TSA plates without streptomycin sulfate for *D. fermentens*. The plates were incubated for 2–3 days at 30 °C.

Soil microcosms at 20% moisture content

To evaluate the long-term survival of the LKA1 transformant in a typical vineyard soil, PVC containers (18 cm in diameter) were filled with ~1.2 kg of typical vineyard soil (consisting of 1/3 peat moss, 1/3 bark, 1/3 fine sand; pH (KCl) 6.2; density, 550.8 kg/m^3) (Newcellar, South Africa) and inoculated with the VIN13 or LKA1 strain. Yeasts were cultivated overnight at 30 °C in 100 mL YPD medium (containing 1% yeast extract, 2% glucose, 2% peptone from casein) and centrifuged at 5000 r/min for 5 min. Cells were washed twice with sterile water and resuspended in a volume of water to a final soil moisture content of 20%. VIN13 and LKA1 were inoculated at a concentration of 4.20×10^7 and 2.98×10^7 CFU/mL, respectively. The soil was watered on a weekly basis to a moisture content of 20%, and sampled at several intervals. Sampling involved collection of 10 g composite soil aliquots from the upper 5 cm of soil in each container, which were added to Erlenmeyer flasks containing 90 mL Ringer's solution. Serial dilutions were then prepared as described earlier, plated in duplicate onto YM agar plates supplemented with 12% ethanol (to prevent fungus growth and enrich *S. cerevisiae* growth), and incubated at 30 °C for 2 to 3 days. Yeast-like colonies were subsequently replica plated onto lysine agar (Biolab) and Phadebas (Phadia AB) agar plates. After 14 weeks, the sampled soil was added to Erlenmeyer flasks containing 90 mL of either Colombard grape must or 1% YM medium for enrichment purposes. These Erlenmeyer flasks were incubated for 1 day at room temperature without shaking before serial dilutions were made and the yeast numbers determined on YM agar plates.

Continuous-flow cells filled with sand

To further investigate the behaviour of yeast in the sand columns, flow cells filled with sand were used to visualize yeast–sand interactions. The channels of Perspex flow cells (Wolfaardt et al. 1994) were filled with autoclaved sand and covered with a glass cover slip (No. 1 thickness, 75 mm × 50 mm). Each flow-cell channel was connected with autoclaved silicone tubing (1.6 mm internal diameter) to a reservoir containing either Ringer's solution or 1% YM medium. Each flow cell consisted of 6 flow channels (each 310 mm long × 40 mm wide × 2.2 mm deep). These sand-filled flow chambers were sterilized for 1 h with 3.5% (*v*/*v*) sodium hypochlorite and rinsed overnight with sterile growth medium. Aliquots of 300 μL of overnight VIN13, LKA1, and *C. laurentii* cultures grown in full-strength YM medium (1% in the case of *C. laurentii*) were used to inoculate duplicate flow chambers. The overnight cultures were washed once with sterile distilled water (5000 r/min for 5 min) before resuspension in either Ringer's solution or 1% YM medium. After 4 h, flow was resumed through the channels at a flow rate of 2.3 mL/h, using a Watson-Marlow 205S multichannel peristaltic pump. One mL of effluent from each channel was collected at daily intervals for 7 days, serially diluted, and plated in duplicate onto YM agar plates to determine suspended cell numbers. Biofilms were allowed to develop for periods of 3, 5, or 7 days before the channels were stained with CalcofluorTM White M2R (Molecular Probes; Eugene, Oregon) at a concentration of 25 μmol/L. Images were randomly captured with epifluorescence microscopy at 600× magnification. Channels were also stained with the FUN-1TM yeast viability probe (Molecular Probes), at a concentration of 40 μmol/L, after each experiment of biofilm development.

A Nikon Eclipse E400 epifluorescence microscope, equipped with excitation/barrier filter sets of 465-495/515-555 nm (Texas Red) and 540-580/600-660 nm (FITC), as well as a multipass filter set for viewing DAPI, was used for in situ visualization of biofilm formation on the sand. Images were captured with a COHU high-performance CCD camera (Model No. 4912-5010/0000) and a Nikon (Coolpix 9909) digital camera. A SEM (LEO 1430 VP) was used to obtain high-magnification images of biofilm formation on sand granules that were collected from the flow channels after 4 and 17 days. Samples were gold-coated with an Edwards S150A sputter coater.

Yeast behaviour in an aqueous environment

Biofilm formation by yeast isolates

Flow cells were also used to observe the behaviour of the test strains in an aqueous environment. In this case, sterile flow cells were irrigated with 1% YM medium or 10% artificial winery effluent (AWE). The AWE contained (per L) 1.7 g yeast nitrogen base without amino acids (Difco) and ammonium sulfate, 5 g $(NH_4)_2SO_4$, 1.8 g glucose, 1.8 g fructose, 1 mg butanol, 1 mg citric acid, 2 mg malic acid, 2 mg tartaric acid, 2 mg lactic acid, 1.24 mg propanol, 3.8 mg isoamyl alcohol, 0.25 g acetic acid, 4 mg ethyl acetate, 8 mg propionic acid, 1 mg valeric acid, 0.5 mg hexanoic acid, 0.7 mg octanoic acid, and 10 mg ethanol, with the pH adjusted to 4 using NaOH. Individual flow chambers were inoculated in duplicate with 300 μL of overnight cultures of VIN13 and LKA1, grown in full-strength YM medium, and *C. laurentii*, grown in 1% YM medium. After washing once with distilled water, the yeast cultures were suspended in 1% YM medium to serve as inocula. When flow cells were irrigated with 10% AWE, the flow chambers were inoculated in duplicate with 400 μL of overnight cultures of VIN13 and LKA1, which were suspended in 10% AWE after washing. Flow of the irrigation medium was resumed at a flow rate of 2.3 mL/h after 4 h and kept at room temperature (22 °C). Biofilms were stained with CalcofluorTM White M2R and FUN-1TM (Molecular Probes) to determine cell viability using epifluorescence microscopy at the end of the experiments. Standard procedures were followed to enumerate cell numbers in the effluent.

In addition to microscope analysis, a photometric approach was applied to quantify and compare biofilm formation of VIN13, LKA1, and *C. laurentii*. Parallel-plate flow cells with an internal volume of 12 mL were used to measure the accumulation of biomass on glass surfaces. In essence, this approach measures light that passes through the flow cell. Biofilm accumulation is recorded in real time as a consequence of the lowered intensity of forward scattered light (Saftic et al. 2005). Data were recorded at 5 min intervals for 9 days, and stored with a designated program executed in Lab View Student Edition, version 3.1 (National Instruments; Berkshire, UK). Preparation of flow cells and inoculation were performed as described for conventional flow-cell experiments.

Behaviour of industrial yeast strains in a biofilm community setting

Flow cells were irrigated with 10% AWE, and replicate flow chambers were inoculated with 400 μL of a mixed-microbial community. The community consisted of 1 mL of an overnight culture of each of the yeasts (*Schizosaccharomyces pombe*, *S. ludwigii*, and *K. apiculata*) and bacteria (*M. nematophilum*, isolate CT05, and *Pseudomonas* sp. AEBL3). The yeasts were cultivated in YM medium and the bacteria in 10% tryptone soy broth (Biolab), after which they were washed once in sterile distilled water and resuspended in 10% AWE. Flow was continued at a rate of 4.1 mL/h for 5 days before flow was arrested, and 400 μL of an overnight culture of LKA1 was inoculated in duplicate. Effluent was collected on days 3, 5 (before the addition of LKA1), and 10, serially diluted, plated onto YM and Wallerstein Laboratory nutrient agar plates (Difco), and incubated for 4–5 days at 30 °C. Colony colour and morphology on Wallerstein Laboratory nutrient agar plates were used to distinguish between the different yeast strains. On day 10, the diluted effluent was plated on Phadebas agar plates to distinguish LKA1.

Results and discussion

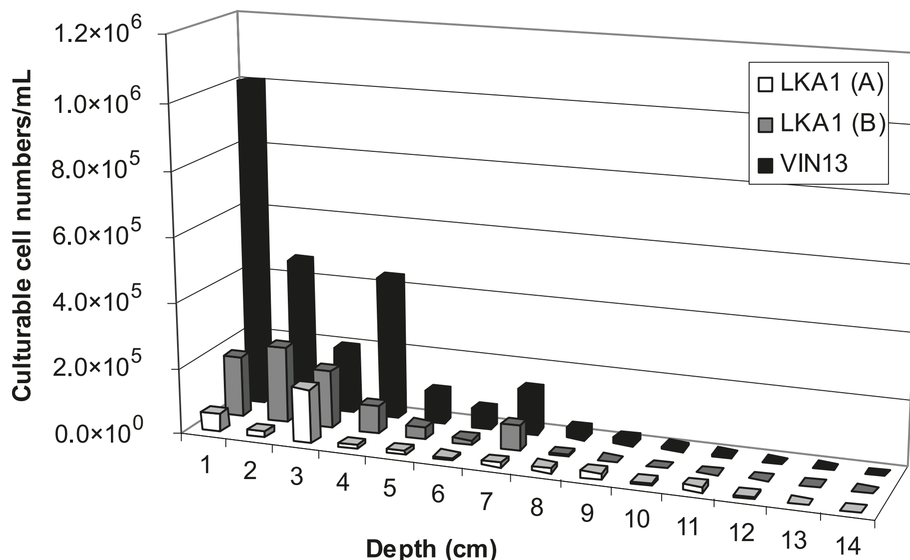
Behaviour of wild-type and GM wine yeasts in sand columns

No breakthrough of VIN13 or LKA1 cells occurred through the sand columns and, even after passage of up to 36 PV (~290 h), yeast cells could not be detected in the effluent collected during replicate experiments. The columns were dissected in 1 cm sections at the end of each experiment to assess the fate of the cells in the saturated sand. The majority of cells was found in the first 6 to 7 cm of the columns, and did not spread farther than 11 cm from the column inlet (Fig. 1).

The soil yeast *C. laurentii* and bacterium *D. fermentens* were included in the comparison between *S. cerevisiae* strains VIN13 and LKA1 in the sand matrix. Duplicate column experiments with *C. laurentii* showed a rapid breakthrough by this yeast; in both cases, a breakthrough of cells was observed after 1 PV (~9 h) (Fig. 2A). This figure also shows that when the LKA1 transgenic strain was co-inoculated with *C. laurentii*, it had a breakthrough (migration through the sand profile) similar to that seen when it was introduced in pure culture, indicating that the presence of the GM wine yeast did not have an influence on the movement of the soil yeast through the sand.

Dissection of the columns and subsequent plating revealed that *C. laurentii* cells were distributed along the length of the column, whereas LKA1 cells were primarily detected in the first 2 cm (Fig. 2B). The higher numbers of *C. laurentii* demonstrate the competitive advantage that *Cryptococcus* has over LKA1 in soil. This observation may be because soil is the natural habitat for *C. laurentii*, but also because of the known ability of *Cryptococcus* spp. to produce antimicrobial substances that can inhibit the *Saccharomyces* strains. Support for this is the observation that, when introduced in pure culture, LKA1 migrated as far as 11 cm through the column, compared with 2 cm when co-introduced with *C. laurentii.* Considering the possibility that *S. cerevisiae* originated from the hybridization of other *Saccharomyces* species in man-made environments, it is likely that *Cryptococcus* may have evolved more effective strategies for dis-

Fig. 1. Distribution of a wild-type commercial *Saccharomyces cerevisiae* VIN13 wine yeast and a genetically modified VIN13 strain, LKA1, through sand columns, showing that the cells were attenuated in the sand. A constant flow of the irrigation medium was maintained in duplicate experiments through the columns (0.125 pore volume (PV)/h). No yeast cells were detected in the effluents, even after extended periods of flow (≤36 PV). This graph shows data for VIN13 after 36 PV (290 h), for LKA1 after 17 PV (137 h) (A) and for LKA1 after 9 PV (76 h) (B).



tribution and proliferation in soil than *Saccharomyces*. This is especially interesting when the fact that the 2 yeasts have cells of similar size is taken into account. Overall, there was not a difference between the mobility of the GM and non-GM *Saccharomyces* strains in saturated sand.

As with *C. laurentii*, the bacterium *D. fermentens* could be detected in the column effluent after 1 PV, and when the columns were dissected at the end of each experiment, cells were found distributed along the full length of the columns (Fig. 2C). When mass balances were performed, the total numbers of cells detected in the columns inoculated with *C. laurentii* and *D. fermentens* were higher than the cell numbers introduced to the respective columns. A possible explanation for this observation is that both of these organisms are well adapted to proliferate under oligotrophic conditions; therefore, the Ringer's solution and impurities associated with sand were sufficient to support prototrophic growth by these organisms.

The survival of GM wine yeast in soil over time

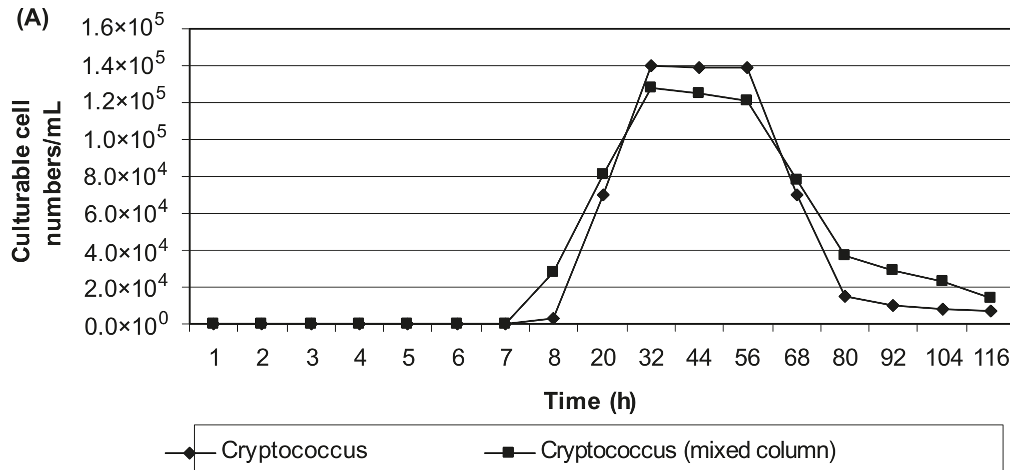
The transgenic LKA1 strain and the VIN13 control strain were detected in the soil in comparable numbers 2 weeks after inoculation (Table 1). However, after longer periods of incubation (7–12 weeks), the organisms were either not detected or were present in numbers that were too low for enumeration. This result is in agreement with a number of studies that have investigated the environmental fate of bacterial inoculants in soils (Van Elsas et al. 1998). Bacteria introduced to soils generally respond with a decline in population density, which often results in their persistence at low densities or complete loss. Several biotic and abiotic factors that can affect this behaviour have been reviewed by Van Veen et al. (1997) and Stotzky (1980, 1995). Similarly, an analysis of yeast population variations from year to year indicated that permanent colonization of commercial wine yeasts in vineyards did not occur, but instead that these strains were subject to natural fluctuations of periodic appearances and disappearances, like autochtonous strains (Valero et al. 2005). These authors showed that the dissemination of commercial yeasts in vineyards was restricted to short distances and limited periods of time, and was mainly favoured by the presence of water runoff (Valero et al. 2005).

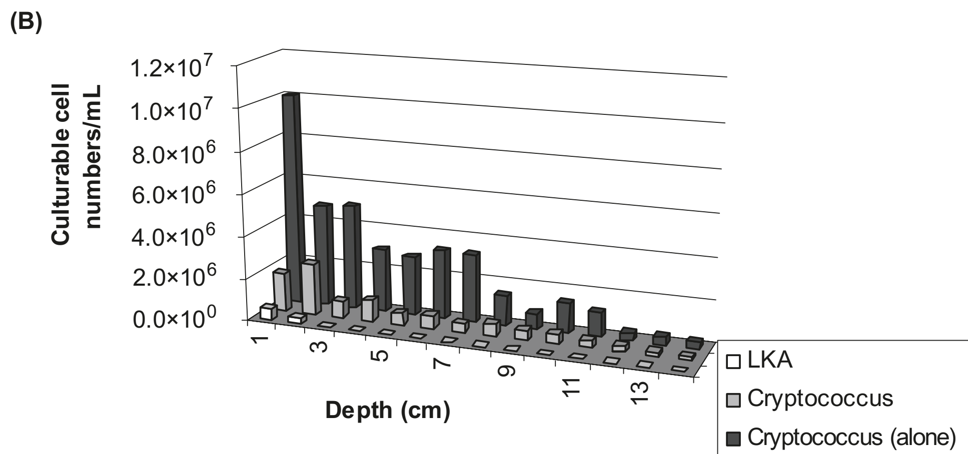
Following the failure to detect introduced yeasts after prolonged incubation in the soil, we decided to use an enrichment strategy, primarily to assess whether there were any surviving cells in the soil. Both strains could be enriched from the soil after 14 weeks when Colombard must was used as enrichment medium. However, after 16 weeks, no statistically meaningful data could be obtained and, therefore, 1% YM medium was used for enrichment on week 18. These results indicate that cells of both the GM and non-GM *Saccharomyces* wine yeasts remained viable in vineyard soil over an extended period of time, although the majority of cells lost their culturability.

Biofilm formation by wine and soil yeasts

The data presented in Figs. 1 and 2 suggest that the test yeast strains attached to sand grains during the column studies. Therefore, the subsequent experiments focused on biofilm formation by these organisms. Initially, this involved the packing of flow cells with the same sand and irrigation with Ringer's solution, similar to the column experiments. Qualitative observations with an epifluorescent microscope showed that both VIN13 and LKA1 attached to the sand grains in pure culture, but they were noticeably less extensive than the biofilms formed by *C. laurentii*, an observation that was supported by environmental SEM (Figs. 3A–3C). Because of the uneven nature of the attachment surface of sand grains, and the inherent difficulty in accurately determining the extent of biofilm formation, the latter was not quantified. However, replicate experiments strongly sug-

Fig. 2. Mobility and survival of the soil yeast *Cryptococcus laurentii* in saturated sand. (A) In contrast with the wild-type and genetically modified *Saccharomyces cerevisiae* wine yeast strains (VIN13 and LKA1), *C. laurentii* broke through after 1 PV (9 h). (B) Distribution of *C. laurentii* and the LKA1 transgenic wine yeast through sand columns after 14 PV (108 h), showing that, similar to its breakthrough profile (A), the presence of LKA1 had little effect on the distribution of *C. laurentii*. In contrast to the *S. cerevisiae* wine strains, *C. laurentii* was found throughout the length of the column, either when co-inoculated with LKA1 or inoculated on its own, supporting the breakthrough data shown in (A). (C) Similar to *C. laurentii*, *Dyadobacter fermentens* was distributed throughout the length of the column and broke through after 1 PV (results not shown). Column was run for 19 PV (145 h).





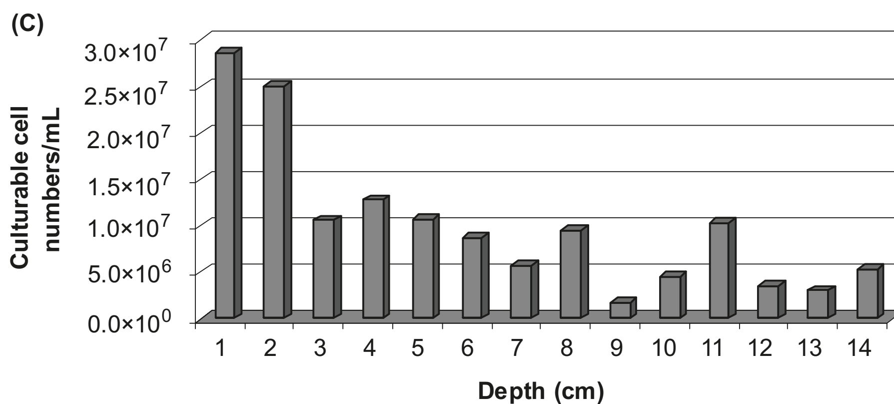


Table 1. Detection of the VIN13 control yeast and the LKA1 genetically modified *Saccharomyces cerevisiae* wine yeast in vineyard soil over time.

	Concn.		
Time	VIN13 (CFU/mL)	LKA1 (CFU/mL)	Enrichment
Week 2	3.75×10^4	3.15×10^4	Not performed
Week 7	SI	SI	Not performed
Week 10	SI	SI	Not performed
Week 12	ND	ND	Not performed
Week 14	+	+	Colombard must
Week 16	–	+	Colombard must
Week 18	+	+	1% YM medium

Note: CFU, colony-forming unit; +, presence indicated by enrichment method; –, not detected, despite enrichment method; ND, not detected; SI, numbers too low to be statistically meaningful.

gested that the biofilms formed by *C. laurentii* were more expansive than those formed by the GM and non-GM wine yeasts when cultivated under the same conditions. Biofilm formation by *C. laurentii*, known for the production of extracellular polymeric substances, was been demonstrated in a related study (Joubert et al. 2006). In our study, introduction of 1% YM medium as irrigation medium resulted in thicker biofilms in all cases, indicating that all 3 test strains actively formed biofilms and associated extracellular polymeric substances, as revealed by routine staining with CalcofluorTM White. Staining with FUN-1 TM showed that, similar to *C. laurentii*, both VIN13 and LKA1 contained a high percentage of viable cells, whereas microcolony formation was demonstrated by ESEM (Figs. 3D–3E). These observations provided further proof that the wine yeasts are capable of a biofilm mode of existence, whereas biofilm formation in Ringer's solution without an exogenous carbon source showed that active metabolism is not a requirement for adherence, as originally suggested by Reynolds and Fink (2001). When co-cultured, LKA1 could not easily be detected among the *C. laurentii* cells, although it was present in the flow-cell effluent. There was no indication that LKA1 had a negative effect on the biofilm formation ability of *C. laurentii*.

Active biofilms release cells into the suspended phase. In this study, relatively high numbers of cells were found in the flow-cell effluent, as shown in Fig. 4. The data also showed that the expression of the heterologous α-amylase gene in wine yeast did not improve the reproductive success or cell release by the LKA1 transgenic strain, compared with the non-GM VIN13 strain. Overall, it was observed that there were either no significant differences in the biofilm densities of VIN13 and LKA1 on sand particles, or that LKA1 formed less dense biofilms than VIN13, as was the case when 1% YM medium was used as flow medium.

Conventional flow cells were also utilized to compare the biofilm formation potential of LKA1 with VIN13 and with *C. laurentii*. Similar to observations made in the sand-filled flow cells, it was found that biofilms formed by *C. laurentii* were noticeably denser than those formed by the *Saccharomyces* wine yeasts; a difference that was observed for the duration of the flow-cell experiments, which lasted up to 8 days. Generally, there was not a significant difference in the numbers of culturable cells in the respective flow-cell effluents (Fig. 5). An intersting result is that the biofilm-to-planktonic cell yield of *C. laurentii* was most similar to the 2 *Saccharomyces* wine yeasts, despite the fact that *C. laurentii* formed more extensive biofilms (Fig. 5A).

It should be noted that flow-cell dilution rates exceeded the μ_{max} values of all strains used in this study by at least 15 times; therefore, it is assumed that planktonic growth did not have a noticeable contribution to the effluent cell numbers. Cells numbers in effluent from conventional flow cells suggest that both VIN13 and LKA1 formed stable biofilm, and that the numbers in their respective effluents were similar when grown on 1% YM medium or 10% AWE (Fig. 5). This comparable biofilm-to-planktonic yield under noticeably different nutrient regimes demonstrates the buffering capacity of biofilms, and suggests that, in addition to their role in survival, biofilms may potentially play an important role in microbial proliferation; phenomenon that warrants further investigation when the environmental fate of GMOs is of concern.

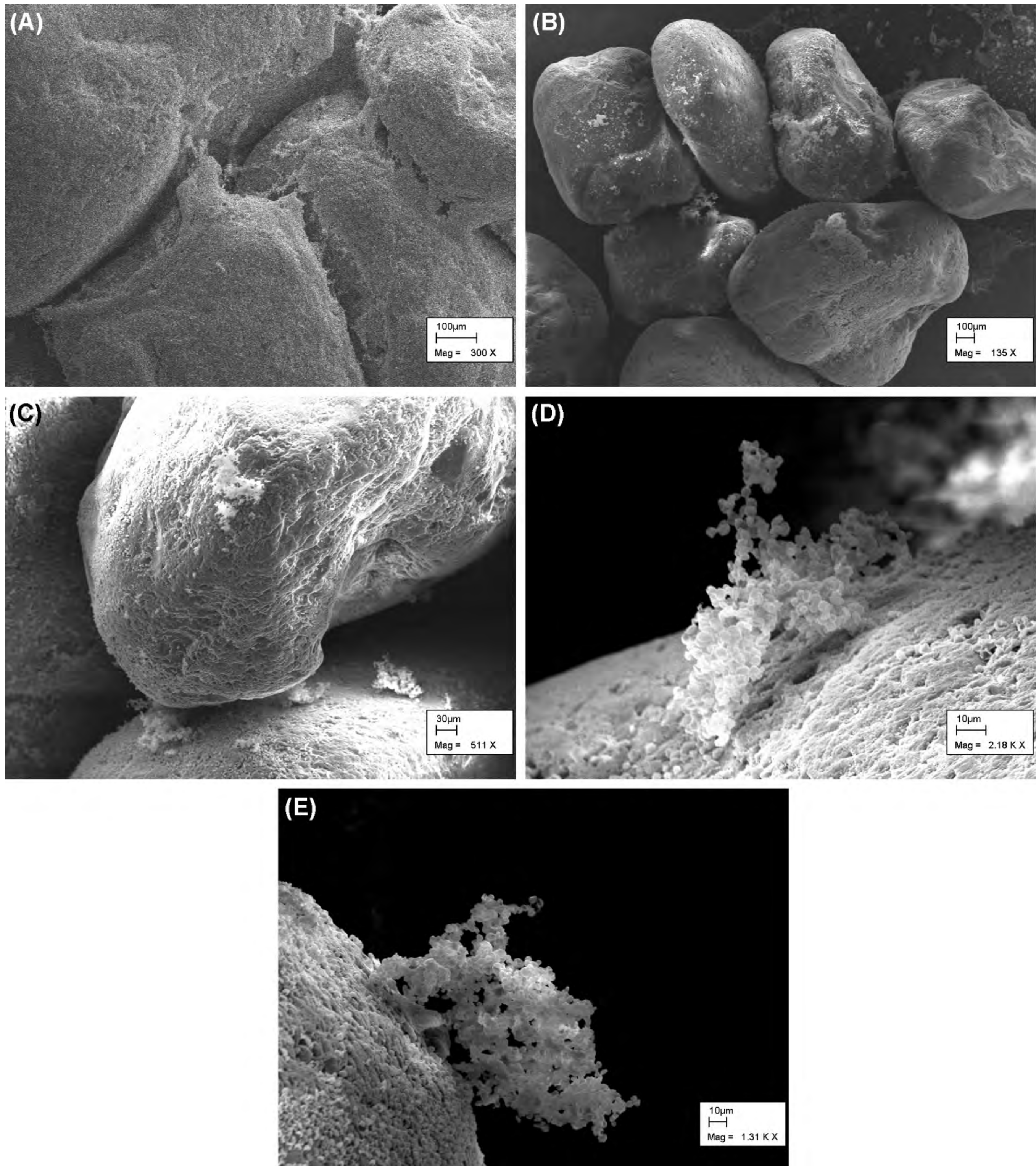
To address the inherent bias of microscope observations, owing to the small field of observation, and to obtain quantitative data related to biofilm formation, an optical large area photometer was used to monitor biofilms of the 3 test organisms (VIN13, LKA1, and *C. laurentii*) (Fig. 6). Biofilms formed by *C. laurentii* and VIN13 were relatively stable, with slight variations in optical density readings. Biofilm formation for LKA1 initially showed more fluctuation and had occasional sloughing events, as shown in Fig. 6, after ~90 h of cultivation. In general, because of the short duration of most biofilm studies, there is relatively little information on the stability and reproducibility of biofilm structure. Others have shown variability in bacterial biofilm profiles when pure cultures were cultivated under carefully controlled experimental conditions (Lewandowski et al. 2004; Bester et al. 2005). It is probable that biofilm function is maintained by a dynamic attachment–detachment behaviour, which may be amplified by experimental conditions.

It has been reported that haploid *S. cerevisiae* cells adhere better to plastic surfaces than diploid cells (Reynolds and Fink 2001). This could be due to the reduced expression of *FLO11* in diploids, compared with haploids, since it has been shown that *FLO11* expression decreases as ploidy increases (Galitski et al. 1999). If ploidy plays a significant role, this may have implications in terms of the ability of laboratory strains, as opposed to industrial yeast strains, to form biofilms. Both *S. cerevisiae* wine yeasts used in this study (VIN13 and LKA1) are diploid, and it is possible that the soil yeast, *C. laurentii*, is polyploid. Little information is available in the literature on the potential link between ploidy and biofilm formation, and this is therefore an area that should be considered when evaluating the ability of environmental yeasts to survive in biofilm mode.

The influence on an established biofilm community of the addition of the GM wine yeast

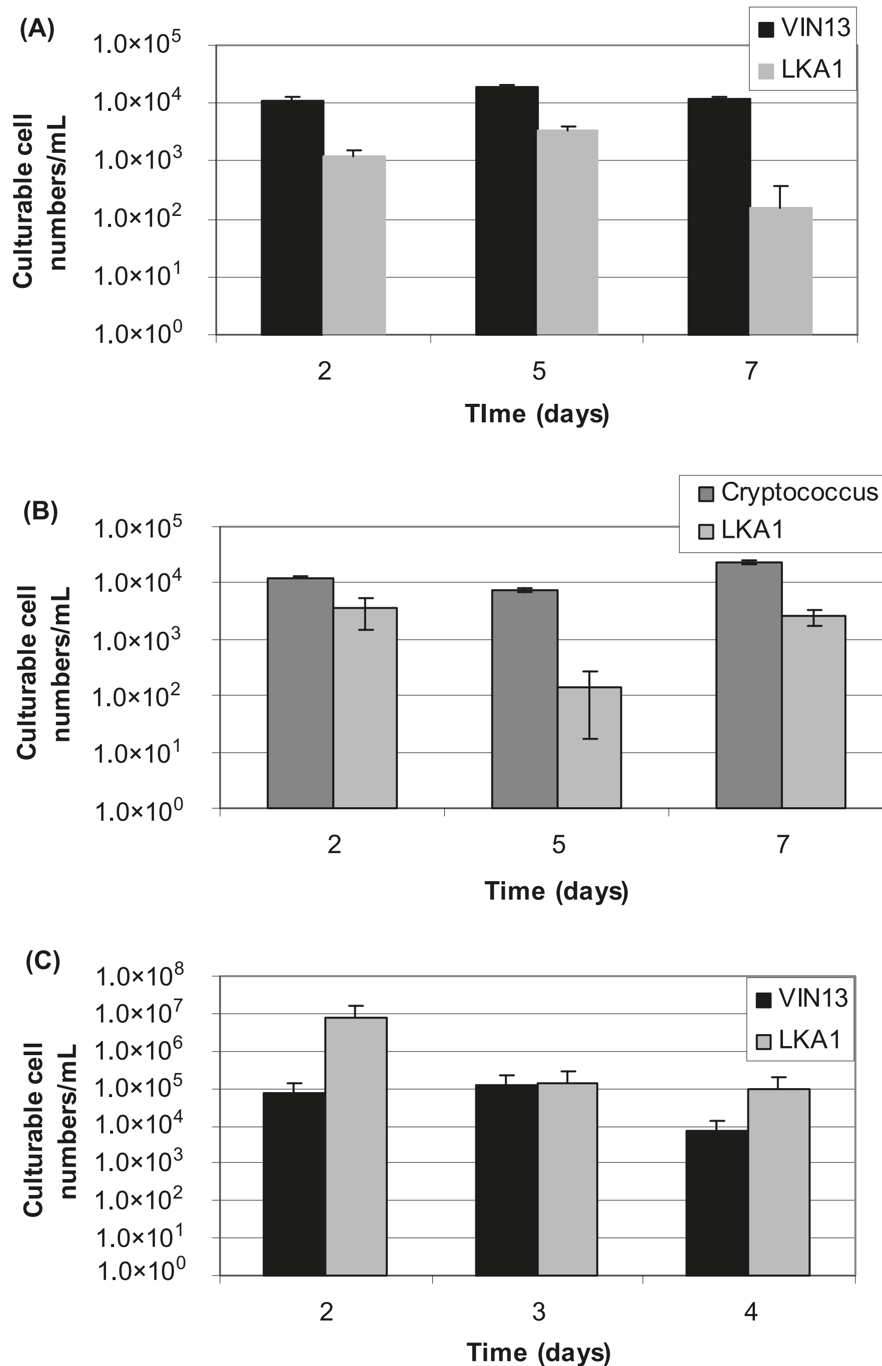
Different yeasts (*Schizosaccharomyces pombe*, *K. apiculata*, *S. ludwigii*) and bacteria were co-inoculated and allowed to form biofilms. No traces of *Schizosaccharomyces pombe* could be found in effluent collected on the third day, and it was assumed that all *Schizosaccharomyces pombe*

Fig. 3. Scanning electron microscopy (SEM) micrographs showing biofilm formation by (A) *Cryptococcus laurentii*, (B) *Saccharomyces cerevisiae* VIN13, and (C) *S. cerevisiae* LKA1 on sand. Confluent basal layers and microcolony formation by (D) LKA1 and (E) VIN13 demonstrate the ability of these wine yeast strains to form biofilms on geological material.



cells were washed out by that time. Wallerstein Laboratory nutrient agar was used to differentiate the different yeast genera in the effluent, since each genus displays unique colony morphology when grown on this medium. *Kloeckera apiculata* was shown to readily form biofilms, and light microscopy images demonstrated that *K. apiculata* prevailed in high numbers in the biofilm community. However, light microscopy also revealed that LKA1 did not incorporate well into a stable biofilm community when introduced after 5 days of biofilm development. Very few LKA1 cells could

Fig. 4. Numbers of culturable cells in the effluent of sand-filled flow cells. (A) Pure cultures of VIN13 and LKA1 in flow cells irrigated with Ringer's solution. (B) Co-culture of *Cryptococcus laurentii* and the LKA1 irrigated with Ringer's solution. (C) Pure cultures of VIN13 and LKA1 in flow cells irrigated with 1% YM medium.



be visualized in the mixed-community biofilms, although LKA1 was still detected at a concentration of 3.00 × 10^4 CFU/mL in effluent collected on day 10 (Fig. 7). The numberof bacterial members of the biofilm community in the effluent remained stable over time, suggesting that the bacteria played a role in maintaining biofilm integrity, a function that they probably also have in nature. In contrast, the numbers of *K. apiculata* and *S. ludwigii* cells in the effluent showed noticeable fluctuations. Overall, these observations showed that, similar to the parental strain, the addition of LKA1 to the stabilized biofilm communities did not disrupt the biofilms.

Conclusions

The public perception of potential health and environmental risks associated with GM food and beverage products has, so far, outweighed perceptions of possible benefits (Smith 1998; Pretorius 2000). The ongoing controversy is often fuelled by the emotional arguments of various interest

Fig. 5. Numbers of culturable wild-type VIN13 and transgenic LKA1 wine yeast cells in the effluent of conventional flow cells irrigated with (A) 1% YM medium or (B) 10% artificial winery effluent.

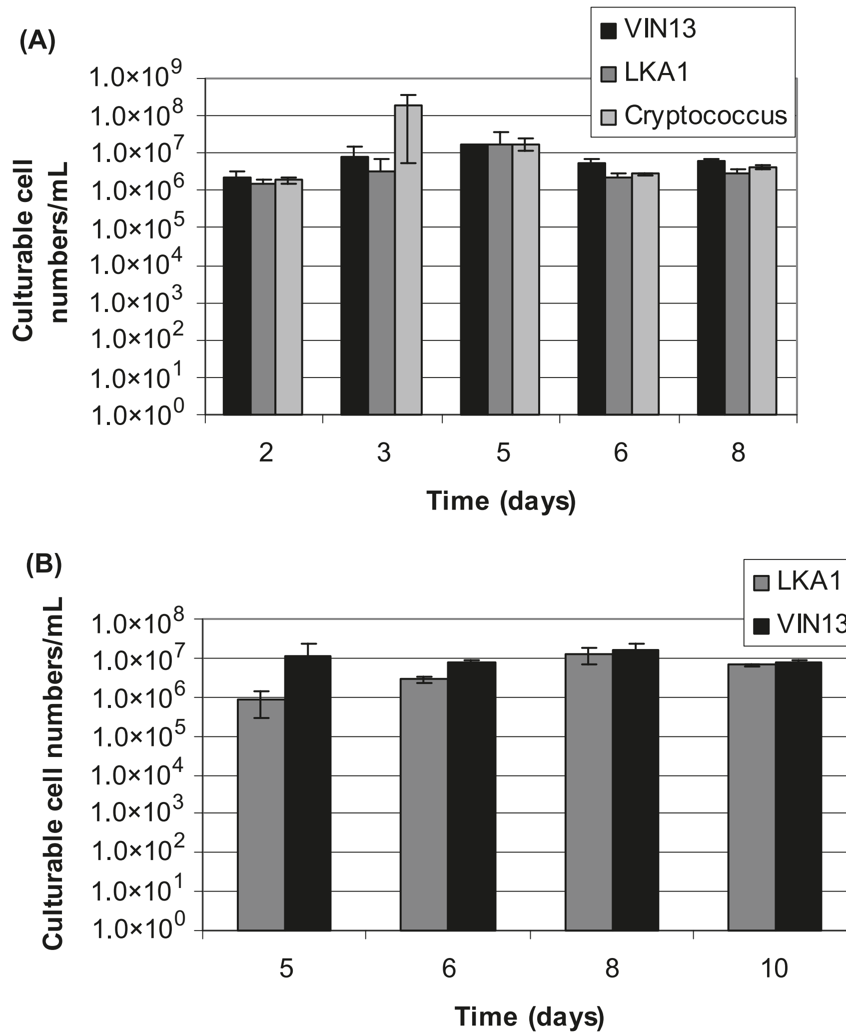
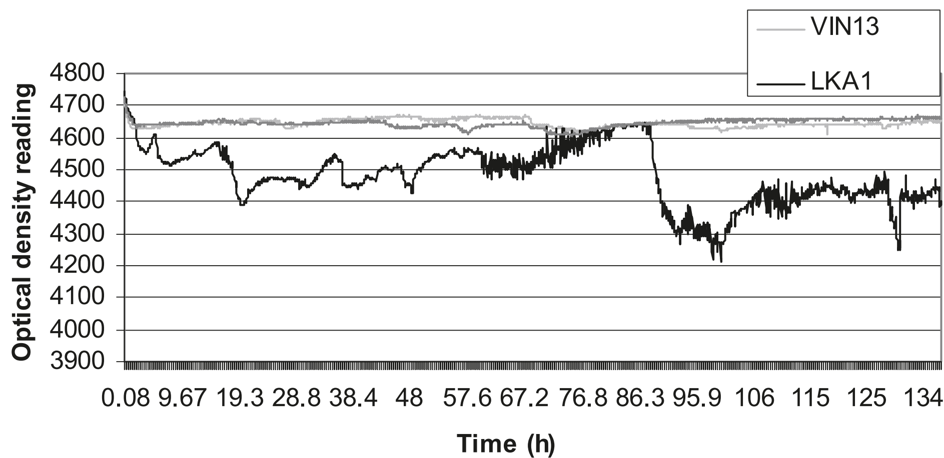


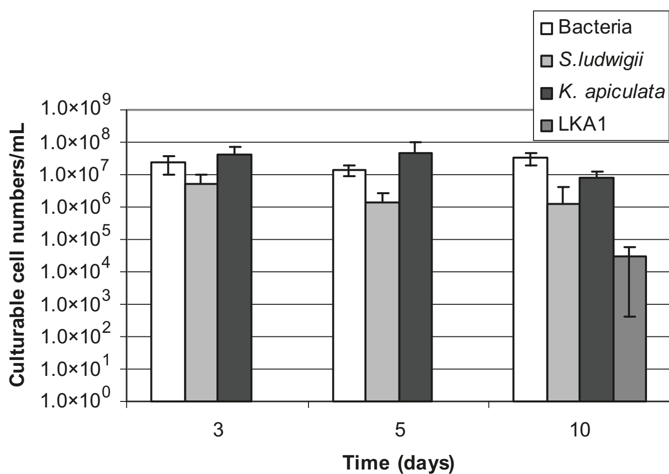
Fig. 6. Comparison of biofilms formed in parallel plate flow cells irrigated with 1% YM medium by VIN13, LKA1, and *Cryptococcus laurentii*, as measured with an optical large area photometer.



groups. Meanwhile, the recent commercialization of malolactic and low-urea GM wine yeasts in the United States and Canada has made the development of scientifically sound risk assessments more urgent in the global wine sector.

To the best of our knowledge, this study is a first attempt

Fig. 7. Detection of different yeast strains, together with the transgenic LKA1 wine yeast (introduced on day 5), in the effluent of a conventional flow cell irrigated with 10% artificial winery effluent.



to establish an objective risk-assessment procedure for the release of GM wine yeast into the environment. In the procedure, it will be essential to apply principles, such as those used here, to monitor the growth patterns, spread, and impact of a GM wine yeast in minicosms that are consistent with standard scientific method, which reject emotion in favour of objective measurement and rationality, and which move forward by subjecting themselves to critical scrutiny. Trustworthy, scientifically sound risk-assessment systems and processes might help swing the current GMO debate from one of being pro or con GM technology to one concerned with evaluation of individual technology packages, conducted on a case-by-case basis (Pretorius 2000; Pretorius and Høj 2005).

Based on our findings and observations that biofilm formation appears to be an important means of habitation for *S. cerevisiae* in nature, we recommend that this mode of growth by GM wine yeast be included when the growth patterns, spread, and effect of transgenic wine yeast are monitored in future risk-assessment systems and processes. The fact that we have shown that *S. cerevisiae* is able to form stable biofilms may open the possibility of exploiting such models in clinical research on yeast pathogenesis and antifungal therapy.

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Research Article

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Oral administration of baker's yeast (*Saccharomyces cerevisiae*) acts as a growth promoter and immunomodulator in *Labeo rohita* (Ham.)

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Abstract

The effect of baker's yeast (*Saccharomyces cerevisiae*), in the diet of the Rohu (*Labeo rohita*) innate immune response were investigated. Indian major carps *Labeo rohita* were fed with four different diets for eight weeks: a formulated diet as control diet and the same diets supplemented with 5%, 7.5% and 10% baker's yeast as an experimental diets. After every fifteen days interval different growth parameters (such as ADG, SGR, FCR and PER), Serological parameters (such as TSP,TSA, TSG and A:G), different Hematological parameters (such as TLC,TEC, Hct, MCV and MCH) and different Non- specific immunological parameters (such as PR, PI, Respiratory Burst activity) were evaluated during experimental trial. At the end of the experimental period, fishes of all the tanks were challenged with pathogenic bacteria *Aeromonas hydrophila*. The results show that, yeast cell wall able to enhance the innate immunity and also have a positive co-relation with growth parameters. Through the absorption of yeast wall particle, the immune function and disease resistance of the entire organism is stimulated. These results support the possible use of baker's yeast as natural immunostimulants in common fish diets.

Keywords: Immunostimulant; *Labeo rohita*; *Saccharomyces cerevisiae*

Introduction

Several whole microorganisms, live or not, such as bacteria, fungi or algae, increase disease resistance in mammals and fish [1,2]. In fish, as in other aquatic organisms, the whole microorganisms administered have mainly been bacterial species, which in the form of feed additives, have been shown to improve the intestinal microbial balance and increase the health status of fish, seemingly by colonising the gut and acting as antagonists to pathogens and so increasing resistance to pathogens [1,3,4].More recently, other whole microorganisms have been tested for their possible immunostimulant properties in fish. Thus, the oral administration or injection of the yeasts *Saccharomyces cerevisiae* or *Candida utilis* has been shown to increase both humoral (myeloperoxidase and antibody titer) and cellular (phagocytosis, respiratory burst and cytotoxicity) immune responses, and to increase or confer resistance against pathogenic bacteria in channel catfish, rainbow trout or gilthead seabream [5-8]. Possible use of baker's yeast in fish diets has many advantages. Firstly, they can be produced rapidly, easily and inexpensively and, at the same time, they are very stable and can be recycled from other industries. They are also natural substances so no negative effects may be expected either to the animals or to the environment. Moreover, there is no need to isolate their components, which consists mainly of cell wall sugars (β-glucans, mannoproteins and chitin), all are well-proved immunostimulant compounds.

The yeasts (*Saccharomyces cerevisiae*) has been used in gilthead seabream was Lyophilized form, which is not easily available to the farmer and also very costly but the baker's yeast, which is directly used in the bakery industry is low cost material. With this aim, the present paper discusses the effects of the dietary intake of the baker's yeast as supplementary feed and possible difference with the use of control diets are also established.

Material and Methods

Fingerlings of the species were obtained from a carp culture farm at the vicinity of Midnapore town has an initial measurement of 12.0±0.2g. Fish were (12.2 ± 0.22g) released into continuous flow glass aquaria (76 X 41 X 41 cm^3 area; 200.00 l capacity) after acclimatization for 15 days to prevailing laboratory condition of water temperature (31-33 °C) and pH (7.42 –7.53). Studies were conducted at room temperature for 60 days. The water quality (pH· DO, Alkalinity, Ammonia) of the experimental aquaria was monitored periodically once in a week following the methods of APHA (1998) and maintained at normal level.

Preparation of experimental feeds

The four prepared feeds (Cont., Exp-1, Exp-2, Exp-3) were formulated using locally available ingredients (mustard oil cake, rice polish, fish meal and tapioca powder). Feed formulation was done basically by "Square method" using determined values of protein content of the different ingredients. Proportion of each ingredient required was calculated precisely providing allowance for the premix. Locally available Baker's Yeast (*Saccharomyces cerevisiae*) was used as an immune stimulant. Baker's Yeast were diluted in water and supplemented @ 5%, 7.5% and 10% in the feed Exp-1, Exp-2 and Exp-3 respectively; where as control feed was not supplemented with *Saccharomyces cerevisiae*. Feeds were pelleted separately with local made (Kolkata, India) hand pelletiser. The pellets were dried in a thermostatic oven (M/S Modern Industrial Corporation, Mumbai, India) at 37°C and less then 10% moist [9, 10] and stored in an air tide jars at room temperature. Proximate composition of the four prepared feeds (Cont., Exp-1, Exp-2, Exp-3) were detailed in (Table 1).

Growth performance and conversion ratio

Fishes were fed twice daily at 8.00 and 16.00 h with ration size maintained with 6% of their body weight in two equal portions. The

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net weight was recorded every 15 days with an electronic and feed quality was readjusted after every weighting period of 15 days. For evaluating the dietary performances, the nutritional indices like live weight gain (LWG), Net weight gain (NWG), Average daily growth (ADG), Protein efficiency ration (PER), Specific growth rate (SGR) and Protein efficiency ratio (PER) were used. Two fishes of each group were terminated through overdose anaesthetiasation by MS222 (Sigma chemicals, India) [11] at the end of the experiment, and stored at –20 ºC until analysis.

Proximate analysis

Proximate analyses of ingredients, feeds and body carcass were determined following the method provided in AOAC (1990). Moisture content was determined gravimetrically in a hot air oven at 100+ 10 ºC for 24 h. Crude protein contents were determined by the micro-kjeldahl method. Crude lipid contents were determined by the soxhlet extraction method using petroleum ether (Boiling point: 40-60ºC) in the electro-thermal soxhlet apparatus. After extraction of lipid the defattend samples were used for the estimation of crude fiber following Patra [12]. Ash content was estimated by inserting samples in a muffle furnace at 500+ 50ºC for 10 h.

Biochemical analysis

DNA (Deoxy-ribo nuclic acid) and RNA (Ribo nuclic acid) contents from liver (hepatopancreas) tissues were estimated as per the scheme given by Munro and Fleck (1969). Tissue was taken from fishes and homogenized with 0.25 m sucrose solutions. 250 µl homogenate and 500 µl 5% TCA mixed thoroughly centrifuged and wait for 15 minutes and then precipitate was dissolved in PCA (several time in different concentration) and centrifuged with 5000 rpm (two times). The ultimate supernatant was used for RNA and DNA estimation by UV spectrophotometer.

Enzyme assay

Two specimen from each of the replicate were sampled both the initial stage and at the end of a 60 days experimental period. The fishes were dissected and the liver was cleaned and removed and pieces of liver was thoroughly washed in ice chilled double distilled water and subsequently collected in ice-cooled Petri dishes, weighed and cut into small pieces. A 5% homogenate was prepared in potter- Elvehjen homogenizer with neutral glass powder and ice cold 0.1 M phosphate buffer (pH-7.4) and centrifuged at 2,500 rpm for 15 minutes in a refrigerated centrifuged. The supernatant was used for the GOT and GPT activity following the method of Bernfeld [13].

Initially and after completion of the study, experimental fishes from different feeding trials were separately pooled and liver was dissected out for ACP and ALP activity estimation. After weighting the whole quantity of proportionate quantity, 10 % sucrose and 1 % tritonex mixture were added and the contents homogenized in a tissue homogenizer. The samples were then put in appendrop tube and cold centrifuged (at -4 °C) at 11000 rpm for 15 minutes. The supernatant was collected as sample for determining the ACP [14] and ALP [15] activity.

Study of blood parameters

Blood samples were collected by heparinized syringe from caudal vein for haematology. EDTA (Ethyline diamine tetraacetic acid) was used as anticoagulant. 1.0 mg EDTA ml^{-1} of blood or 1 drop of 1.0% solution 5 ml^{-1} of blood was used for haematology. Haematological parameters were estimated according to the method of Wintrobe [16]. MCV, MCH, MCHC were calculated by using standard formulae (Decie and Lewis 1991). Blood samples were collected in the laboratory for serological diagnosis by syringe from caudal vein and heart. Determination of the Total Serum Protein (TSP) and albumin by Gornall's biuret method [17].

TSG = TSP- TSA

(Where TSG = Total Serum globulin, TSP= Total Serum Protein, TSA= Total Serum albumin.)

Determination of immunity level

On day 60, blood was collected form fishes of each group. Part of the blood was heparinised and the rest was allowed to clot for serum samples, which were preserved at -20ºC for further analysis. Immediately after collection, the heparinised blood samples of each group were pooled to three aliquots. Part of the blood was analysed for leucocrit value in duplicate per sample [18]. The rest of the heparinised blood was immediately used for the phagocytic assay [8, 19]. Blood was collected from the fish by using a 0.2 ml glass syringe rinsed with an anticoagulant. Then the blood was transferred into the heparinised vial and mix properly. 0.1 ml of freshly prepared NBT solution was added to 0.1 ml of the heparin mixed blood and 15 µl of stimulant solution in the incubating bottle. The bottles were incubated at 37ºC for 10 minutes and at 26ºC for another 10 minutes. 50-70 µl of this blood was transferred onto a clean slide and makes a thick smear with a spreader slide. The slides were air dried and stain with Wright's stain. For staining with the Wright's stain, first flood the slide with 1 ml of the staining solution for 30 seconds then 1 ml of distilled water was added and keep for another 30 seconds. The slide was then pour off the stain and dried. Then the slide was then under oil immersion lens at 100 X. The positive cells had the violet coloured formazan granules in the cytoplasm. The percentage of the positive cells gave the idea about the non-specific immune status of the organism.

Challenge trial

Rohu fishes (*Labeo rohita*) of medium size (435±28g) were collected from a semi-intensive culture pond at the University campus. The intestine were gently excised & cut open with a pair of sterile scissors. The non-adherent micro floras of the intestine were isolated by three times washed with sterile solution & homogenized with 10 ml distilled water in stomacher bags. The presumptive numbers of micro flora were determined by the spread plate technique using nutrient agar. The pathogenic strain *Aeromonas hydrophila* (AH1) were isolated by the method of Kaneko [20], which had been cultured and maintained in the *Aeromonas* selective medium (M884, Hi – Media).

After 60 days feeding trial, fishes of each experimental group were released in four aseptic tanks. Different water quality parameters (Temperature pH, Dissolve Oxygen, Alkalinity, Ammonia) were maintained in normal level. Different experimental feeds and control feeds were provided twice daily according to 6 % of their body weight. The fishes in each treatment were challenged with *Aeromonas hydrophila* (AH1). Fishes in all replicates were immersed in a suspension of *Aeromonas hydrophila* (AH1), ~ 10^5 CFU ml^{-1} according to Austin et al. [21]. This was followed by a second immersion ~ 10^7 CFU ml^{-1} after 7 days Austin *et al.* [21]. The survivability of the fishes was recorded against pathogenic strain for last 10 days.

Statistical analyses

As all the above analyses were carried out on pooled samples of a given lot, standard deviations of means were calculated. However, for evaluating the dietary performances, nutritional indices, enzymatic

activity and RNA: DNA ratio, different haematological, serological, immunological parameters and challenge trials; correlation and regression test were performed through SPSS software package. Significant differences between the means of the treatments were tested by Duncan Multiple Range Test [22] through SAS software package [23].

Results

Locally available ingredients (Mustard oil cake, Rich polish and Fish meal) were used for preparing experimental feeds for *Labeo rohita*. Except control feed (Control), other three experimental feeds (Exp-1, Exp-2 and Exp-3) were supplemented with baker's yeast (*Saccharomyces cerevisiae*) and it was replaced with equal amount of rich polish. All the four experimental feeds are isocaloric and isonitrogenous. The average crude protein percentage of the feeds were 32.18± 0.22 and the crude protein percentage were around 7.67±0.09 (Table 1).

After 60 days feeding trial initial and final carcass composition of *Labeo rohita* in relation to various feeds was presented in (Table 2). The carcass composition of the test animals revealed an apparent increase in the final carcass protein and lipid over the initial carcass protein and lipid. Significantly highest carcass protein (12.01±1.29) and lipid (3.16± 0.31) was recorded in feed Exp-1 fed fishes as compare to other experimental and control feeds. This was clearly indicating that, the enhancement of carcass composition with the increasing supplementation of yeast at a specific level (5% supplementation). Perhaps it was due to the enzymatic activity in the gut and there by nutrients are spread for the growth and it make overall well being for the fishes.

Maximum weight gain of 135.18±1.21(of 10 fishes) was obtained in feed Exp-1 fed fishes followed by Exp-2 (109.88±1.36) and Exp-3 (102.86±1.96) and control (92.82±1.29) respectively (Table 3, Figure 1). There were significant differences (P≤0.05) in the different growth parameters among the different experimental feed fed fishes (Table 3). Significantly lowest (P≤0.05) FCR (3.54±0.033) was observed in Exp-1 fed fishes; it was also observed that significantly higher SGR was obtained from feed Exp-1 fed fishes (1.27±0.04). It indicates that Exp-1 feed shows better utilization of nutrients than the other feeds. Similarly significantly (P≤0.05) highest PER values (6.46±0.11) was obtained

Proximate composition of feeds	Diets			
	Control	Exp –1	Exp –2	Exp –3
Moisture(%)	7.18[a]	7.23[a]	7.19[a]	7.28[a]
Crude protein(%)	32.83 ± 0.13[a]	32.18 ± 0.22[a]	31.85 ± 0.32[a]	31.53 ± 0.32[a]
Crude lipid (%)	7.92 ± 0.07[a]	7.67 ± 0.09[a]	7.54 ± 0.11[a]	7.41 ± 0.23[a]
Ash(%)	11.69 ± 0.16[a]	10.62 ± 0.08[a]	10.08 ± 0.06[a]	9.55 ± 0.19[a]
Energy (KJ/g)	9.35 ± 0.004[a]	9.43 ±0.002[a]	9.46 ±0.003[a]	9.50 ±0.008[a]

[*] On dry matter basis.
Results are means of five separate determinations (Mean ± SEM)
Figures having same alphabets in the different rows are significantly different (p ≤ 0.05)

Table 1: Proximate composition (%) of different experimental diets[*] for *Labeo rohita.*

Carcass Composition (%)	Experimental tanks				
	Initial	Final			
		Control	Exp –1	Exp –2	Exp –3
Crude protein	7.75 ± 0.86[a]	9.96 ± 1.05[b]	12.01 ± 1.29[c]	10.40 ± 0.30[b]	10.25 ± 0.65[b]
Crude lipid	2.83 ± 0.71[a]	2.90 ± 0.29[a]	3.16 ± 0.31[a]	2.93 ± 0.93[a]	2.99 ± 0.12[a]
Ash	12.63 ±0.062[a]	12.55 ± 0.089[a]	12.89 ± 0.073[a]	12.55 ±0.062[a]	12.68 ±0.071[a]

Results are means of five separate determinations (Mean ± SEM).
Figures having different alphabets (superscribed) in the same row are significantly different (p ≤ 0.05)

Table 2: Initial and final carcass composition of fingerlings of *Labeo rohita* after 60 days experimental trial on four different dietary treatment.

Diets	NWG (g)	ADG (g)	SGR (%)	FCR	PER	Hb (g %)	TLC ($\times 10^3 mm^{-3}$)	TEC ($\times 10^3 mm^{-3}$)	Hct (%)	Leucocrit value
Control	92.82 ± 1.29[a]	1.54 ± 0.002[a]	0.95 ± 0.30[a]	4.64 ± 0.18[a]	4.35 ± 0.14[a]	7.1 ± 0.91[a]	13.8 ± 0.76[a]	1.30 ± 0.15[a]	27.21 ±1.02[a]	37.10 ± 2.05[a]
Exp –1	135.18 ± 1.21[b]	2.25 ± 0.001[b]	1.27 ± 0.41[a]	3.54 ±0.33[b]	6.46 ± 0.11[b]	8.2 ± 0.17[b]	22.2 ± 0.57[b]	1.76 ± 0.11[b]	35.34 ± 1.25[b]	59.20 ± 0.33[b]
Exp –2	109.88 ± 1.36[c]	1.83 ± 0.007[c]	1.13 ± 0.39[a]	4.39 ± 0.34[a]	5.30 ± 0.32[c]	7.9 ± 0.21[c]	18.3 ± 0.76[c]	1.52 ± 0.06[c]	28.32 ± 1.41c	47.50 ± 1.34[c]
Exp –3	102.86 ± 1.96[d]	1.71 ± 0.011[d]	1.10 ± 0.30[a]	4.36 ± 0.44[a]	5.02 ± 0.19[d]	7.5 ± 0.20[d]	16.7 ± 0.87[d]	1.43 ± 0.14[d]	32.18 ±1.30[c]	42.60 ±2.13[d]

Figures having different alphabets (superscribed) in the same column are significantly different (p ≤ 0.05)

Table 3: Different growth parameters, haematological parameters and serological parameters after 60 days feeding trial on *L. rohita* with yeast supplemented diets.

from Exp-1 fed fishes, which indicate better utilization of protein for growth and metabolism.

Significantly (P≤0.05) highest Alkaline phosphatase (ALP) (8.62 ± 0.04) and Acid Phosphatase (1.48 ±0.02) activity was observed in Exp-1 feed fed fishes (Table 4) following Exp-2 and Exp-3 feed fed fishes. Similarly highest GPT (0.068±0.003) and GOT (0.042 ± 0.002) values were registered in feed Exp-1 fed fishes, where as lowest in feed control fed fishes (0.055±0.001 and 0.032 ±0.003). Significantly (P≤0.05) higher RNA: DNA ratio (2.10 ± 0.003) was observed in fishes fed with Exp-1 feed and least was recorded (1.54 ±0.001) in control feed treated fishes (Table 5) after 60 days feeding trial. It was also observed that RNA: DNA ratio of fishes increased in all the treatments over the initial RNA: DNA ratio (1.02 ±0.010).

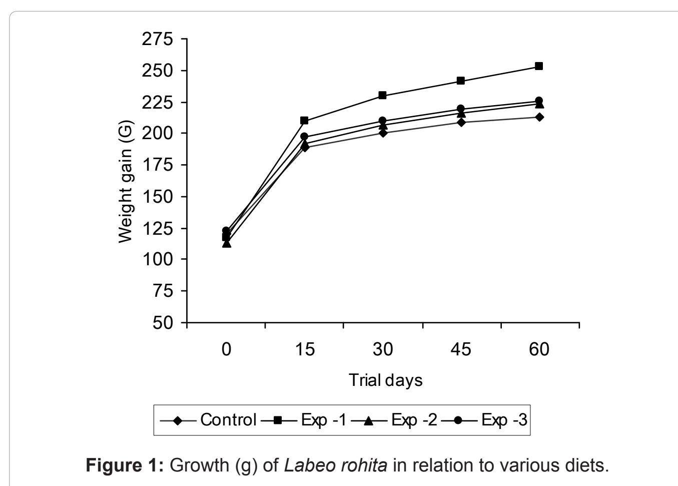

Figure 1: Growth (g) of *Labeo rohita* in relation to various diets.

Treatment	ALP	ACP	GOT	GPT
Control	1.08 ±0.01^{a}	0.40±0.001 ^{a}	0.032±0.02 ^{a}	0.055±0.001 ^{a}
Exp –1	8.62±0.04^{b}	1.48±0.02 ^{b}	0.042±0.06 ^{b}	0.068±0.005 ^{b}
Exp –2	5.29±0.02^{c}	1.01±0.001 ^{c}	0.039±0.01 ^{c}	0.062±0.002 ^{c}
Exp –3	5.10±0.03^{c}	0.73±0.003^{d}	0.035±0.04^{d}	0.059±0.006^{d}

ALP (Alkaline phosphatase); ACP (Acid Phosphatase); GOT (glutamate-oxaloacetate transaminase) (EC 2.6.1.1); GPT (glutamate-pyruvate transaminase) (EC 2.6.1.2)

Table 4: Activities of ALP. ACP , GOT and GPT in the intestine of *Labeo rohita* after 60 days feeding trial.

Treatment	Initial	Final
Control	1.02 ± 0.010^{m}	1.54 ± 0.001$^{a\ n}$
Exp –1		2.10 ±0.003$^{b\ o}$
Exp –2		1.98 ±0.005$^{c\ p}$
Exp –3		1.82 ±0.000$^{c\ q}$

Results are means of three separate determinations (Mean ± SEM),
Values with the same superscript in the different rows are not significantly different (p < 0.05)
from each other.
RNA/DNA : Initial value = 1.02± 0.010

Table 5: Initial and final RNA/DNA ratio in the muscle of *Labeo rohita* after 60 days feeding trial.

Treatment	Albumin: Globulin	Phagocytic Ratio	Phagocytic Index	NBT cells
Control	3.00 ± 0.04^{a}	15 ± 0.49^{a}	1.52 ± 0.12^{a}	48.5± 0.32^{a}
Exp –1	2.34 ± 0.03^{b}	63 ± 1.22^{b}	2.34 ± 0.09^{b}	57.0±0.61^{b}
Exp – 2	2.52 ± 0.06^{c}	42 ± 1.27^{c}	2.15 ± 0.09^{c}	52.5±0.24^{c}
Exp – 3	2.49 ± 0.09^{c}	18 ± 0.86^{d}	1.49 ± 0.20^{a}	49.5±0.23^{d}

Results are means of five separate determinations (Mean ± SEM)
Figures having different alphabets (superscribed) in the same column are significantly different (p ≤ 0.05)

Table 6: Effect of *Yeast (Saccharomyces cerevisiae)* on A:G ratio & non specific Immunity levels of *Labeo rohita* after 60 days feeding trial.

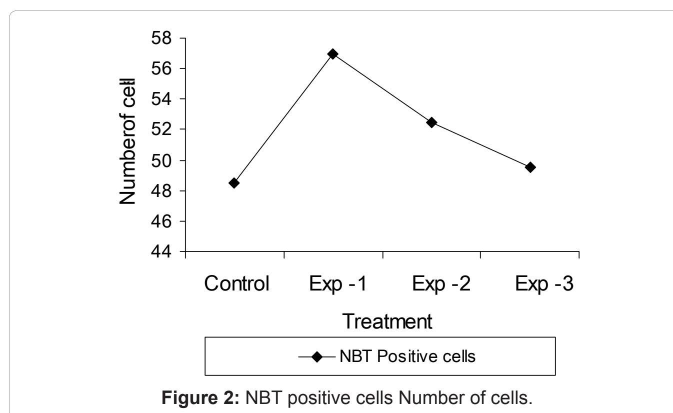

Figure 2: NBT positive cells Number of cells.

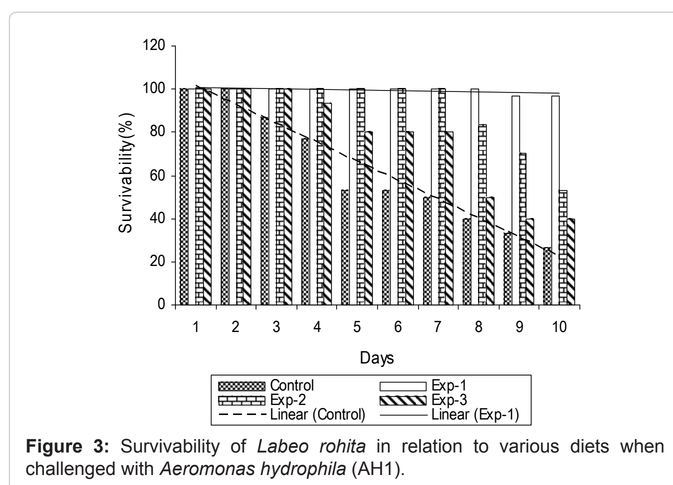

Figure 3: Survivability of *Labeo rohita* in relation to various diets when challenged with *Aeromonas hydrophila* (AH1).

Different Haematological and serological parameters of *Labeo rohita* after 60 days feeding trial were presented in (Table 3). Significantly (P≤0.05) highest TEC, Hb and HCT and lowest Albumin: Globulin ratio were observed in feed Exp-1 fed fishes, where as significantly (P≤0.05) lowest TEC, Hb and HCT and highest Albumin: Globulin ratio were recorded in control feed fed fishes after 60 days feeding trial.

The effect of yeast (*Saccharomyces cerevisiae*) on non-specific immunity was observed. Significantly (P≤0.05) highest phagocytic ratio (63 ±1.22), phagocytic index (2.34±0.09) and leucocrit value (59.20±0.57) were recorded in Exp-1 feed fed fishes, where as lowest of all these were recorded (9±0.49, 1.52 ±0.12and 37.10 ±2.05 respectively) in control feed fed fishes (Table 6). Similar trend was observed in case of number of NBT positive cells. Significantly (P≤0.05) highest number of NBT positive cells (57.0 ± 0.61) were observed in Exp-1 feed treated fishes and least one (48.5±0.32) recorded in case of control feeds of *Labeo rohita* (Table 6, Figure 2).

After 60 days feeding trial all treated and controlled fish faced challenge trial with *Aeromonas hydrophila* (AH1) for 10 days. After 10 days challenge trial significantly (P≤0.05) highest (96.66%) survivability was observed in Exp-1 feed fed fishes followed by Exp-2 (53.33%), Exp-3 (40%) and control (26%) feed treated fishes (Figure 3).

Discussion

In the last two decades, many substances have proved their

usefulness in fish culture because of their properties to stimulate the immune system & increase disease resistance. Among these immunostimulants the role of isolated β-glucans , chitin or vitamins is well documented [24-34]. However, the use of whole organisms instead of their isolated components has hardly been evaluated. In this way whole yeast cells (mainly *S. cerevisiae)*, Which represent a major commercial source of β-glucans , have recently been described as good immiunostimulants in fish [6,8].

Charlon and Bergot [35] achieved more than 89 % survival and good growth of carp larvae; feed exclusively on dry diets having yeast powder and pork / beef freeze dried liver. Alami, Durante et al [36] reported 87 % survival and FCR of 0.62 in common carp larvae reared on liver , yeast and commercial trout starter feed . Singh et al. [37] also found that yeast increased the rate of feed intake and conversion efficiency. Mohanty *et al* ., reported 100 % survival using a liver based diet containing goat liver , Cod liver oil , Vitamin and mineral mixture with average growth of 133.3 mg / fry in Rohu (*Labeo rohit*) raised from spawn to fry .

In the present study, *S. cerevisiae* are also found to stimulate the digestion through the supply of digestive enzymes and certain essential nutrients to the animals. Immunostimulants, particularly *Saccharomyces cerevisiae* producing several enzymes, which is not, produces by the host. Similar observation was also reported by Swain et al. [38]. A complex polysaccharide including cellulose are better utilized by the host in the presence of direct feed microbes like *Aspergillus oryza* , *Saccharomyces cerevisiae*.

Furthermore, it was reported that yeast in the diet improves feed efficiency, organic phosphorus (phytic acid) utilization and fibre digestion [38]. It also reported that supplementation of immunostimulants in feed improves the nutrition by hydrolytic enzymes including amylase and proteases, the production of vitamins such as biotin and vitamin B_{12} [39-44] and the host immunity [45]. It indicate that a given amount of immunostimulant elicit more than one protective response by the host.

Dietary intake of immunostimulants by fish has definite advantages and it is a useful method of exposure in large scale fish culture [8], particularly lypopolysaccharides of yeast cell wall was evaluated by many investigators like Ortuno et al. [6]; Sakai [28]; Sahoo and Mukherjee [46]. It is recognized that immunostimulants enhance the host defence system against pathogens by increasing phagocytosis , antibody production , leucocrit level and reduced A:G (Sakai, 1999) . A comparative study done by Ortuno et al. [6] revealed that oral administration of whole yeast *Saccharomyces cerevisiae* enhances the cellular innate immune response. Notably that all kinds of cellular parameter such as phagocytic index, respiratory burst activity, number of erythrocytes, lymphocytes were also enhanced but not the humoral one.

Blood is a pathophysiological reflector of the whole body and therefore, blood parameters are important in diagnosing the status of fish health (Pecie and Lewis, 1991), particularly when some additives used in the feed. In this present study it was observed that all the blood parameters in all the treatments were similar to standard (Banerjee et al., 2002) and Exp-1 fed fishes showed superior as compare to others, indication of the blood parameters revealed the positive impact, but also demonstrated a stable physiological reflection of the whole body [47]. In this study, a superior growth performance in terms of weight gain percentage, specific growth rate was recorded in *L. rolita* fishes fed with Exp-1 feed as compare to other feed fed fishes. These observations determine the optimum doses of yeast supplementation in feed. This might be helpful for optimum dietary utilization.

In this study, although all the feeds were isonitrogenous but the concentration of immunostimulant i.e. Baker's yeast (*S. cerevisoae*) in Exp-1 feed might be helpful for proper nutrient utilization. Whole body carcass composition was higher in Exp-1 feed fed fishes as compare to control one, which could have also noted to the overall low feed utilization level. RNA: DNA is known to provide dependable indication of growth trend [48-50]. The ratio was greatest in the fish fed Exp –1 feed with higher dietary utilization and best growth. Bazaz and keshavnath (1993) found higher RNA: DNA in better growing fish fed with oil supplemented diets using equal level of crude protein. The present study also reports such a finding, where all the feeds are isocaloric and isonitrogenous but 5% supplementation of Baker's Yeast (*S. cerevisiae*) incorporated feed (feed Exp-1) exhibit better growth as well as better RNA:DNA ratio. The highest level of GOT and GPT as well as ALP and ACP were found in Exp-1 feed fed fishes and lowest in control. Most of the amino acids normally found in protein undergo transamination reaction and transaminases are localized in both citosol and mitochondria (Wada and Marino, 1964), which is induced by high protein diet [51], thus a positive correlation between the immunostimulant concentration at a specific level and the GOT, GPT level in the liver could be observed.

The result obtain in this study not only support the use of Baker's yeast for better growth, and proper nutrient utilization but also it act as an immunostinunlant by stimulating the immune response. The activation mechanisms involved are known to be related to the carbohydrates, derived from the yeast cell wall. -glucans/whole yeast added to feed stimulates the phagocytic activity, respiratory burst activity and increase protection after challenge with pathogenic bacteria, similar finding ware reviewed by Robertson, 1999.

In this study oral administration of Baker's Yeast (*S .cerevisiae*) stimulate the non specific immunity level as measured through enhanced phagocytic activity, leucocrit level, respiratory burst activity and reduced A: G. Although enhanced leucocrit value does not necessarily relate to an immunostimulatory action of Baker's Yeast . The possible role of yeast as an imunostimulant due to its cell wall which composed of Lipopolysaccharide, such as glucan, which enhanced phagocytic activity of macrophages and globulin level as observed in the present experiment, the phagocytic indices seemed to be reliable indices of a hightened immunostimulatory response. Similar observation also observed by Swicki et al. [8]; Esteban et al. [31]; Orturo et al. [6]; Radriguezatal [7] in Rainbow trout and gilthread seabream. Albumin – Globulin ratio is a measurable humoral component at the non-specific defences. The reduction of A: G, might be due to the increase of Total serum globulin level with Significance protective mechanisms for fish [46]. Fish produce reactive oxygen species, which are considered to be toxic for fish bacterial pathogens [52,53] and are generated by phagocytes after stimulation by a variety of agents. In this experiment higher respiratory burst activity (O_2^- production) was increased in all the experimental diets as compare to control diets, but highest number of NBT positive cells were observed in Exp-1 feed fed fishes, an effect also seen in rain bow trout (Siwicki at al,1994), Seabream (Ortuno et al., 2002) and turbot [54]. After challenge trial with *Aeromonus hydrophila*, highest survivability observed in Exp-1 fed fishes, the enhanced protection conferred by glucan is not surprising, similar report has been reported to induce resistance in carp (*Cyprinus carpio*) against *E. tada* [55]. Evidence suggests that glucan enhance disease resistance by stimulating non-specific components of fish immune system [56,57].

To conclude the present results provide evidences that Baker's yeast

(*S. cerevisiae*) added in a common fish diet, exhibit better growth, better nutrient utilization and activate the innate immunity, as well as increase the survivability of *L. rohita*. Optimal doses and administration time have been established in an attempt to provide a useful approach for protecting culture fish against infectious diseases.

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Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides

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Abstract

The objective of this study was to determine the effect of two levels of inclusion of mannan oligosaccharides derived from the outer cell wall of a select strain of *Saccharomyces cerevisiae* (Bio-Mos, Alltech Inc, USA) on growth, feed utilization, immune status and disease resistance of European sea bass (*Dicentrarchus labrax*). Specimens of 35 g at initial density of 3 kg/m^3 were fed during 67 days at 0‰, 2‰ and 4‰ dietary MOS level of inclusion in a commercial sea bass diet. Food conversion rate, specific growth rate, whole body biochemical composition, phagocyctic index of head kidney macrophages, NBT index, lysozyme and alternative complement pathway (ACP) activities as well as gut and liver histological structure were evaluated. Growth significantly increased at both MOS dietary inclusion levels. Histological features of the liver showed lower lipid vacuolization and regular-shaped morphology of hepatocytes around the sinusoidal spaces denoting a better utilization of dietary nutrients. No differences were found on gut histological evaluation. Statistical differences ($P < 0.05$) on the phagocytic index were denoted with the inclusion of 4‰ Bio-Mos group. A positive correlation was found between the levels of lysozyme and alternative complement pathway activities in blood and the level of inclusion of MOS in diets.

After the feeding trial, a cohabitation challenge test and direct gut inoculation were also performed with the pathogen *Vibrio alginolyticus* in a ratio 3:1. Twenty-one days post-challenge the number of cohabitant fish infected in the control group reached 33% comparing with none on the 0.4‰ MOS group. Finally, new fish were infected with *V. alginolyticus* by gut canalisation. After

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24 h post-infection no significant difference was denoted between groups and 48 h post-infection total infected fish in the control group was twice that of the 2‰ and 4‰ MOS groups.
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1. Introduction

Bacterial disease outbreaks impose a significant constraint in fish and shellfish production [1,2]. In commercial farms, unfavourable environmental conditions (availability of iron, osmotic strength, oxygen levels, pH, water quality, temperature) or poor management practices (inadequate nutrition, overcrowding, overfeeding) may stress the fish causing them a growth rate reduction and immune suppression, making them more susceptible to disease outbreaks [2–4]. This is particularly significant for European sea bass (*Dicentrarchus labrax*), which despite its importance for Mediterranean aquaculture where it reaches up to 20% of total fish production, is a species very sensitive to stressors and infections may occasionally cause important losses [5].

The increasing economical and social concern to decrease the use of antibiotics and other therapeutic chemicals used in fish farming has encouraged more environmentally friendly approaches to disease control [2,3]. For instance, there is a great interest in the use of different products and organisms to control potential pathogens by competitive exclusion (probiotics and prebiotics), what has been successful in preventing disease outbreaks in other areas of animal production [3,6–8]. Some of these products derive from *Saccharomyces cerevisiae*, like Bio-Mos® (Alltech, Kentucky) which is composed by a fraction of its cell wall rich in mannan oligosaccharides (MOS). The benefits of this product have been reported in a broad number of poultry species, where it improves feed efficiency [9–13], promotes growth and seems to modulate immune response and preserve intestinal wall integrity. However, studies of MOS effect in fish are very scarce. Common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) growth and feed utilization significantly improved by feeding MOS supplemented diets [14]. Besides, MOS feeding markedly reduced mortality and improved antibodies levels, bactericidal and lysozyme activity and alternative complement pathway activity (ACP) [14]. MOS supplementation to *Artemia* improved intestinal microvilli structure in white sea bream larvae (*Diplodus sargus*) and induced a higher resistance to handling and stress [15].

In view of this evidence for other fish species, inclusion of MOS on diets for European sea bass could improve stress and disease resistance together with growth and feed utilization. Hence, the objective of this study was to evaluate the effect of dietary inclusion of MOS on the rearing performance of on-growing European sea bass and its implications in certain immune parameters and resistance to infections.

2. Materials and methods

2.1. Diets

Three isonitrogenous and isoenergetic diets, based in a commercial formulation, were designed to contain 0‰ (Control), 2‰ (BM2) and 4‰ (BM4) MOS (Bio-Mos, Alltech Inc., USA) replacing standard carbohydrates. Diets covered nutritional requirements for this species [5] and were manufactured by a commercial feed producer (Graneros de Tenerife, Tenerife, Spain) with the composition indicated in Table 1.

2.2. Experimental conditions

2.2.1. Experiment I

Eight hundred commercially reared European sea bass juveniles from a population which had not suffered apparent diseases originated from a local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain), arrived at the main facility of the Canarian Institute of Marine Science (ICCM) on July 1st 2005. Fish were maintained in stocking tanks

Table 1
Main ingredients if the experimental diet

Ingredients	Diet (% dry weight)
Fish meal[a]	51.50
Soybean meal	9.78
Wheat	7.50
Wheat gluten	7.50
Corn meal	6.53
Fish oil[b]	12.69
Fats and oils	2.03
Mineral mix[c]	1.43
Vitamin mix[d]	1.03
Antioxidant (BHT)	0.01

[a] Peruvian fish meal (65% protein).
[b] Peruvian fish oil.
[c] TROUW Seabream/Seabass (0.8 g), Choline chloride (0.17 g) and Inositol (0.06 g).
[d] TROUW Seabream/Seabass (1 g) Calcium carbonate (0.2 g), Potassium monophosphate (0.19 g) and NaCl 97% (0.04 g).

and fed a commercial extruded diet for 3 weeks (19–20.5 °C) until being well adapted to the environmental conditions (3 kg/m^2 stoking density). Afterwards, 792 sea bass juveniles were randomly distributed in 9 indoor, cylindroconical 1000 l fibreglass tanks at an initial stocking density of 3 kg/m^3 (88 fish/tank). Fish average initial weight (g $\pm$ SD) and length (cm $\pm$ SD) were 33.75 ± 7.69 and 13.46 ± 0.97, respectively ($n = 792$). Tanks were supplied with filtered sea water (1.39 l min^{-1}), at a temperature of 20.5–23.4 °C, and natural photoperiod (12L:12D). Water dissolved oxygen was kept at 8.0 ± 0.2 ppm. Fish were manually fed until apparent satiation with one of the three experimental diets for 9 weeks (3 times a day, 6 days a week). Each diet was assayed by triplicate.

Feed intake was calculated daily and growth parameters were determined at day 0, 36 and 67. Food conversion ration (FCR) defined as the amount of food ingested divided by the generated biomass, specific growth rate (SGR) defined as: [(ln final weight − ln initial weight)/number of days] $\times$ 100 and relative growth (%) defined as: [(final weight − initial weight)/initial weight)] $\times$ 100. Nine fish at the beginning of the trial and 15 fish per tank at day 67 were sampled, after 24 h fasting, for body proximal composition and studies of hepatic and enteric tissue morphology. Blood samples were taken from 15 fish for plasma and serum collection at day 67 to determine NBT, lysozyme and ACP activities. In addition, the head kidney was removed from 15 fish per dietary treatment to determine phagocytic activity of macrophages.

2.2.2. Experiment II

After 9 weeks of feeding the experimental diets, 60 fish per diet from Experiment I were randomly selected and distributed among 12 indoor cylindrical 500 l fibreglass tanks in the pathogens incubation facility (3 kg of fish/m^3, 20 fish/tank). Each group of fish were fed the same diet that they were fed in Experiment I. Initial fish average weight (g $\pm$ SD) and length (cm $\pm$ SD) were 99.37 ± 21.84 and 18.71 ± 6.30, respectively. A bacterial analysis was performed before the beginning of the challenge test to ensure that fish were not infected by bacteria. Two groups of 20 fish from each dietary treatment (Control, BM2, BM4) were exposed to a pathogen by cohabitation (3:1, healthy: infected) with fish intraperitoneally infected (ip) (2.4×10^8 cfu ml^{-1} per fish) with *Vibrio alginolyticus*. Another two 20 fish groups per treatment were used as non-infected controls cohabitating (3:1) with fish intraperitoneally injected with sterile saline solution. Fish injected with *V. alginolyticus* or sterile saline solution were marked by Visible Implant Elastomers (VIE, Northwest Marine Technologies, Shaw Island Washington, WA, USA) [16]. Water temperature ranged from 22 to 23 °C during the trial and artificial light photoperiod was adjusted to 12L:12D. Fish were manually fed their respective experimental diets until apparent satiation for 21 days (3 times a day, 6 days a week). At the end of this challenge test all fish were slaughtered and microbiological analysis of head kidney, spleen and liver were conducted in order to determine the infection spread grade.

2.2.3. Experiment III

In a third experiment, 80 fish per diet from Experiment I were randomly selected and distributed among 12 indoor cylindrical 500 l fibreglass tanks in the pathogens incubation facility (3.5 kg of fish/m^3, 20 fish/tank). Each group of fish

were fed the same diet that they were fed in Experiment I. Initial fish average weight (g ± SD) and length (cm ± SD) were 98.23 ± 5.63 and 18.51 ± 0.30, respectively. A bacterial analysis was performed before the beginning of the challenge test to ensure that fish were not infected by bacteria. Two groups of 20 fish from each dietary treatment (Control, BM2, BM4) were inoculated via anal canalisation with *V. alginolyticus* (2.4×10^8 cfu ml^{-1} · per fish). Another two 20 fish groups per treatment were inoculated via anal canalisation with 1 ml of sterile saline solution. Water temperature ranged from 22 to 23 °C during the trial and artificial light photoperiod was adjusted to 12L:12D. Fish were manually fed their respective experimental diets until apparent satiation for one week (3 times a day, 6 days a week). After 24 h and 48 h post inoculation, 33% of fish population was randomly selected and bacterial characterization of head kidney, spleen and liver was performed in order to determine the pathogen's capacity to pass through the intestinal epithelium. Seven days after experimental infection, gut and liver samples were taken for histological studies.

2.3. Biochemical analysis

Feed and fish biochemical composition were conducted following standard procedures [17], dry matter content was determined after drying the sample in an oven at 105 °C to constant weight, ash by combustion in a muffle furnace at 600 °C for 12 h, protein content (Nx6.25) was determined by Kjeldahl method and crude lipid was extracted following the method of Folch et al. [18]. Fatty acids from total lipids were prepared by transmethylation as described by Christie [19] and separated by gas chromatography under the conditions described by Izquierdo et al. [20], being quantified by FID and identified by comparison to external standards. All analysis were conducted in triplicate.

2.4. Histological studies

On Experiment I, liver and gut samples were taken after 67 days of the feeding trial. Samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination [21]. Micrographs of liver were taken from the paraffin sections at a final magnification of 400× using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Hepatocellular area as well as maximum and minimum longitude of cells taking hepatocyte nuclei as reference were measured with an analySIS® (Image Pro Plus®) software package using arbitrary units. For each diet, twenty-seven micrographs originating from 9 fish ($n = 27$) were analyzed. On Experiment III, seven days after experimental infection, gut and liver samples were taken for histology analysis and routinely processed.

2.5. Blood collection and sample preparation

Blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. No anaesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. The

Table 2
Growth performance, nutrient utilization and somatic parameters of European sea bass fed commercial extruded diets with different levels of MOS inclusion

	Days of feeding	Control	BM2	BM4
Average Weight (g)	0	34.01 ± 7.47	34.04 ± 7.56	33.56 ± 7.78
	36	59.17^a ± 2.80	63.30^b ± 2.56	62.17^b ± 3.95
	67	93.17^a ± 0.06	102.41^b ± 0.90	102.38^b ± 5.10
Long (cm)	0	13.48 ± 1.03	13.55 ± 0.92	13.41 ± 0.95
	36	15.76^a ± 0.05	16.26^b ± 0.05	16.50^b ± 0.29
	67	18.16 ± 0.13	18.73 ± 0.15	18.63 ± 0.25
Condition factor (k)	0	1.38 ± 0.17	1.35 ± 0.11	1.39 ± 0.13
	36	1.51 ± 0.01	1.47 ± 0.02	1.48 ± 0.02
	67	1.58 ± 0.03	1.56 ± 0.03	1.58 ± 0.02
Relative growth (%)	36	73.18^a ± 0.90	84.62^b ± 3.38	83.81^b ± 8.71
	67	56.90^a ± 1.63	58.57^b ± 1.97	59.75^b ± 2.99

Different letters within a line denotes significant differences ($P < 0.05$). Control = 0‰ Bio-Mos; BM2 = 2‰ Bio-Mos; BM4 = 4‰ Bio-Mos. Values expressed in mean ± SD.

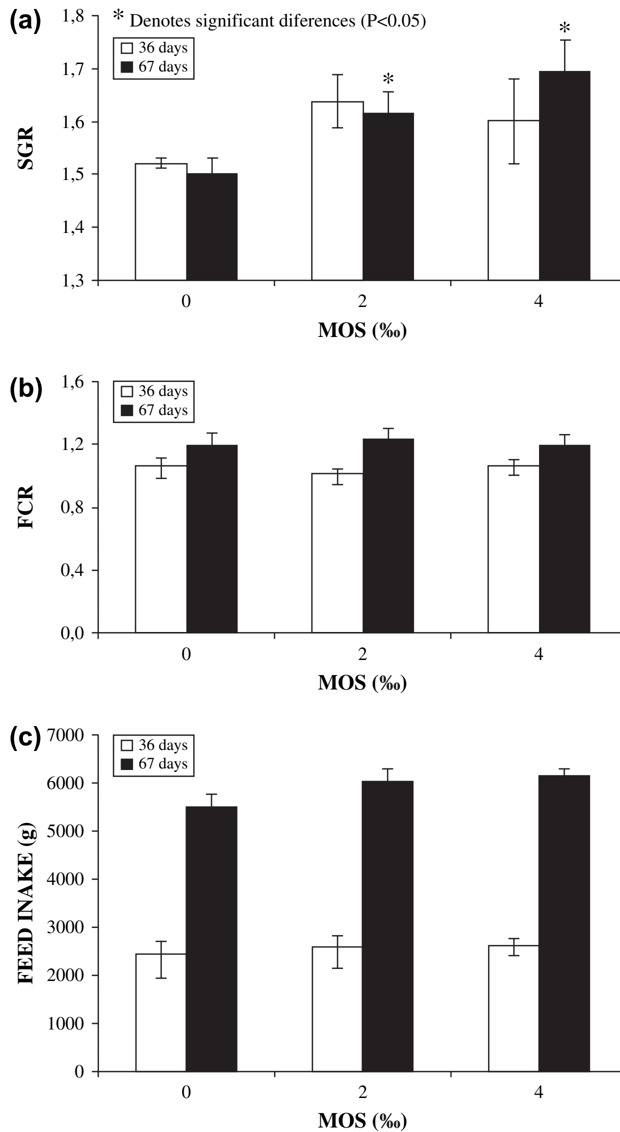

Fig. 1. (a) Growth performance (SGR); (b) Feed efficiency (FCR); and (c) feed intake for European sea bass fed at different levels of MOS inclusion during Experiment I. Data represent the mean ± SD. ($n_{36\ days} = 264$; $n_{67\ days} = 210$). Significant differences ($P < 0.05$) among treatments for a given feeding period are indicated by (*).

Table 3
Whole body final proximal composition (g/100 g dry weight) of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I ($t = 67$ days)

	Diet		
	Control	BM2	BM4
Crude protein	46.96 ± 2.10	44.74 ± 1.08	44.19 ± 1.12
Crude lipids	44.81 ± 3.54	43.56 ± 2.5	42.99 ± 3.35
Ash	3.60 ± 0.65	3.06 ± 0.27	2.48 ± 0.14
Moisture	63.12 ± 2.35	63.16 ± 1.00	62.77 ± 0.86

Different letters within a line denotes significant differences ($P < 0.05$). Control = 0‰ Bio-Mos; BM2 = 2‰ Bio-Mos; BM4 = 4‰ Bio-Mos. Values expressed in mean ± SD.

Table 4
Composition of main fatty acids groups (g fatty acids/100 g total fatty acids) for whole body composition of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I ($t = 67$ days)

	Diet		
	Control	BM2	BM4
Saturated	27.080 ± 0.805	26.224 ± 0.332	27.981 ± 1.595
Monosaturated	31.059 ± 0.154	29.640 ± 1.076	31.217 ± 1.828
Σn-3	24.585 ± 0.114	23.582 ± 1.360	24.586 ± 0.878
Σn-6	8.496 ± 0.586	8.486 ± 0.370	7.820 ± 0.613
Σn-9	19.886 ± 0.033	18.823 ± 0.573	19.884 ± 1.266
Σn-3 HUFA	19.796 ± 0.073	18.995 ± 1.105	19.767 ± 0.726

Different letters within a line denotes significant differences ($P < 0.05$). Control = 0‰ Bio-Mos; BM2 = 2‰ Bio-Mos; BM4 = 4‰ Bio-Mos. Values expressed in mean ± SD.

first aliquot of blood was transferred to a coated Eppendorf with lithium heparin as anticoagulant and used for NBT (Nitro Blue Tetrazolium) index, measuring the activity of circulating neutrophils spectrophotometrically as described by Siwicki et al. [22], measuring the reduction of NBT to formazan in presence of oxygen radicals. The second aliquot was divided in two portions, transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at −80 °C for ACP and lysozyme activities determination.

2.6. Serum analysis

Alternative complement pathway was performed as described by Sunyer and Tort [23] for gilthead sea bream (*Sparus aurata*) using rabbit RBC. The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results are presented as ACH50 units ml^{-1}.

Lysozyme level in blood serum was determined by turbidimetric assay according to the method described by Anderson and Siwicki [24] using hens egg white lysozyme (Sigma) in PBS as a standard. Results were presented as Lysozyme units ml^{-1}.

2.7. Macrophage phagocytic activity

Head kidneys of five fish per diet were removed and macrophages were isolated by density gradient centrifugation technique as follows. Following collection, head kidneys were homogenized in supplemented Minimal Essential Medium (MEM) (10 μl ml^{-1} heparin; 100 μl ml^{-1} penicillin/streptomycin; 2% foetal bovine serum) and filtered through a nylon membrane (100 μm mesh syze, Sigma). Resulting cell solution was layered onto preformed gradient of 51%–34% Percoll in 10% Hank's Balanced salt solution (HBSS) and centrifuged at 800 × g for 30 min at 4 °C. The interface layer was harvested with a Pasteur pipette, diluted in 1 ml of supplemented MEM and recentrifuged at 800 × g for 5 min at 4 °C to remove residual Percoll. The resulting pellet was diluted in 1 ml of supplemented MEM and viability and concentration of macrophage cell suspensions were determined in trypan blue. The final pellet with more than 93% viability was resuspended in supplemented MEM adjusting the final concentration to 10^6 macrophages ml^{-1}. Macrophage solution was incubated with *V. alginolyticus* as described by Esteban and Meseguer [25] for *Vibrio anguillarum*. Phagocytic activity was measured as described by Blazer [26]. One hundred macrophages per fish were counted and the phagocytic activity was determined as the percentage of macrophages with phagocytic ability.

2.8. Bacterial isolation and biochemical characterization

Samples of head kidney, liver and kidney from dead fish were cultured on TSA (Tryptone Soybean Agar, Cultimed) and incubated at 25 °C for 24–48 h. All strains isolated were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests using previously methods [27], in order to identify *V. alginolyticus* present in the fish.

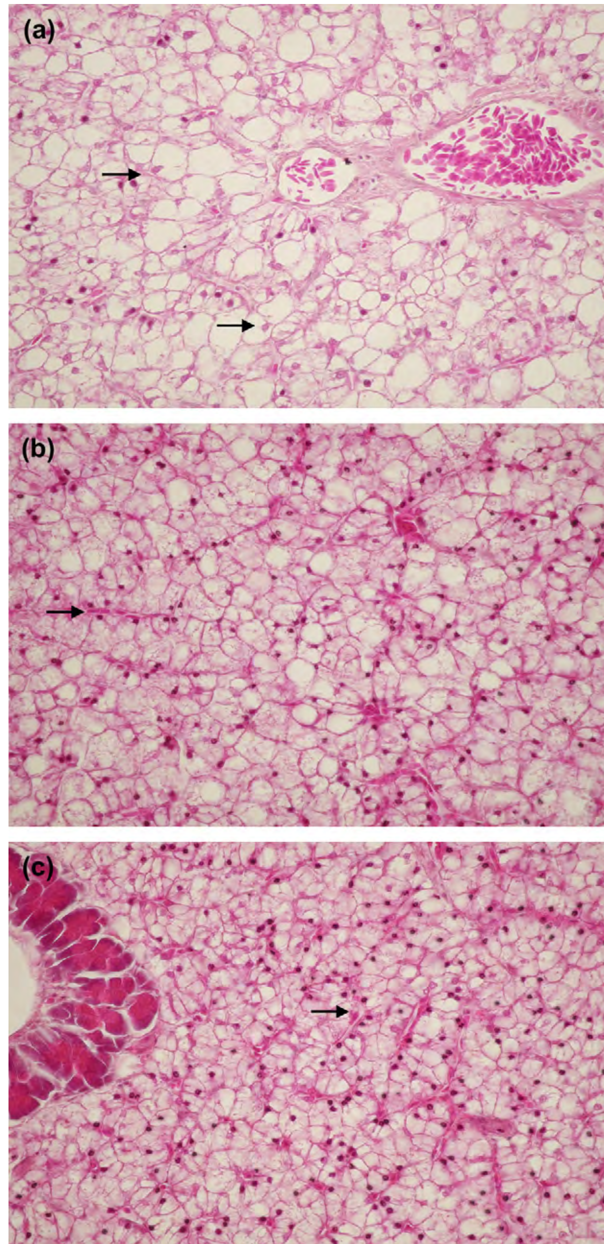

Fig. 2. Hepatocytes (H&E. ×400) from fish fed. (a) Control diet showing foci of swelling hepatoyctes characterized by cytoplasm vacuolization and nuclei displaced to periphery cellular; (b) BM2 diet with lower number of swelling hepatocytes; and (c) BM4 diet with a regular morphology of the hepatocytes located around sinusoidal spaces.

2.9. Statistical analysis

Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf [28]. Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of MOS inclusion. When F values showed significance, individual means were compared using Tukey's test for multiple means comparison. Significant differences were considered for $P < 0.05$.

3. Results

3.1. Experiment I

No mortalities were registered during the feeding experiment. Results for growth, biometry and feed utilization are shown in Table 2 and Fig. 1. After 36 and 67 days of feeding, fish fed with diets BM2 and BM4 showed a significantly higher body weight and relative growth. Specific growth rate followed a similar trend (Fig. 1). Total length was also significantly higher in fish fed MOS containing diets after 36 days of feeding. Feed conversion ratio (FCR) was not significantly affected by MOS inclusion, but a positive correlation was found between the total amount of ingested food and the level of inclusion MOS inclusion ($y(t_{36}\text{ days}) = 88.493x + 2366.6$, $R^2 = 0.9197$; $y(t_{67}\text{ days}) = 321.2x + 5255.2$, $R^2 = 0.869$). No significant differences were found on the condition factor (K) (Table 2). MOS supplementation did not affected whole fish proximate composition (Table 3) or fatty acid composition (g fatty acids/100 g total fatty acids) (Table 4).

Morphological analyses for fish fed diets containing MOS resulted qualitatively in a regular-shaped morphology of the hepatocytes around sinusoidal spaces and a reduction on the lipid vacuolization of the cytoplasm that decreased the number of hepatocytes with the nuclei displaced to the cellular periphery. This effect was more pronounced in fish fed with the higher concentration of MOS (Fig. 2). According to these histological findings, quantitative morphometric analysis of the liver revealed significant differences ($P < 0.05$) in the cells parameter studied. Thus, hepatocytes of fish fed BM4 presented significantly lower hepatocellular area as well as maximum and minimum cell length (Table 5). No differences were found on histological evaluation of guts of fish fed with the different diets.

Phagocytic activity of head kidney macrophages against *V. alginolyticus* significantly increased ($P < 0.05$) in fish fed the highest MOS inclusion level (4‰) (Fig. 3). Resulting in a positive correlation between the phagocytic activity and the dietary MOS inclusion levels ($y = 4.33x + 19.043$, $R^2 = 0.9742$). No concluding results were obtained for neutrophils potential of reduction because significant differences were found between all treatments (Fig. 3). No significant differences ($P < 0.05$) were found on lysozyme and ACH50 (Fig. 3), although a positive correlation was observed between the level of MOS inclusion and the level of these parameters in fish serum, ($y_{\text{lysozyme}} = 22.285x + 389.89$, $R^2 = 0.869$) ($y_{\text{ACH50}} = 7.925x + 89.903$, $R^2 = 0.9074$).

3.2. Experiment II

Despite all the intraperitoneally infected fish dying within 72 h, there were no cohabitation mortalities during the experiment. The grade of horizontal transmission was determined by the presence or absence of the pathogen in different internal organs, but the pathogen was only recovered on head kidney. After 21 days of challenge test the number of fish infected by *V. alginolyticus* was significantly ($P < 0.05$) reduced by the dietary MOS supplementation (Fig. 4). VIE mark allowed the correct identification of all injected fish.

3.3. Experiment III

No mortalities were registered in this experiment. The grade of pathogen infection was determined by the presence or absence of the pathogen in internal organs and the pathogen was only recovered on head kidney. A 10% of infected

Table 5
Results of quantitative image analysis (μm) of hepatocytes from European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I ($t = 67$ days)

	Diets		
	Control	BM2	BM4
Maximum longitude	$2.030^{ab} \pm 0.451$	$2.165^{a} \pm 0.498$	$1.610^{b} \pm 0.311$
Minimum longitude	$1.416^{a} \pm 0.240$	$1.352^{a} \pm 0.261$	$1.087^{b} \pm 0.256$
Area	$2.577^{a} \pm 0.887$	$2.758^{a} \pm 0.781$	$1.711^{b} \pm 0.612$

Different letters within a line denotes significant differences ($P < 0.05$). Different letters within a line denotes significant differences ($P < 0.05$). Control = 0‰ Bio-Mos; BM2 = 2‰ Bio-Mos; BM4 = 4‰Bio-Mos. Values expressed in mean ± SD.

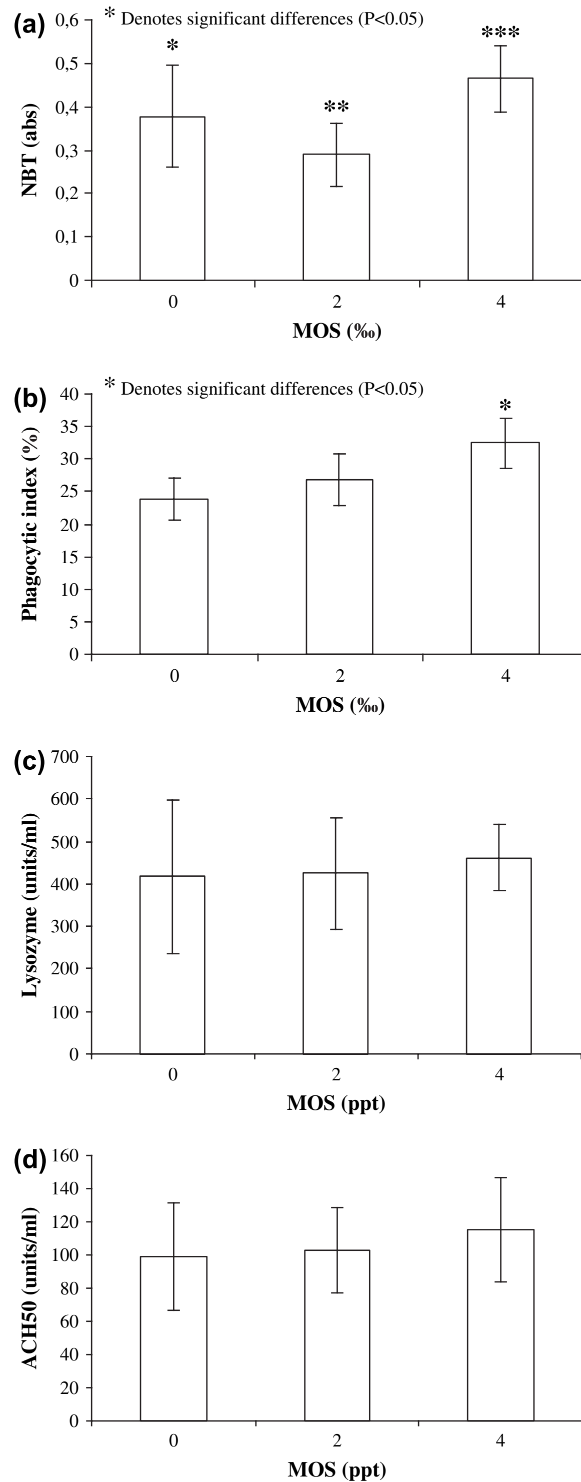

Fig. 3. Influence of MOS on total (a) phagocytic activity of head kidney macrophages ($n = 24$); (b) reduction potential of circulating neutrophils ($n = 24$); (c) lysozyme activity ($n = 24$); and (d) alternative complement pathway activity ($n = 24$) in European sea bass during Experiment I (mean ± SD; $n = 24$). Significant differences ($P < 0.05$) among treatments are indicated by (*).

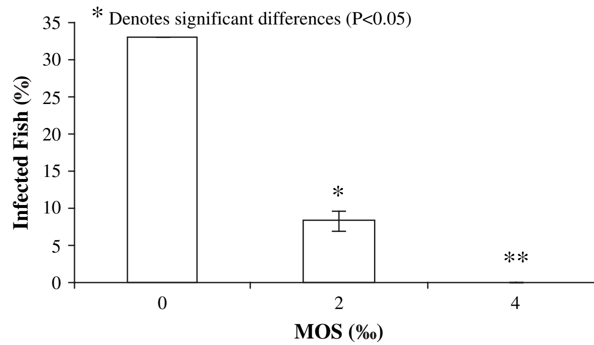

Fig. 4. Presence of *Vibrio alginolyticus* presence on head kidney of European sea bass fed different levels of MOS inclusion and submitted to infection by cohabitation in Experiment II (mean ± SD; $n = 60$). Significant differences ($P < 0.05$) among treatments are indicated by (*).

fish were found in all pathogen induced fish at 24 h postinoculation regardless of the dietary treatment. However, after 48 h the number of infected fish found in the control treatment was twice in the fish fed MOS (Fig. 5).

4. Discussion

In the present study, despite inclusion of dietary MOS at 2‰ and 4‰ did not significantly affect diet proximate composition, fish fed MOS supplemented diets showed a significant growth improvement. Similar effects have been described for broiler chickens [29], pigs [30,31], and common carp or rainbow trout [14]. Improved growth may be related with an enhanced amino acid absorption as it has been shown in chicken [13] and it is suggested by the improvement in the functional integrity of the enterocyte membrane in MOS fed animals [11,32]. In sea bass, dietary MOS did not affect fish gut at an optical microscopy level. However, some studies have reported a better microvilli alignment when MOS was fed to fish [15] or broiler chickens [13], where a significant increased jejunal villus height was described.

A positive correlation was also observed for sea bass between the dietary MOS inclusion level and feed intake, in agreement with the results found in neonatal dairy calves [33] and senior dogs [34]. Besides increasing feed intake, MOS has been found also to reduce by 20% cholesterol absorption in rats [35] and increase bile salt excretion [36,37]. In turn, both reduced cholesterol absorption and increased bile salt excretion could reduce liver cholesterol in relation with the reduced liver fat deposition found in sea bass hepaocytes in the present experiment. This is in agreement with the results obtained by Young et al. [38], who reported a significant reduction of fat deposition in liver and a significant hypocholesterolemic effect in liver and eggs after fed laying hens 0.1% Bio-Mos.

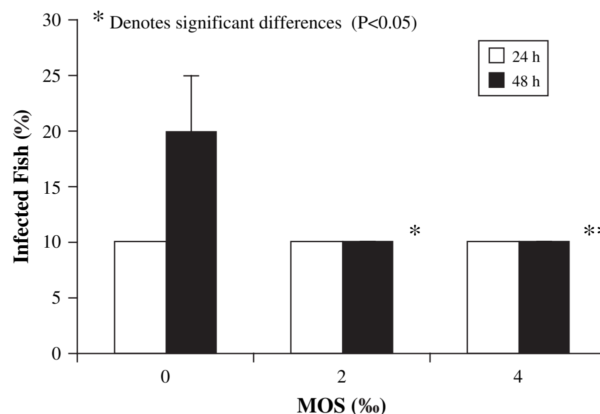

Fig. 5. Presence of *Vibrio alginolyticus* presence on head kidney of European sea bass fed different levels of MOS inclusion and submitted to infection by anal canalisation in Experiment III (mean ± SD; $n = 60$). Significant differences ($P < 0.05$) among treatments are indicated by (*).

In the present study, dietary MOS incorporation at 4‰ significantly improved head kidney macrophages phagocytic activity. Comparable results have been obtained for sea bass by Montero et al. [39], who observed an improvement on immune status and stress resistance in fish fed with a mixture of MOS-β-glucans for 60 days. A dose-dependant enhancement of phagocytic activity by MOS has been also found in chicken macrophages [40], that could be related to the presence of a mannose receptor involved in microbe recognition and phagocytosis in the absence of specific opsonization [41]. The mannose receptor is the main molecule involved in antigen recognition and the binding process in antigen presenting cells [42]. Another possible action mechanism of MOS on the immune system could be the stimulation of mannose binding lectin (MBL) by liver secretion. This protein binds the capsule of bacteria and triggers the complement cascade [43]. In this respect, the results of the present study showed a trend to increase in the alternative complement pathway activity by elevation of MOS dietary levels. This is in agreement with the results obtained by Staykov [14] who found higher levels of bactericidal activity, lysozyme, antibody levels and alternative complement pathway activity in rainbow trout and common carp fed MOS. Therefore, MOS could activate and facilitate antigen processing and serve to stimulate the initial stages of the immune response [44]. Nevertheless, MOS effect on immune response could be related with other factors. For instance, a selective absorption of tryptophan by gut brush-border membrane has been reported in chicks fed MOS [13] and certain studies show that high doses of tryptophan cause abnormal white blood cell accumulation in tissues [45,46], evidencing the active role that tryptophan or its metabolites play in modulating immune system activity. Hence, further studies must be conducted to understand the role of MOS in fish immunomodulation.

Despite all of the intraperitoneally infected fish rapidly dying due to the endotoxic hiperagude shock produced by *V. alginolyticus* extracellular products (ECPs) [47], no mortality was detected in cohabitants. In other species such as chinook salmon (*Oncorhynchus tshawytscha*) exposition to an *Aeromonas salmonicida* challenge showed a direct relationship between a rapid death of the experimentally infected fish and disease occurrence [48]. Hence, the lack of mortality in cohabitant fish seems to be related to a low density of bacteria released to the water from donors, being the receptors asymptomatic infected fish. However direct gut inoculation of *V. alginolyticus* showed the reduction in the number of infected fish by feeding MOS. Among other carbohydrates, mannose constitutes an important surface component of cells. Mannose-specific lectins are utilized by many gastrointestinal pathogens as a means of attachment to the gut epithelium [49]. These molecules function as adhesins mediating the binding of some bacteria as *Campylobacter jejuni* [50] and *Aeromonas hydrophila* [51] to epithelial cells, and the interaction of bacteria with phagocytic cells [52,53]. Some studies reported that mannose inhibited the *in vitro* colonization of the chicken small intestine by *Salmonella typhimurium* [54] and reduced caecum colonization by *S. typhimurium* following oral inoculation [11].

In summary, these results showed that dietary incorporation of MOS at 0.4% enhances sea bass growth, activates its immune system and increase its resistance to a bacterial infection directly inoculated in the gut, one of the main sites of infection in fish [55]. Further experiments must be conducted to clarify the action mechanisms of MOS, as well as the optimum feeding period and dose.

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Dietary administration of beta-mercapto-ethanol treated *Saccharomyces cerevisiae* enhanced the growth, innate immune response and disease resistance of the rainbow trout, *Oncorhynchus mykiss*

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The effects of dietary whole cell yeast (*Saccharomyces cerevisiae*), n-3 HUFA-enriched yeast and treated yeast cells with beta-mercapto-ethanol (2ME) on immunity, growth performance and disease resistance to *Yersinia ruckeri* were investigated in *Oncorhynchus mykiss*. During 30 days, juvenile rainbow trout were fed diets supplemented with different forms of yeast at 5×10^7 CFU g^{-1} or a control diet. After the feeding trial, remaining fish of each treatment were challenged by pathogenic *Yersinia ruckeri* and kept under observation for 14 days to record clinical signs and daily mortality rate. Yeast supplementation in all treatment groups significantly promoted the growth performance compared to control group. A significantly increase was also observed in immune responses in juvenile fish fed 2ME-treated yeast diet. More ever, the lowest fish mortality was obtained in this treatment group. The present results show that a diet supplemented with 2ME-treated yeast stimulates the immune system and growth of juvenile rainbow trout thus enhancing their resistance against *Y. ruckeri*.

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1. Introduction

The intensification of fish culture has led to high number of infectious and non infectious disease outbreak. In rainbow trout farming, one of the most prominent bacterial diseases is caused by *Yersinia ruckeri*. Rainbow trout affected by Yersiniosis mainly show hemorrhages in various tissues and organs, especially around the mouth, in the gills, muscles, peritoneum, body fat, viscera and low intestine. Meanwhile, sever inflammations of the distal portion of the intestine and vents are common. This pathogen is probably persistent in the environment and is horizontally transmitted [1]. In order to improve health in the fish rearing, several alternatives such as improved husbandry, nutrition, water quality, lower stocking density, use of vaccines and different stimulants such as probiotics have been proposed [2].

Probiotics are live microbial feed supplements with beneficial effects on host by producing inhibitory compounds, competition for chemicals and adhesion sites, immune modulation and stimulation, and improving the microbial balance [3]. In deed, there has already been intensive research on probiotics for use in aquaculture. Baker's yeast (*Saccharomyces cerevisiae*) is a natural product from the baker's industry that contains various immunostimulating compounds such as β-glucan, nucleic acids, mannan oligosaccharides and chitin, and has been proved to enhance the immune responses and growth in fish [4]. Previous studies were conducted to determine the effects of whole cell yeast, partially autolyzed yeast or some components isolated from whole cell yeast such as β-glucan or nucleic acids in fish species [4–9]. There are few information regarding the potential of chemically treated baker's yeast with 2ME on gnotobiotic artemia and Indian white shrimp *Fenneropenaeus indicus* health [10,11] but the effect of chemically treated baker's yeast with beta-mercapto-ethanol (2ME) and comparison with whole cell yeast and n-3 HUFA-enriched yeast has not been studied in fish species. Thus, the aim of this study was to evaluate the effects of dietary whole cell yeast (*S. cerevisiae*), n-3 HUFA-enriched yeast and treated yeast cells with 2ME on some immune parameters, growth performance and protection against pathogen *Y. ruckeri* in juvenile rainbow trout.

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2. Materials and methods

2.1. Fish

Rainbow trout of 20 ± 3 g average weight were obtained from a commercial fish farm in Urmia, Iran. Healthy fish were kept in an indoor cement tanks for 10 days for acclimation to the laboratory conditions. Fish were randomly distributed at 42 fish per tank and maintained in continuously aerated free-flowing dechlorinated fresh water at 14 ± 2 °C. Fish were fed with commercial pelleted diet (Faradaneh, Iran) at 3% of body weight daily. Analysis of pelleted diet is shown in Table 1.

2.2. Preparation of *S. cerevisiae* mixtures

Three forms of baker's yeast were used in this study: 1) Whole cell yeast without any treatment, 2) 2ME-treated yeast, 3) n-3 HUFA-enriched yeast.

For preparing 2ME-treated yeast, yeasts cells were grown according to the procedures described by Soltanian et al. (2008) [10], using minimal yeast nitrogen base culture medium (YNB). In the stationary growth phase (after 3 days) yeast cells were harvested by centrifugation (800×*g* for 10 min). Then chemical (2ME) treatment was applied on yeast cells. First, yeast cells were suspended at a concentration of 200 mg wet weight ml in a sterilized medium containing Na_2EDTA (0.05 mol/L) and Tris-buffer ((0.2 mol/L); pH 8). After addition of 2ME (2% volume/volume) the yeast cells were incubated for 30 min at 30 °C. Pretreated yeasts were collected and washed with protoplasting medium comprising a phosphate–citrate buffer (KH_2PO_4 0.08 mol/L; Na3citrate 0.016 mol/L; pH 5.8) and KCl (0.6 mol/L). Finally, yeast cells were washed thrice with filtered (0.2 μm) seawater. Yeast cell concentrations were determined with a Burker haemocytometer. Yeast suspensions were stored at 4 °C until the end of each experiment (maximum storage of 1 week).

Yeast enrichment was performed by oil rich in n-3 HUFA. The fatty acid composition was analyzed by gas chromatography (DANI 1000, Italy) using a BP × 70 capillary glass column (0.32 mm × 50 m, SGE Analytical Science Australia) after esterification in acetylchloride/methanol mixture [12]. Analysis of fatty acid composition is shown in Table 2. For yeast enrichment, protocol by Aoki et al. (2004) with small modification was used [13]. Briefly, 1 g of *S. cerevisiae* was inoculated at 25 °C to 700 ml of a medium consisting 5% glucose, 2% yeast extract, 0.5% choline, 1% K_2HPO_4, and 3% n-3 HUFA and the culture was incubated at 25 °C and agitation was performed at 150 rpm for 2 days. The cells were harvested by centrifugation and then washed with ethanol and normal saline. The wet cells were stored at −20 °C and used for feeding to fish.

2.3. Diet preparation and feeding trial

Three experimental diets were formulated to be supplemented at 5×10^7 CFU per g of diets. After spraying the yeast on commercial feed, pellets were dried at room temperature for 2 h and then they were stored at 4 °C for further use. Each diet was fed to triplicate tanks twice daily for a period of 30 days. After feeding trial, diets were replaced by normal pelleted diet without any yeast.

Table 1
Composition of the basal diet used throughout the experiment.

Proximate analysis (%)	FFT_1	FFT_2
Dry matter	89	89
Crude protein	40	40
Crude lipid	14	14
Ash	10	10
Fiber	3.5	3.5
Phosphorous	1.2	1.2

Table 2
Fatty acid composition of whole cell *S. cerevisiae* and n-3 HUFA-enriched *S. cerevisiae*. Each value (X ± SD) is the average performance of five samples for fatty acid analysis.

Fatty acids	Whole cell *S. cerevisiae*	n-3 HUFA-enriched *S. cerevisiae*
14:0	4.5 ± 0.07^a	3.8 ± 0.07^a
14:1n5	3.8 ± 0.21^a	3.4 ± 0.056^a
16:0	29.6 ± 0.91^a	9.5 ± 0.98^b
16:1n7	0	0
18:0	7.4 ± 0.14^a	2.3 ± 0.57^b
18:1n9	11.85 ± 0.67^a	4.75 ± 0.31^b
18:1n7	11.85 ± 0.33^a	9.95 ± 0.12^a
18:2n6cis	7.7 ± 0.98^a	5.3 ± 0.18^a
18:3n3	0.87 ± 0.42^a	0.9 ± 0.063^a
20:0	0^a	0.89 ± 0.056^b
18:3n6	0	0
18:4n3	0	0
22:0	0	0
20:3n6	0.34 ± 0.007^a	0^b
20:3n3	0.52 ± 0.021^a	0^b
20:4n6	0.56 ± 0.028^a	0.48 ± 0.021^a
20:5n3	0.64 ± 0.021^a	17 ± 0.98^b
22:5n6	0	0
22:6n3	1.82 ± 0.035^a	30.7 ± 0.98^b
24:0	0	0

The same superscript alphabets in the same row are not significantly different at $P < 0.05$.

2.4. Fish performance

5 fish from each individual tank were randomly selected and factors such as total length, total weight, viscera and carcass weights were determined. Condition factor, weight gain and specific growth rate were calculated as following:

Condition factor (CF) = $W/L^3 \times 100$, where W is final weight (g), L is total length (cm).

Weight gain (WG) = $(W_2 - W_1) \times 100/W_1$, where W_1 and W_2 are the initial and final weight (g), respectively.

Specific growth rate (SGR) = $100 (\ln W_2 - W_1)/T$; where W_1 and W_2 are the initial and final weight (g), respectively, and T is the number of days in the feeding period.

2.5. Fish dissection and microbiology

5 fish from each tank were sampled one day before start feeding, then at 30 and 45 days post start feeding. After euthanasia with Clove powder (200 mg ml^{-1}) disinfection of fish external surface was carried out. Cultivable yeast associated with the intestine was counted by the methods of Andlid et al. (1995) [14]. Briefly after dissection of midline in ventral surface, gut sections between pyloric caeca and 1 cm before anal pore were gently removed. Intestinal contents were homogenized, diluted and spread on Sabouraud dextrose agar with antibiotics to count yeast.

2.6. Hematological measurements

9 fish from each group were anaesthetized with clove powder (200 mg l^{-1}) and blood was collected by caudal vein puncture in heparinised syringes. Hematocrit values (Ht) were determined after sampling by placing fresh blood in glass capillary tubes and centrifuging for 5 min in a microhematocrit centrifuge. Haemoglobin (Hb)

level was determined calorimetrically by measuring the formation of cyanomethaemoglobin using a commercial kit. Red blood cells (RBCs) and white blood cells (WBCs) were counted under a light microscope using a Neubauer haemocytomter after dilution with phosphate-buffered-saline. Differential leukocyte counts (neutrophil, lymphocyte, monocyte) were determined using blood smears under a light microscope. Cells were identified on the basis of morphology and cell ultra structure as documented in previous fish leucocyte studies [15].

2.7. Immunological assays

3 fish from each tank were sampled one day before start feeding, then at 30 and 45 days post start feeding. Blood was collected by vein puncture using syringes coated with heparin and transferred immediately into sterile tubes and allowed to clot at room temperature for 1 h samples were kept at 4 °C for 5 h. The sera were separated by centrifugation (1500×*g* for 5 min at 4 °C) and stored at −20 °C until required.

2.7.1. Lysozyme activity

Lysozyme activity in serum was determined according to the method of Demers and Bayne (1997) [16] based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodiekticus* (Sigma). The dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 20 μg ml^{-1} (in 0.1 M phosphate–citrate buffer, pH 5.8) were considered as the standard. This along with the undiluted serum sample (25 μl) was placed into wells of a 96-well plate in triplicate. 175 μl of *M. lysodiekticus* suspension (75 μg ml^{-1}) prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm at approximately 20 °C using a microplate reader. The equivalent unit of activity of the sample as compared to the standard were determined and expressed as μg ml^{-1} serum.

2.7.2. Complement activity

Alternative complement activity was assayed based on the hemolysis of rabbit red blood cells (RaRBC) as described by Waley and North (1996) [17]; Boesen et al. (1999) [18] and Amar et al. (2000) [19]. The RaRBC were washed three times in ethylene glycol tetraacetic acid–magnesium–gelatin veronal buffer (0.01 M EGTA–Mg–GVB, pH 7) and the cell numbers were adjusted to 2×10^8 cells ml^{-1} in the same buffer. At first, the 100% lysis value was obtained by adding 100 μl of the above RaRBC to 3.4 ml distilled water. The hemolysate was centrifuged and the optical density (O.D.) of the supernatant was determined at 414 nm using a spectrophotometer (Awareness, USA). Following, the test sera were diluted (100 times), different volumes ranging from 100 to 250 μl (total volume was adjusted to 250 μl with the buffer) was allowed to react with 100 μl of RaRBC in small test tubes. This mixture was incubated at 20 °C for 90 min with intermittent mixing, following which 3.15 ml of 0.85% NaCl solution was added and the tubes were centrifuged at 1600×g for 10 min at 4 °C and the O.D. of the supernatant was measured as mentioned above. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added on a log–log graph. The volume yielding 50% haemolysis was used for determining the complement activity of the sample as follows:

$$\text{ACH50 (Units ml}^{-1}) = \text{K} \times (\text{reciprocal of the serum dilution}) \times 0.5$$

where K is the amount of serum (ml) giving 50% lysis and 0.5 is the correction factor since the assay was performed on half scale of the original method.

2.7.3. Serum total antibody level

Serum total immunoglobulin was assayed following the method of Siwicki et al. (1994) [20]. After dilution of serum samples with 0.85% NaCl (100 times), total protein content was determined by Bradford method (Kruger, 1996) [21]. 100 μl of total serum samples were mixed with an equal volume of 12% solution of polyethylene glycol (Sigma) in wells of a 96-well microtiter plate. After 2 h of incubation at room temperature, plate was centrifuged at 5000×*g* at 4 °C. The supernatant was diluted 50 times with 0.85% of NaCl and the protein content was determined by Bradford method [21]. This value was subtracted from the total protein level and the result was equal to the total immunoglobulin concentration of the serum that was expressed as mg/ml.

2.8. In situ Y. ruckeri challenge

After 30-day feeding trial, 30 fish from each dietary treatment (10 fish per each tank) were obtained, anaesthetized with clove powder (200 mg lit^{-1}). Then, the fish were challenged by I.P. injection with 0.1 ml of a suspension of *Y. ruckeri* ($BCCM^5$/LMG3279) (1×10^7 cells ml^{-1}). Dead and moribund fish were removed and examined microbiologically for up to 14 days. More ever, agglutination test was performed on samples for confirmation.

2.9. Statistical analysis

All the measurements were made in triplicate. The results were subjected to analysis of variance (ANOVA) followed by least significant differences (Tukey) test. Correlation coefficients were significant with $P < 0.05$.

3. Result

After the 30 day feeding period, all treatment groups exhibited significantly higher final weight than those fed the control diet ($P < 0.05$). Fish supplemented with whole cell yeast and 2ME-treated yeast diets showed the highest final weight followed by group supplemented with n-3 HUFA-enriched yeast in comparison with control group ($P < 0.05$) (Table 3). No significant difference ($P > 0.05$) was observed in fish total length because of yeast supplement, and its range was 14.9–16.1 cm. Viscera weight was significantly high in all treatment groups, meanwhile carcass weight were not enhanced when yeast was included in fish diet ($P > 0.05$). Moreover, weight gain was significantly enhanced when yeast was included in fish diet in all forms ($P < 0.05$). Specific growth rate was also enhanced in all treatment groups while no significant

Table 3
Growth performance and survival rate of rainbow trout fed diets with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SD) is the average performance of fifteen fish/treatment for growth performance and thirty fish/treatment for survival rate for a period of 30 days.

Items	Control	Whole cell yeast	2ME-treated yeast	n-3 HUFA-enriched yeast
Final weight (g)	42.9 ± 0.8^a	54.1 ± 1.4^b	53.1 ± 1.6^{bc}	51.9 ± 1.4^c
Total length (cm)	14.9 ± 0.5^a	16.1 ± 0.5^a	15.5 ± 1.5^a	15.0 ± 1.2^a
Viscera weight (g)	13.1 ± 0.7^a	15.7 ± 0.8^b	15.5 ± 1.1^b	14.8 ± 1.2^b
Carcass weight (g)	85.4 ± 1.3^a	84.8 ± 1.5^a	84.5 ± 1.1^a	84.9 ± 1.1^a
Weight gain (%)	118.8 ± 4.3^a	175.9 ± 7.3^b	171.1 ± 7.7^{bc}	164.4 ± 7.5^c
Specific growth rate (%/day)	1.13 ± 0.02^a	1.46 ± 0.03^b	1.44 ± 0.04^{bc}	1.40 ± 0.04^c
Condition factor	1.30 ± 0.13^a	1.30 ± 0.15^a	1.47 ± 0.41^a	1.57 ± 0.38^a
Survival (%)	46.6 ± 3.56^a	51 ± 6.76^a	66.6 ± 4.87^b	48 ± 2.0^a

The same superscript alphabets in the same row are not significantly different at $P < 0.05$.

differences in condition factor were observed between all groups ($P > 0.05$).

The yeast counts in trout intestine were not significantly different among treatments (Table 4). One day before start feeding, yeast was not detectable in all groups while at 30 day post feeding the count was maximum in whole yeast treatment group then the count decreased by the end of feeding on day 45 in all groups.

Hematocrit was not significantly affected by addition of yeast to diets ($P > 0.05$) in all treatment groups (Table 5). Total leukocytes besides neutrophil counts were significantly higher in 2ME-treated yeast group ($P < 0.05$) than in the control group but significant decrease was shown in lymphocyte count in this group in comparison with control group ($P < 0.05$). Meanwhile fish fed 2ME-treated yeast diet had significantly elevated monocyte count than other groups ($P < 0.05$).

Fish fed the 2ME-treated yeast-supplemented diet showed a significant increase in lysozyme activity on day 30 of treatment compared with the control group (Fig. 1). No significant differences in the serum lysozyme activity were observed between yeast-supplemented diets in other days. Complement activity showed the same parent with significant increase on day 30 in fish received 2ME-treated yeast diet (Fig. 2). Treatment with whole cell yeast and n-3 HUFA-enriched yeast did not affect the complement activity. Total antibody titer of rainbow trout was not significantly affected by the dietary supplementation of baker's yeast in different forms during the study (Fig. 3).

In Table 3 the survival rate of fish challenged with virulent strain of *Y. ruckeri* is shown. During 14 days of challenge, significantly the lowest mortality ($P < 0.05$) was observed in 2ME-treated yeast group (66.6 ± 4.87%). Difference in other groups was not significant even though minor decrease in mortality was observed in these treatment groups compared with control group ($P > 0.05$).

4. Discussion

In the present study the supplementation of *S. cerevisiae* in all 3 forms improved the growth in rainbow trout. This result agree with that obtained with administration of whole cell yeast, enriched yeast and 2ME-treated yeast in fish and crustaceans [8,10,11,13,22]. The improved fish growth in treatment groups may possibly be due to improved nutrient digestibility. As shown in this study highest growth rate was shown in 2ME-treated yeast group. This treatment breaks disulphide linkages between mannoprotein molecules of the yeast cell wall, giving rise to a more open structure in the cell wall and possibly facilitating the action of the digestive enzymes on yeast biomass [11]. It is noteworthy to ponder that viscera weight without fat content significantly increased in all treatment groups but carcass weight did not change compared to control group. There is no available data to study the weight of viscera and carcass in fish administrated with yeast but in the present study it was shown that the effects of all 3 forms of yeast on carcass weight were not efficient.

Table 4
Count of yeast (CFU g^{-1}) associated with the intestine of rainbow trout fed diets with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SD) is the average performance of fifteen fish/treatment for a period of 30 days.

Treatment	Times (Day)		
	−1	30	45
Control	Nd	Nd	Nd
Whole cell yeast	Nd	6.7×10^{5a}	>100
2ME-treated yeast	Nd	4.1×10^{5a}	>100
n-3 HUFA-enriched yeast	Nd	5.3×10^{5a}	>100

The same superscript alphabets in the same column are not significantly different at $P < 0.05$.
Nd: Not detectable.

Table 5
Haematological parameters in rainbow trout fed diets with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SD) is the average performance of nine fish/treatment for a period of 30 days.

Parameters	Control	Whole cell yeast	2ME-treated yeast	n-3 HUFA-enriched yeast
Hematocrit (%)	64.4 ± 1.1^{a}	64.6 ± 1.1^{a}	66.2 ± 1.0^{a}	63.2 ± 0.8^{a}
Total leukocyte count ($\times 10^4$ mm^{-3})	3.45 ± 1.03^{a}	3.21 ± 1.52^{a}	4.13 ± 0.3^{b}	3.91 ± 1.65^{ab}
Neutrophils (%)	5.0 ± 0.7^{a}	5.2 ± 1.0^{a}	8.0 ± 1.2^{b}	6.0 ± 0.7^{ab}
Lymphocytes (%)	91 ± 1.4^{a}	91.2 ± 1.3^{a}	86.0 ± 1.2^{b}	90.0 ± 1.0^{a}
Monocytes (%)	3.0 ± 1.0^{a}	2.4 ± 1.1^{a}	5.8 ± 0.8^{b}	3.6 ± 1.1^{a}

The same superscript alphabets in the same row are not significantly different at $P < 0.05$.

The massive association of yeast with the intestine seemed limited to the last day of feeding trial on day 30 in all treatment groups while in control group this maximum was not reached suggesting the colonization of yeast in all treatment groups on day 30. Similar result was observed in previous studies [7,23,24] after feeding this yeast in rainbow trout feeding.

Haematological parameters of fish blood are useful tools that aids in diagnosis of disease. It can also be used to study immnuopotentiators. Haematological parameters showed significant changes in 2ME-treated yeast group in comparison with control group. Reduced hematocrit values may indicate that fish are not eating properly or are suffering from infections [25]. The hematocrit values did not show any significant changes in treatment groups compared with control group while elevated level of total leukocytes besides different cell types (neutrophils and monocytes) were observed in 2ME-treated yeast treatment group. These cell types are the main phagocytic cells that are active for phagocytosis which is an indicator of immune response.

Current study showed that 2ME-treated yeast treatment group was the most effective one in enhancing serum lysozyme and complement activity. Neutrophil of rainbow trout is the main cell type for production of lysozyme. As shown in this study the significant increase in neutrophil count was just observed in this treatment group that is in parallel with increase in serum lysozyme activity. However, contrary results have been reported regarding the

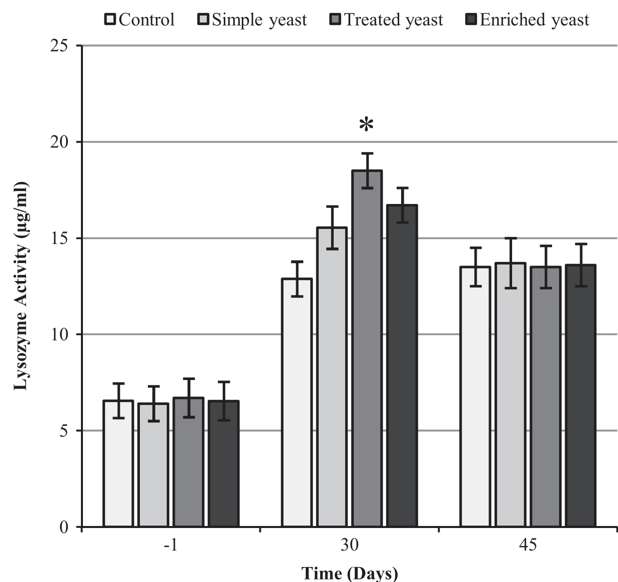

Fig. 1. The Lysozyme activity of rainbow trout fed with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SE) is the average performance of nine fish/treatment for a period of 30 days. Significance at $P < 0.05$.

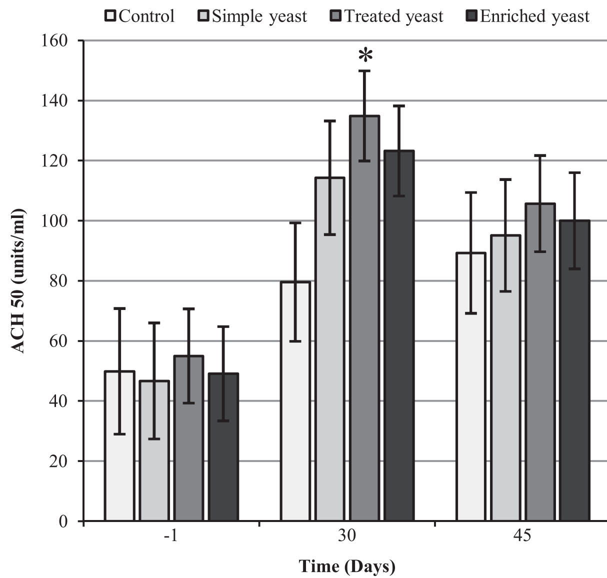

Fig. 2. The Complement activity of rainbow trout fed with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SE) is the average performance of nine fish/treatment for a period of 30 days. Significance at $P < 0.05$.

influence of yeast whole cells and their constituents on serum lysozyme activity in carp [4]; gilthead sea bream [26]; salmon [27] and trout [28]. The alternative pathway of complement activity is a powerful innate mechanism for protecting fish against potentially invasive organisms [9]. Our results showed that 2ME-treated group exhibited significant increase in alternative complement pathway activity. Contrary result was observed in grouper that supplementation with 10^5 and 10^7 CFU kg^{-1} of whole yeast cell exhibited significant increase in this parameter [9]. This immunostimulation can be mainly attributed to the enhanced availability of yeast cellular constituents in 2ME-treated yeast treatment group. In other treatment groups insignificant increase of these parameters were shown on day 30 compared with control group. It can be concluded that availability of cellular constituent in whole cell yeast was not

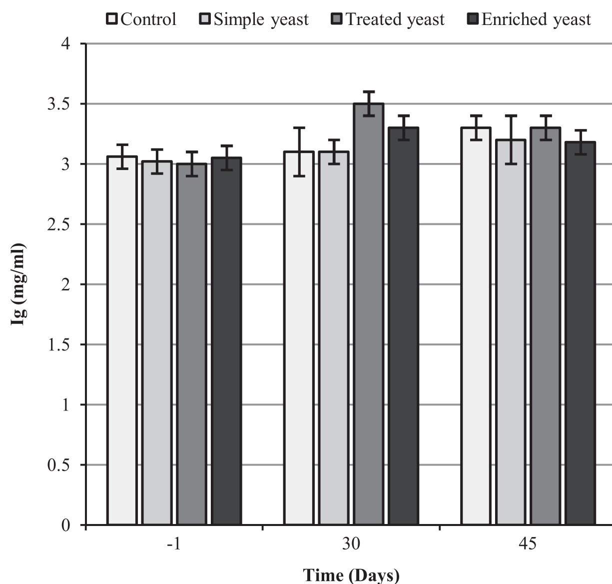

Fig. 3. The total antibody level of rainbow trout fed with whole cell yeast, n-3 HUFA-enriched yeast and treated yeast with 2ME. Each value (X ± SE) is the average performance of nine fish/treatment for a period of 30 days. Significance at $P < 0.05$.

convenient enough to stimulate the immune system. By the way, it was shown that rainbow trout and other freshwater fish species have the ability to synthesis 20:5n3 and 22:6n3 from the precursor 18:3n3. On the other hand, high dietary HUFA promote an increase of oxidative processes in fish [29]. Application of the convenient antioxidant can be helpful to decrease the oxidative stress. In the present study the whole cell *S. cerevisiae* played a role as antioxidant [30] so some minor positive effects of enrichment in fish growth was observed.

Plasma proteins include the humoral elements of the nonspecific immune system such as immunoglobulin. In our study, total immunoglobulin level of serum was not affected by administration of yeast in all 3 forms. Immunostimulants can have a contradictory effect on these parameters [31]. In Nile tilapia, *Oreochromis niloticus*, after feeding the fish with Chinese herb and boron this parameter similarly did not change significantly. In rainbow trout, plasma total protein level was significantly increased after feeding the fish with various herbal extracts [32].

The results of the present study demonstrated that in general fish fed with 2ME-treated yeast-supplemented diet enhanced the survival of rainbow trout against *Y. ruckeri* compared with other groups. Similarly, dietary 2ME-treated *S. cerevisiae* enhanced resistance against white spot virus in Indian white shrimp, *Fenneropenaeus indicus* [11] in comparison with untreated yeast feed and control group. Even though yeast cells are rich in nutrients the outer thick mannan layer that gives mechanical strength to the cell wall hinders its digestibility and absorption [11] so the stimulation of immune system and consequently protection against disease agents will dwindle.

In general it seems that great proportion of whole cell live yeast used in this study was lost through faeces as undigested and unutilized. By the way, HUFA-enrichment of this yeast was not efficient enough for augmentation of immunity in rainbow trout. Treating *S. cerevisiae* with 2ME improved the digestibility leading to a better utilization of yeast cell nutrients.

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Assessment of environmental impact of commercial wine yeast in vineyard ecosystems

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Introduction

Modern winemaking practices and diversification of wine products involve an increasing quest for specialised wine yeasts. During the last two decades, considerable efforts have been made to improve wine yeast strains through the use of new biotechnologies. Today, about 50% of European wine production is based on the use of active dried yeast. Commercial yeasts are classically used in winemaking without any special containment and are released into the environment in large numbers with various effluents and by-products. The behaviour of these yeasts in natural habitats is practically unknown, as well as their potential impact on the natural microflore. There is very little available data that could contribute to the evaluation of the importance of starter yeast dissemination and permanence in the vineyard (Frezier and Dubourdieu, 1992; Vezinhet *et al.* 1992; Guillamón *et al.*, 1996). Recently, a large-scale biogeographical study in South African vineyards was carried out in five areas situated in the Coastal Region vineyards of the Western Cape. Commercial yeasts were recovered in 3 of 13 samples (van der Westhuizen *et al.*, 2000a and 2000b).

In the present study a large-scale sampling plan was established with the aim of evaluating the industrial starter yeasts' ability to spread and survive in nature. This study provides a consistent assessment of potential environmental risks associated with the use of genetically engineered winery yeast strains using commercial wine yeast as a model.

Material and Methods

The sampling plan included 36 sites in 6 vineyards, 3 of which were located in the South of France (A, B and C) and 3 in the North of Portugal (D, E and F). The total

duration of these studies was 3 years (2001-2003). The wineries selected had used one or more commercial yeast strains consecutively in the last 5 years. In each vineyard, six sampling points were defined according to the local conditions (size and orientation of the vineyard, predominating wind direction), and the distance between the winery and the sampling sites varied between 20 to 1000 m. In order to evaluate the remanence of commercial yeast over the years, a first sampling campaign was performed before the winery started wine production with the use of commercial yeast strains (pre-harvest campaigns). In a second post-harvest sampling campaign, the grapes were collected after the onset of wine production, in order to evaluate the immediate commercial yeast dissemination from the winery. With the present experimental design, 72 grape samples were collected each year.
From each sampling point, approximately 2 kg of grapes were aseptically collected, and the extracted grape juice was fermented at 20ºC. Daily weight determinations allowed the monitoring of the fermentation progress. The yeast flora was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YPD medium, and after 2 days of incubation 30 randomly selected colonies were collected from each spontaneous fermentation and subjected to molecular identification.

Results and discussion

A total of 198 samples were collected during three consecutive campaigns (2001-2003), 108 of which were taken in France and 90 in Portugal. Of the 198 samples, 126 musts (64%) produced spontaneous fermentations, 20% and 44% in must from pre-harvest and post-harvest campaigns respectively. The percentages of spontaneous fermentations were similar in both countries. A total of 3780 colonies were isolated, of which 2355 were identified as *Saccharomyces* strains. Molecular characterisation of these led to the identification of 296 strains with a genetic profile similar to that of commercial yeasts (Table 1). These strains represent 7.8% of the fermentative yeast community, the majority of which (5.8%) were recovered in post-harvest campaigns. The global data reflects a very different situation. In 4 out of 6 vineyards (3 French vineyards and vineyard F in Portugal) where the sampling sites were placed at a greater distance from the winery, the occurrence of commercial yeast was very low, representing between 0% and 2% of the fermentative

community, and these strains were isolated from only five samples (Table 1). The results were very different in the Portuguese wineries D and E, for which a high number of commercial strains was isolated representing 43 and 10% of the fermentative yeast community respectively.

An overview of the dissemination of commercial strains in relation to their distance from the winery is shown in Figure 1. Ninety four percent of commercial strains were found in a radius of around 10-200 m from the winery and a large majority (78%) was recovered in sites at very close proximity (10-50 m) to the wineries (vineyards D and E). A major proportion (73%) was collected in post-harvest campaigns, indicating immediate dissemination.

As a whole, the evolution of the fermentative yeast communities over the three years studied showed that the same strains were not found in the same sites from one year to another. This indicates that if some of these strains are able to remain in the ecosystem, they are not capable of dominating the natural yeast community of the vineyard.

Conclusion

This study has provided new insight into the impact of commercial yeasts on the communities of fermentative yeast microflore surrounding vineyards. The data show that dissemination of commercial yeasts in the vineyard is restricted to short distances and limited periods of time and largely favoured by the present of liquid effluents. More than 90% of commercial yeasts were found at a radius between 10 and 200 m from the winery and did not become implanted in the ecosystem in a systematic way. Given that they are used in large quantities, commercial strains tend to out-compete autochthonous strains inside the winery (Beltran et al, 2002). In contrast, they do not become implanted systematically in the ecosystem and are not able to dominate the natural microflore although they are subject to natural fluctuations of periodical appearance and disappearance in the same way as autochthonous strains are. Considering commercial yeast strains as an appropriate model system for genetically modified yeast strains, our data also contribute to the in-depth environmental risk assessment concerning the use of such strains in the wine industry.

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Tables and Figures

Table 1: Commercial yeast strains recovered in each vineyard over the 3 years

Vineyards	**A**	**B**	**C**	**D**	**E**	**F**	**Total**
Spontaneous fermentations	19	24	29	16	23	15	126
Isolates	570	720	870	480	690	450	3780
Commercial yeasts strains	0	15*	1	206	54+18*	2	296
% Commercial yeast / nb of isolates	0	2	0.1	43	10	0.5	7.8

*Commercial yeasts initially isolated in the same region

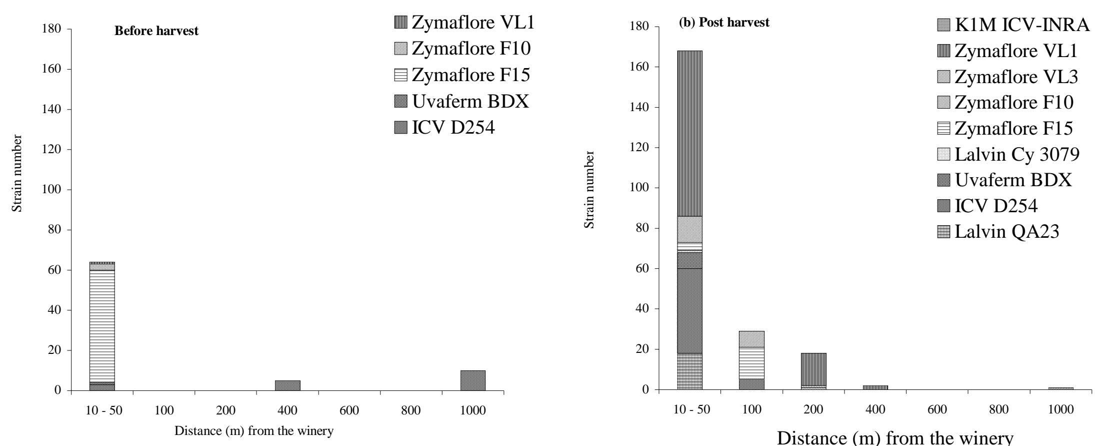

Fig. 1. Overall (three years) distribution of commercial yeast strains according to the distance from the wineries in pre-harvest (a) and in post-harvest (b) campaigns.

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Cross effects of the strain of dietary Saccharomyces cerevisiae and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, Onchorhynchus mykiss, fry

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Abstract: Two strains of Saccharomyces cerevisiae were tested as probiotics for rainbow trout fry, during the first month of feeding. Each strain was introduced into separate diets, at the rate of 106 CFU g− 1 and their effects were compared with those of a control diet. Two rearing conditions were simultaneously compared, to test the adaptability of the probiotic treatment. From start feeding onwards, the water supply came from either spring or river, resulting in two different temperature ranges, 11–11.5 and 7–8 °C respectively. Growth and development were optimal in spring water, while some delay was observed with colder river water. A slight but significant increase in mortality was also observed in the river group. In all groups, the counts of bacteria associated with trout intestine were maximum 10 days post start feeding (dpsf; 107 CFU g− 1). The counts of probiotic yeast were also maximum at 10 dpsf (104–105 CFU g− 1), but the decrease was slower in river than in spring water. An autochthonous yeast, Debaryomyces hansenii, was also retrieved associated to the intestine of the control group in high numbers after 240 degree days of experiment (104–105 CFU g− 1), while the colonization level was significantly less in trout fed the probiotic diets. The effect of the dietary yeast was observed by assaying the activity of three enzymes in the brush border membrane of the enterocytes: alkaline phosphatase (AP), γ-glutamyl-transpeptidase (GGT), and leucine-amino-peptidase N (LAP). At 10 and 20 dpsf, the trout reared in spring water had higher activities of the three enzymes when they were fed the strain S. cerevisiae var. boulardii, suggesting an earlier maturation of the digestive system in this group, compared with trout fed either the other strain of S. cerevisiae or the control diet. The effect was not observed in trout reared in river water with slower growth. Both S. boulardii and D. hansenii seemed to stimulate digestive maturation in fish, but the natural colonization by D. hansenii was likely too late for trout reared at optimal temperature. The supplementation of trout starter diet with S. boulardii may be particularly useful in fast growing conditions.

Keywords: Start feeding; Larval development; Gut maturation; Brush border membrane; Yeast; *Debaryomyces hansenii*

1. Introduction

Many probiotic bacteria have been proposed to improve health in rainbow trout. The strains were generally antagonistic to pathogens (Jöborn et al., 1997; Gram et al., 1999; Robertson et al., 2000; Spanggaard et al., 2001; Irianto and Austin, 2002; Aubin et al., 2005a), and an important feature was the ability to colonise fish gut (Jöborn et al., 1997; Nikoskelainen et al., 2001a, b). The immune system was stimulated in rainbow trout by several probiotics (Irianto and Austin, 2002; Nikoskelainen et al., 2003; Raida et al., 2003; Panigrahi et al., 2004). Inactivated bacterial cells might be also efficient to control furunculosis, but the viability of the probiotics influenced the immune response (Irianto and Austin, 2003; Panigrahi et al., 2005).

Andlid et al. (1995) suggested that yeast isolated from rainbow trout might also improve health, with a particular attention to their colonisation potential. Like probiotic bacteria, *Saccharomyces cerevisiae* var. *boulardii* acted as pathogen antagonist and immunomodulator in mammals (McFarland and Bernasconi, 1993), but the yeast increased also the specific and total activities of digestive enzymes in the brush-border membrane (BBM; Buts et al., 1986, 1999). *S. cerevisiae* var. *boulardii* had some effect on rainbow trout metabolism, since the dietary supply of the yeast increased muscle lipids and red pigmentation, while improving the resistance of trout to *Yersinia ruckeri* (Aubin et al., 2005b; Quentel et al., 2005). It seems that the probiotic efficiency of *S. cerevisiae* is dependent on the strain (Fietto et al., 2004;Van der Aa Kühle et al., 2005).

Considering the presence of autochthonous yeast with probiotic potential in trout intestine, a dietary supply of allochthonous strains might seem worthless. Aubin et al. (2005a) hypothesised that autochthonous *Debaryomyces hansenii* could stimulate the mucosal metabolism in rainbow trout intestine, while interfering with the dietary yeast. However, the occurrence of autochthonous yeast was different in several locations (Gatesoupe et al., 2005a). More generally, the intestinal microbiota of rainbow trout was highly variable in time, and between farms (Spanggaard et al., 2000; Huber et al., 2004; Gatesoupe et al., 2005a, b). Some environmental conditions may account for this variability, for instance the rearing temperature (Lésel, 1990). Consequently, probiotics may serve as a precaution in front of this variability, but at the same time, the efficiency of the treatments should be validated in several rearing conditions.

The present experiment was conducted on rainbow trout fry at start feeding, to compare the effects of two probiotic strains of *S. cerevisiae*, in combination with the cross effects of two rearing conditions, with particular attention to intestinal microbiota, and to the activity of BBM enzymes in trout enterocytes. The effects of the strain already tested on rainbow trout (Aubin et al., 2005a, b) were compared to those of another strain, which was recommended by Lara-Flores et al. (2003) as growth promoter for Nile tilapia.

2. Materials and methods

2.1. Rearing conditions and diets

The strain of rainbow trout (*Oncorhynchus mykiss*), and the general rearing conditions were as described by Aubin et al. (2005a), but two water qualities were compared in the present experiment. All the eggs were incubated and hatched in UV-treated spring water (11.54 ± 0.02°C, mean ± standard error). Ten days post hatching, the fry were dispatched in 18 tanks. Nine tanks were kept receiving UV-treated spring water, while in the nine other tanks, the water supply was shifted to filtered river water within 5 h, resulting in a temperature decrease

from 11.4 to 7.2°C. Then the two conditions differed in their temperature ranges, 11-11.5 and 7-8°C for the spring and the river, respectively.

The diets were prepared with Ecostart® 15, crumble size '01'. The control diet was obtained by coating the pellets with cod liver oil (32 ml kg^{-1}). Two experimental diets SC and SB were prepared with the probiotic yeast *Saccharomyces cerevisiae* strain NCYC Sc 47/g (National Collection of Yeast Culture, Norwich, UK), or *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (*S. boulardii,* Institut Pasteur, Paris, France), respectively. Both strains were obtained as commercial preparations, Biosaf® Sc 47 and Levucell® SB20, respectively. The active dried yeast preparations were powdered by grinding and sifting through 100 μm, then suspended in cod liver oil. The amounts of powder were adjusted in the oily suspensions to obtain a final concentration of ca. 10^6 Colony Forming Units (CFU) of yeast per g of experimental diet, after the pellets had been coated with the shaken suspensions (32 ml kg^{-1}). After coating, the actual counts of yeast on Sabouraud agar with antibiotics (Aubin et al. 2005a) were $(7 \pm 3) \times 10^5$ and $(9 \pm 1) \times 10^5$ CFU g^{-1} of *S. cerevisiae* (diet SC) and *S. boulardii* (diet SB), respectively (mean ± standard error).

One day before start feeding, the fry weighed 60 mg, and they were dispatched in three tanks per treatment, i.e. the three diets crossed with the two water qualities. At start feeding, 423 ± 2 fish were counted per tank, without any significant difference. The trout were weighed at 23 days post start feeding (dpsf), then at 37 dpsf.

2.2 Fish dissection, microbiology, and enzymatic assays

Six fish were sampled in each tank one day before start feeding, then at 10, 20, and 31 dpsf, after 20 h of starvation. After euthanasia and dissection of the intestine, three fish were used for microbiological examination, and the cultivable bacteria and yeast associated with the intestine were counted and characterised by the methods of Aubin et al. (2005a). Briefly, hindgut sections were homogenised, diluted and spread on Petrifilm and Sabouraud agar with antibiotics, to count bacteria and yeast, respectively. After phenotypic characterization with the API system, the 16S rRNA genes were characterised with restriction enzymes, and those corresponding to the dominant genotypes were partially sequenced. The three other fish were used for enzymatic measurements, according to the methods mentioned by Zambonino Infante and Cahu (1999). Three enzymes were assayed in BBM: alkaline phosphatase (AP), γ-glutamyl-transpeptidase (GGT), and leucine-amino-peptidase N (LAP). The maturation of the enterocytes is described by expressing the activities of the BBM enzymes as a ratio to a cytosolic enzyme, leucine-alanine peptidase (Leu-Ala).

2.3 *Statistical analysis*

The mortality was arcsine square root transformed, whereas the bacterial counts were log transformed. The normality and homogeneity of all the data were checked with the tests of Kolmogorov-Smirnov and Bartlett, respectively. When the data passed the tests, they were compared by two-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. Otherwise, they were compared by the Kruskal-Wallis test, followed by the Dunnett's method.

3. Results

Though mortality was low, the survival of trout kept in spring water was significantly higher than that of the other group by the end of the experiment (97.1 ± 0.4% vs. 95.5 ± 0.4%, P=0.01). No cannibalism was observed. Growth was strongly different between the fish reared with the two water qualities, due to temperature gap. At 23 dpsf, the trout reared in spring water weighed 605 ± 6 mg, while those reared in river water weighed 312 ± 3 mg (mean ± standard error). At 37 dpsf, the mean weights were 1293 ± 20 and 547 ± 2 mg for the groups reared in spring and river water, respectively. Neither survival nor growth was significantly affected by the probiotic treatment.

3.1 Intestinal microbiota

The bacterial counts in trout intestine were not significantly different among treatments (Fig 1A and 1B). One day before start feeding, ca. 10^4 CFU g^{-1} were retrieved in the intestine. The counts were maximum at 10 dpsf (10^7 CFU g^{-1}), then they decreased by the end of the first month (10^2-10^4 CFU g^{-1}).
There was no clear effect of the probiotics on intestinal bacteria, but in trout kept in spring water, the bacterial diversity seemed more marked than in trout reared in river water (Fig 1C and 1D). This was observed as early as one day before feeding, 20 h after shifting temperature. At this date, *Rhodococcus* sp. (Table 1) was dominant in trout transferred in river water. The strain was also present in the group kept in spring water, but with other dominant bacteria, *Aeromonas* sp. PL2A1 and *Flavobacterium* sp. *Aeromonas* sp. PL2D1 was dominant at 10 dpsf in both groups, then at 20 dpsf in the river group. *Pseudomonas* sp. was observed in both groups at all dates, but the strain was strongly dominant in the river group at 31 dpsf.
The probiotic yeasts were retrieved associated with the intestine in high numbers at 10 dpsf (10^4-10^5 CFU g^{-1}; Fig 2A and 2B). The counts were still high in the river group at 20 dpsf, while the population decreased to ca. 10^3 CFU g^{-1} in the spring water group. At 31 dpsf, the counts were irregular, and no colony was detected in the group SC in spring water.
An autochthonous strain of *Debaryomyces hansenii* YB3A3 was also observed, as already noted in previous experiments (Aubin et al., 2005a; Gatesoupe et al., 2005a). The association with the intestine was maximum with the control diet at 20 and 31 dpsf in the spring water and river groups, respectively (10^4-10^5 CFU g^{-1}; Fig 2C and 2D). The rearing temperature seemed determinative, and considering that similar numbers of degree days were cumulated at 20 dpsf in the spring water group, and at 31 dpsf in the river group (239 and 243 degree days, respectively), the counts of the autochthonous yeast were compared among dietary treatments at ca. 240 degree days. Even after log transformation the normality test failed, and it was not possible to use the two-way ANOVA. By pooling the replicates fed the same diet till 240 degree days, while disregarding water quality, the Kruskal-Wallis test indicated a significant difference (P<0.05). The counts in the groups fed the probiotics were found significantly lower than the counts in the control group with the Dunnett's method.

3.2 Enzymatic activities

In the spring water group, the specific activity of the three BBM enzymes was significantly higher with diet SB at 10 and 20 dpsf, except for leucine aminopeptidase at 20 dpsf, but the trend was similar (Fig 3). This was no longer observed at 31 dpsf, nor was it observed at any

sampling date in the river group. In this latter group, the activity of the three enzymes was more intense with the control diet at 20 dpsf, and also at the other dates for GGT.
The segmental activity of the three BBM enzymes was expressed as a ratio to Leu-Ala peptidase activity (Fig 4). The spring water group exhibited higher ratios than the river group for the three enzymes at 10 dpsf ($P<0.001$), then at 20 dpsf ($P<0.05$). At 31 dpfs the AP/Leu-Ala and LAP/leu-Ala ratios were still higher in the spring water group ($P<0.05$), but the difference was not significant for GGT. An effect of the diet on enterocyte maturation was noted in spring water. At 10 dpsf, the LAP/Leu-Ala ratio obtained with diet SB was significantly greater than with the other treatments. The same trend was observed with the two other enzymes, though the differences were not significant. At 20 dpsf, the trend was still observed with alkaline phosphatase, but it disappeared at 31 dpsf. Some significant differences were also observed in the river group, but without suggesting any general trend.

4. Discussion

The difference in temperature strongly affected fish growth and metabolism. The effect on intestinal bacteria was less marked, since the counts were similar at each sampling date. In spite of UV treatment, the bacteria associated with the group maintained in spring water were more diverse than those of the group reared in river water. This might be due to the presence of a limited number of psychrophilic strains, while some other ones, still active at 11°C, would become nonculturable after the decrease in temperature. For instance, *Aeromonas* sp. PL2A1 was not retrieved 20 h after shifting temperature, possibly entering into the viable but nonculturable state below 10°C, like *Vibrio vulnificus* (Oliver et al., 1991).
The effect of temperature was more visible with yeast than with bacteria. The massive association of yeast with the intestine seemed limited to the first month of feeding in previous experiments (Aubin et al., 2005a; Gatesoupe et al., 2005a, b). The decrease in counts of the probiotic strains was delayed in the river group, concomitantly with the delayed trout development. This decrease was observed in both groups when 240 degree dpsf were cumulated, which corresponded to the maximum of intestinal colonisation by autochthonous *D. hansenii* in trout fed the control diet. This maximum was not reached in trout fed the probiotic diets, suggesting a competition for space between *D. hansenii* and *S. cerevisiae*.
Dietary *S. cerevisiae* var. *boulardii* increased the activities of BBM enzymes at 10 and 20 dpsf in trout kept in spring water, while the other dietary yeast did not. *S. boulardii* stimulated BBM enzyme activities in mammals (Buts et al., 1986, 1999), but there was no such report with other biovars of *S. cerevisiae*. A strain isolated from rainbow trout intestine, *Debaryomyces hansenii* HF1, stimulated amylase secretion and the activity of BBM enzymes in larval sea bass, whereas *S. cerevisiae* X2180 did not (Tovar et al., 2002). However, some probiotic traits are not specific to *S. boulardii* (Brandao et al., 1998). Growth yield, and resistance to thermal and acidic stresses might be important characteristics of the probiotic strains (Fietto et al., 2004). Adhesiveness did not appear as an essential condition, even though the prerequisites for probiotic yeast have not been clearly identified (Van der Aa Kühle et al., 2005). In sea bass larvae, the colonisation potential could not explain the differences observed between the effects of *D. hansenii* HF1 and *S. cerevisiae* X2180, while the amounts of polyamines produced by the yeast were suspected to play a role (Tovar et al., 2002). The probiotic effects of *D. hansenii* HF1 on sea bass larvae were further confirmed by Tovar-Ramírez et al. (2004). When the diet was supplemented with a suitable amount of the yeast, the sea bass larvae grew faster, with an accelerated pancreatic and intestinal maturation, while survival and conformation were improved. The case of rainbow trout is different because the fry are much more developed than sea bass at first feeding. In the present experiment, the early intestinal maturation of rainbow trout was observed at 10 dpfs, but only

when trout fed *S. boulardii* were kept in spring water. The river temperature was likely below the optimum for trout development, and the probiotic yeast did not appear efficient in that condition. *D. hansenii* YB3A3 colonised naturally the intestine, but not so early as the probiotic yeast, which was brought in high numbers since start feeding. This delay may explain why *S. boulardii* had an effect on BBM activity till 20 dpsf in the spring water group, while the colonisation level of *D. hansenii* was not sufficient yet.
No clear effect was observed in the present experiment with the strain of *S. cerevisiae* that improved growth of Nile tilapia juveniles (Lara-flores et al., 2003). However, the long term effects of the yeast should be further investigated. The supplementation of trout starter diet with *S. boulardii* may be particularly useful in fast growing conditions, where it would be necessary to stimulate the precocious maturation of the digestive function (Tovar et al., 2004). However, this effect was dependent on the rearing conditions, and it should be tested in other locations, especially in those where *D. hansenii* has not been detected. The practical interest of such confirmation lies in regulatory aspects. The authorization of *D. hansenii* as probiotic for fish may be less easy to obtain than that of *S. cerevisiae* strains, which are currently used for mammals.

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Table 1 – Identification of the dominant strains isolated in rainbow trout intestine, with partial sequences of 16S ribosomal RNA gene referenced in nucleotide databases; the identical fragments can be retrieved by reading the published sequences from the first nucleotide to the last one indicated in the last two columns.

Dominant	Reference strain in GenBank			Identical 16S gene fragment		
Strain	Genus	Species	Accession n°	Fragment length (bp)	From	To
5R020	*Rhodococcus*	*erythropolis*	**AF532870**	667	1371	705
5S035	*Flavobacterium*	sp. IsoA1	**AJ319015**	533	148	680
AFT112	*Pseudomonas*	sp. TB2-1-II	**AY599711**	771	16	786
PL2A1	*Aeromonas*	*salmonicida*	**AF200329**	821	119	939
PL2D1	*Aeromonas*	*sobria*	**X74683**	670	151	920
PM3B2	*Arthrobacter*	*bergerei*	**AJ609630**	808	104	911

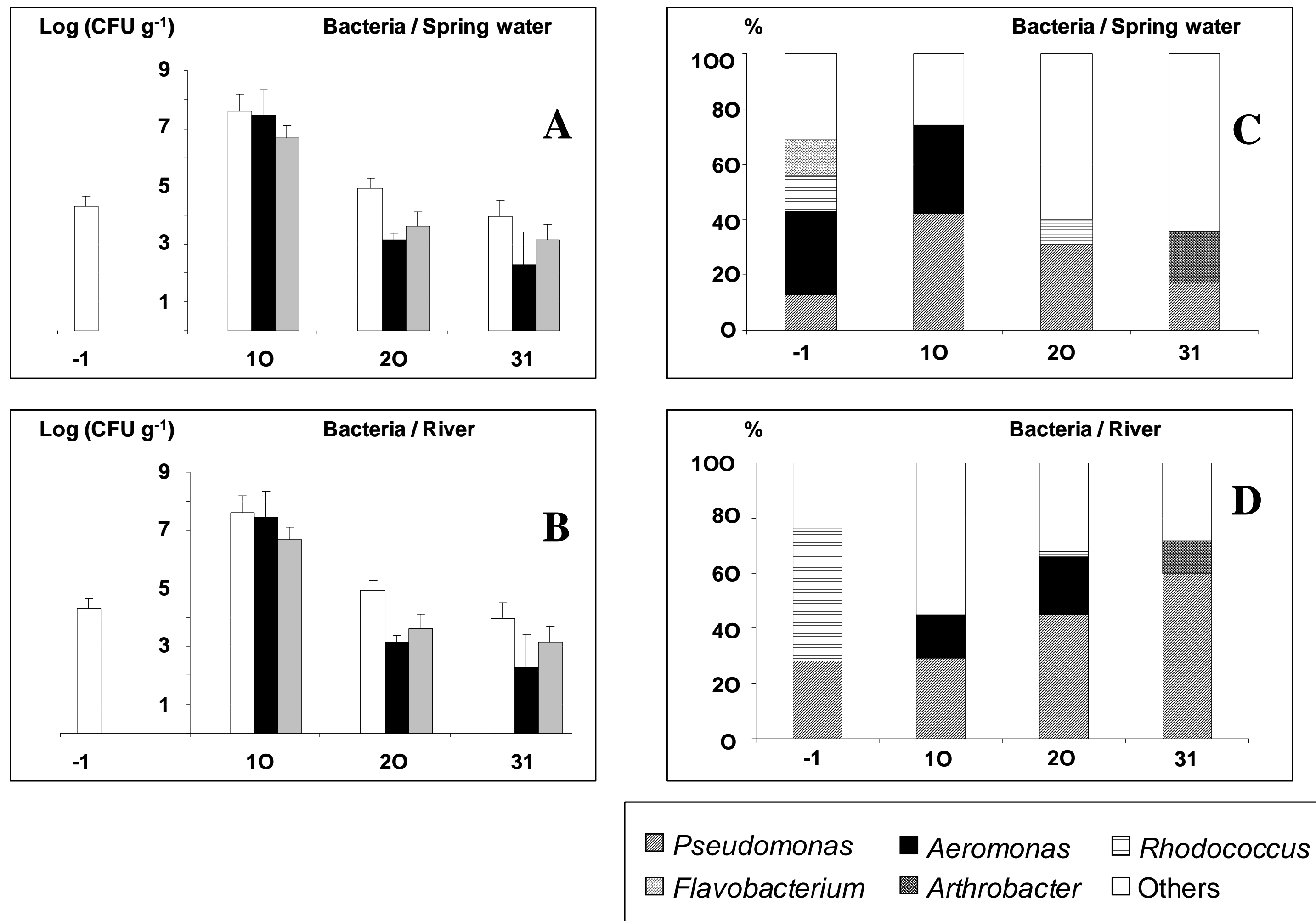

Fig. 1. Left: counts of bacteria associated with the intestine of rainbow trout at –1, 10, 20, and 31 dpsf in log (CFU g^{-1}), either kept in spring water (A) or reared in river water (B); white bars: control group, black bars: group SB, grey bars: group SC. Right: proportions of the dominant genera of bacteria characterised in trout, either kept in spring water (C) or reared in river water (D).

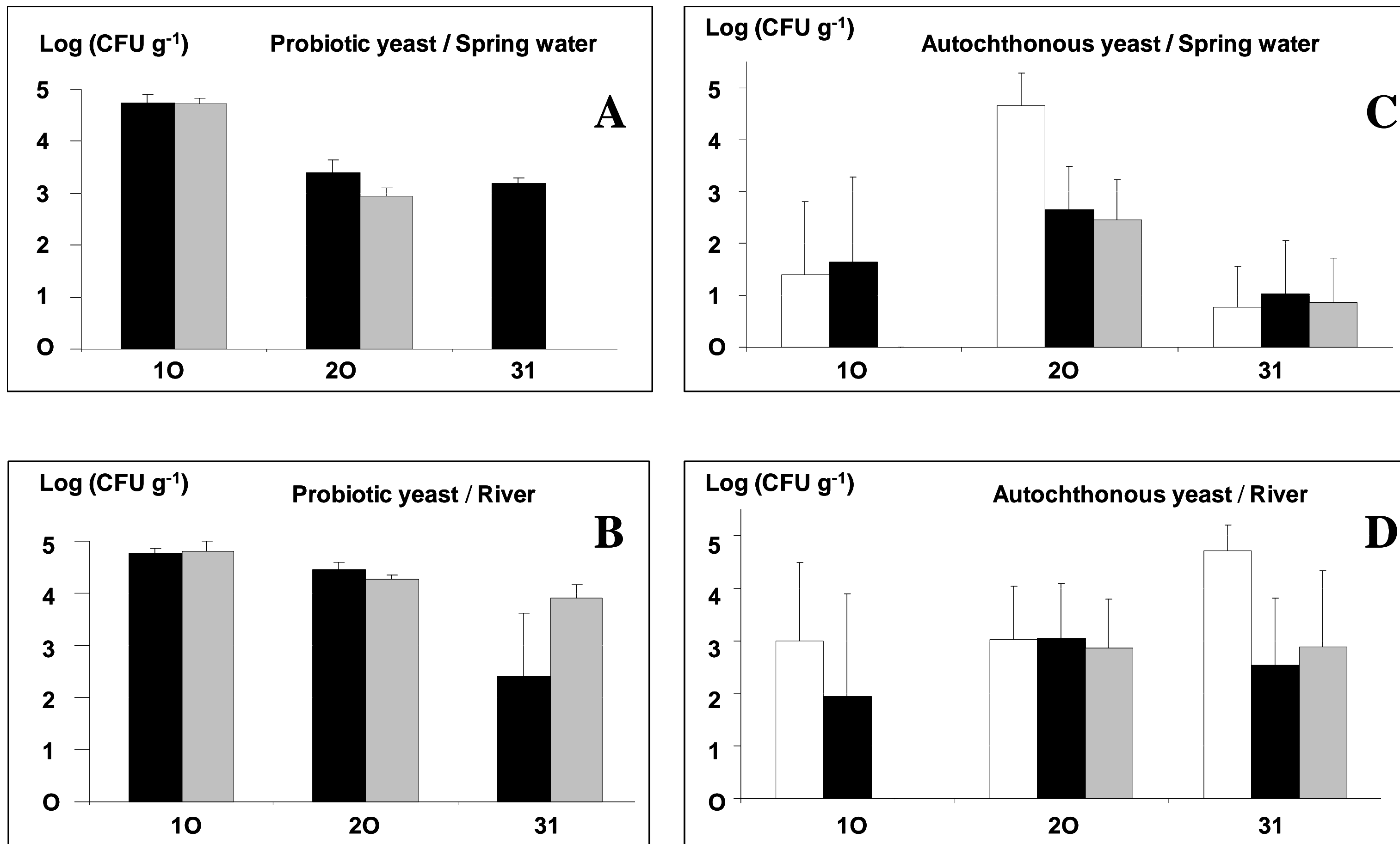

Fig. 2. Left: counts of probiotic yeast associated with the intestine of rainbow trout at 10, 20, and 31 dpsf in log (CFU g^{-1}), either kept in spring water (A) or reared in river water (B); black bars: *S. boulardii*, grey bars: *S. cerevisiae*. Right: counts of autochthonous yeast, *Debaryomyces hansenii*, associated with the intestine of rainbow trout, either kept in spring water (C) or reared in river water (D). white bars: control group, black bars: group SB, grey bars: group SC.

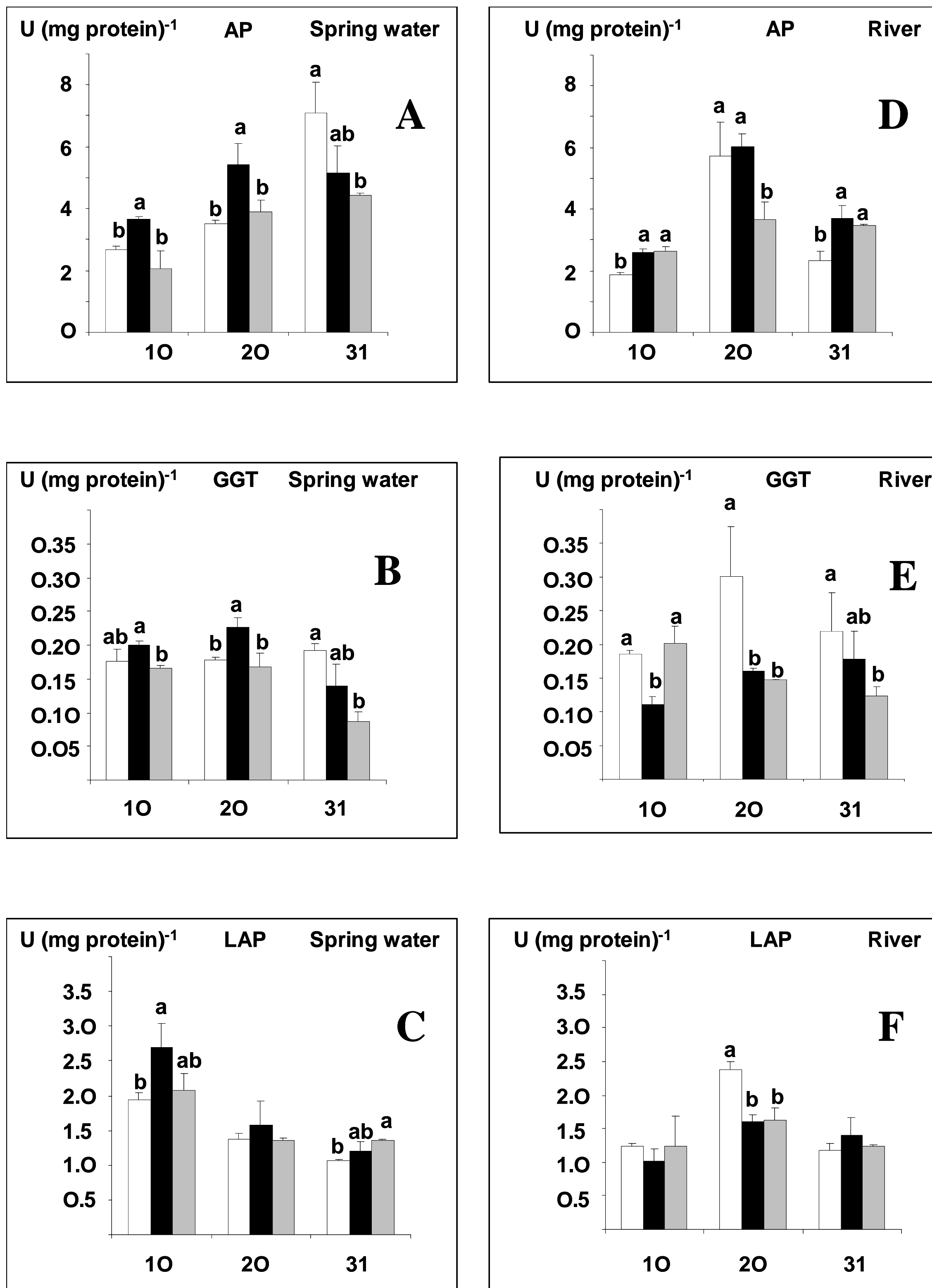

Fig. 3. Specific activity of the three BBM enzymes (in mg protein^{-1}) in trout reared in spring water (left), or in river water (right) at 10, 20, and 31 dpsf; (A), (D): alkaline phosphatase; (B), (E): γ-glutamyl-transpeptidase; (C), (F): leucine-aminopeptidase N; the letters a and b above the error bars indicate the significant differences. white bars: control group, black bars: group SB, grey bars: group SC.

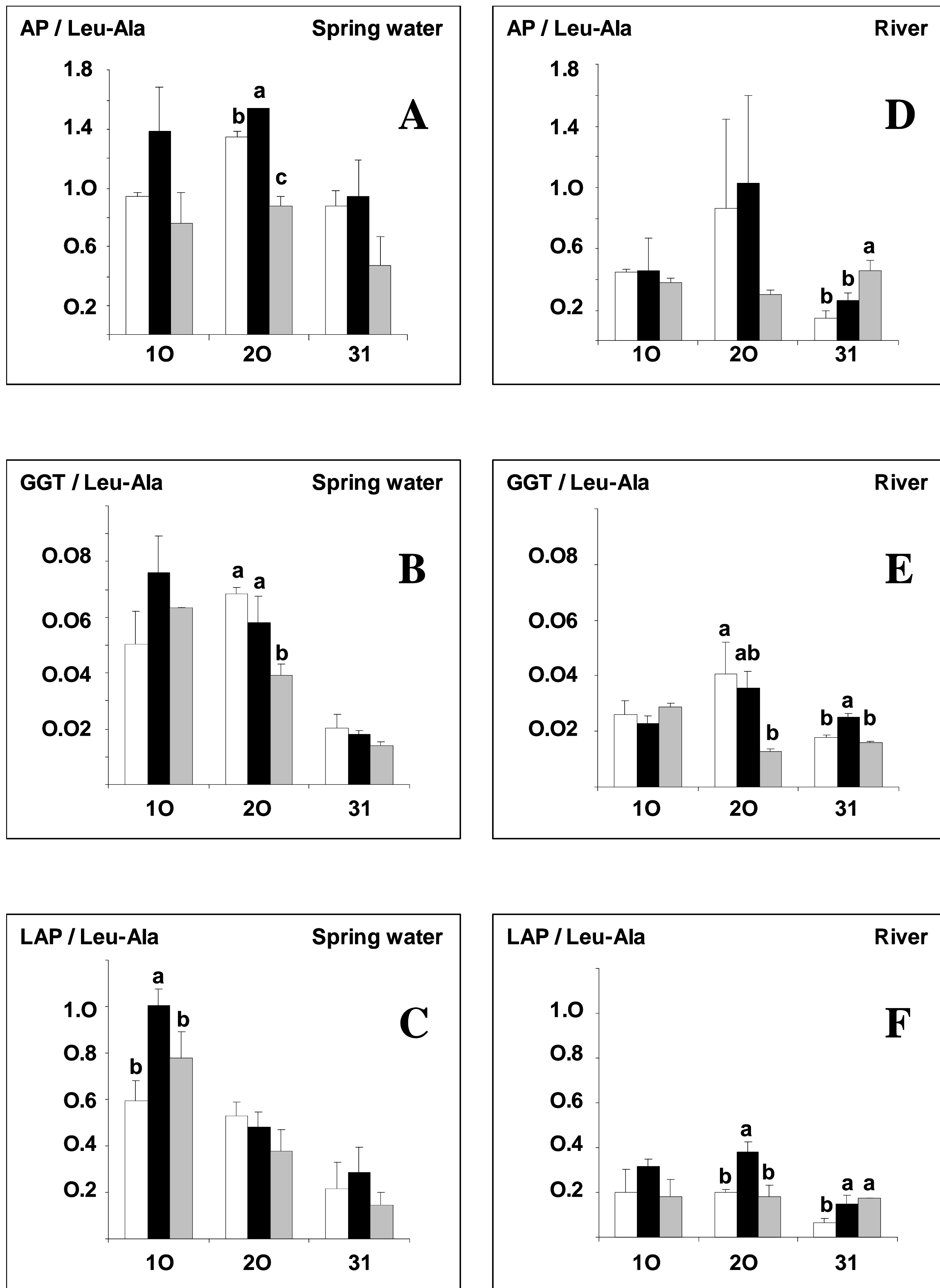

Fig. 4. Activity ratio to leucine-alanine peptidase of the three BBM enzymes in trout reared in spring water (left), or in river water (right) at 10, 20, and 31 dpsf; (A), (D): alkaline phosphatase; (B), (E): γ-glutamyl-transpeptidase; (C), (F): leucine-aminopeptidase N; the letters a, b and c above the error bars indicate the significant differences. white bars: control group, black bars: group SB, grey bars: group SC.

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ORIGINAL ARTICLE

Effect of short-term feeding duration of diets containing commercial whole-cell yeast or yeast subcomponents on immune function and disease resistance in channel catfish, *Ictalurus punctatus*

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Keywords
β-glucan, *Edwardsiella ictaluri*, enteric septicaemia, mannan oligosaccharide, *Saccharomyces cerevisiae*

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Summary

Juvenile channel catfish, *Ictalurus punctatus*, were fed diets supplemented with yeast or yeast subcomponents (YYS) as commercial preparations of *β*-glucan (MacroGard® and Betagard A®), mannan oligosaccharide (Bio-Mos® Aqua Grade), or whole-cell *Saccharomyces cerevisiae* (Levucell SB20®) at the manufacturers' recommended levels. Fish were fed experimental diets for 1 or 2 weeks prior to disease challenge (pre-challenge feeding periods) and sampled at the end of each feeding period to measure haematological and immune parameters and to determine the effects of dietary YYS on resistance to *Edwardsiella ictaluri*, the causative agent of enteric septicaemia disease (ESC). Feeding of experimental diets continued for 3 weeks post-challenge. In channel catfish fed diets supplemented with MacroGard®, Betagard A®, or Levucell SB20®, survival in the 1 week pre-challenge feeding group and antibody titres in the 2 week feeding group were significantly higher post-*E. ictaluri* challenge in relation to catfish fed with the control diet. In fish fed these same three diets, survival to ESC was significantly higher after 1 week vs. 2 weeks feeding, while the antibody response was significantly higher after 2 weeks vs. 1 week. Lysozyme activity was also higher in the 1 week feeding group, but the increased activity was unrelated to diet. Feeding YYS-supplemented diets for a shorter duration of 1 week prior to challenge may prove beneficial in increasing resistance to ESC in channel catfish. However, we cannot discount that feeding YYS diets during the recovery period may have contributed to 'glucan overload' and reduced survival in the 2 week feeding group.

Introduction

Enteric septicaemia (ESC) of channel catfish (*Ictalurus punctatus*), caused by *Edwardsiella ictaluri* (Hawke, 1979), is one of the leading causes of economic loss in the catfish industry and is responsible for approximately $60 million in lost revenue each year (Shoemaker et al., 2002). Increase in infectivity and mortality of channel catfish from ESC have largely been attributed to stress-related reductions in immune function (Klesius et al., 2003; Sink and Strange, 2004). Restriction of feeding, antibiotics and vaccination are currently used to varying degrees to control ESC. However, each of these approaches has its drawbacks, including cost (antibiotics and vaccination), antibiotic resistance, increased labour (vaccination) and lack of effectiveness and growth reduction (feed restriction). The addition of

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immunostimulants, such as β-glucan, to fish diets has been suggested as a cost-effective alternative for prophylactic treatment against disease outbreaks in intensive aquaculture (Sakai, 1999). Feeding channel catfish diets supplemented with immunostimulants prior to exposure to stressful practices or at times of increased disease susceptibility may prove effective in preventing infection from *E. ictaluri* and other pathogens.

Whole-cell yeast and yeast subcomponents (β-glucan and mannan oligosaccharide) (YYS) are often used as immunostimulants in diets of terrestrial and aquatic monogastric animals (Sohn et al., 2000). β-glucans are polysaccharides composed of glucose molecules linked by β-1,3 and β-1,6 or β-1,3 and β-1,4 bonds. β-glucans with β-1,3 and β-1,6 bonding are most commonly found in the cell walls of yeast and mycelial fungi (Verlhac et al., 1998). The health benefits of β-glucans have been studied in many different animals, and dietary β-glucans have been shown to stimulate cellular and humoral immune function and increase disease resistance in many species of fish (Sakai, 1999; Raa, 2000; Dalmo and Bøgwald, 2008). The supplementation of mannan oligosaccharides, a glucomannoprotein complex derived from yeast cell wall, in diets of fish has also shown promise in stimulating the immune response and increasing resistance to disease (Yoshida et al., 1995; Staykov et al., 2005; Peterson et al., 2010). Although there are many examples in the published literature suggesting dietary YYS increases immunity in fish, the results are often equivocal and vary considerably depending on experimental conditions (Raa, 2000).

Dietary YYS supplementation has shown some promise in improving channel catfish immunity, but the results have also been inconsistent. Chen and Ainsworth (1992) reported that catfish injected with yeast glucans showed increased resistance to *E. ictaluri*. Feeding diets supplemented with mannan oligosaccharide has improved resistance of catfish to ESC (Peterson et al., 2010). However, dietary supplementation of β-glucan (Ainsworth et al., 1994; Duncan and Klesius, 1996) or whole-cell yeast (Duncan and Klesius, 1996), while enhancing non-specific immune function, did not increase resistance of catfish to *E. ictaluri*. We previously examined the effect of YYS supplementation (β-glucan, mannan oligosaccharide and whole-cell yeast) in diets of juvenile channel catfish (Welker et al., 2007). Fish were fed experimental diets for 4 weeks followed by feeding the control, basal diet for a 2-week 'recovery' period (4 + 2). Several studies have suggested that feeding of the control/basal diet for 1 or 2 weeks should follow administration of immunostimulant diets for optimal effects on immune function enhancement and disease resistance in fish (Chen and Ainsworth, 1992; Bagni et al., 2000; Couso et al., 2003; Bridle et al., 2005). However, we did not observe any effect of YYS dietary supplementation on immune function or disease resistance in our study using this strategy. The absence of YYS-stimulated immunity may have resulted from a loss of immune cell stimulation during the recovery period or from 'glucan overload'. Elevated levels of YYS intake, either through high dietary concentrations, extended feeding (normally, >4 weeks), or both can cause an overload of glucan receptors on phagocytic cells reducing their ability to phagocytose bacteria in fish (Couso et al., 2003). Decreased immunity through elevated dietary intake of YYS has been reported in seabream (Ortuno et al., 2002; Couso et al., 2003), African catfish (Yoshida et al., 1995), Atlantic salmon (Robertsen et al., 1990) and rainbow trout (Jeney et al., 1998). In channel catfish, feeding YYS diets for extended periods may cause immunosuppression or the immunostimulatory effects may be lost, and shorter feeding duration may be needed to see YYS-stimulation of immunity.

Criticisms of classical frequentist tests of significance (e.g. ANOVA) and use of p-values in data analysis have increased in recent years due to their misinterpretation and inadequacy in addressing the question of whether results are important, valuable, or useful (practical significance) (Gurrin et al., 2000; Dixon, 2003; Kirk, 2007). Traditional p-values reflect only the probability of obtaining a particular pattern of results, or one more extreme, on the basis of a hypothesis (null) that is assumed to be true and therefore, do not sufficiently inform of the impact of treatments on measured variables (Gurrin et al., 2000; Lecoutre et al., 2001; Kirk, 2007). Numerous methods have been suggested to replace traditional frequentist tests of significance (e.g. confidence intervals and effect size). These methods are an improvement over traditional frequentist tests but are unduly affected by sample size, which can be problematic when sample sizes are small (Kirk, 2007). Therefore, a fiducial Bayesian statistical testing procedure (Rouanet, 1996) was conducted to determine the effect of YYS supplementation on immunity and disease resistance in this study. The fiducial Bayesian approach based on non-informative prior probabilities provides intuitive interpretations and extensions of familiar frequentist significance tests yet supplies probability distributions

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for the quantities of interest (in this case, standardized effect size), thereby allowing statements approximately the magnitude of effect and likelihood of occurrence to be made (Gurrin et al., 2000). The standardized effect size is a measure of the magnitude of observed effects, and the Bayesian posterior distribution constructed around each is used to draw inferences to find approximately how large or small the effect may be (Rouanet, 1996). Unlike frequentist measures of effect size, Bayesian methods, such as the one used herein, are not as severely affected by differences in sample size (Bayarri and Berger, 2004).

As glucan overload may have negatively affected immunity in our previous work (Welker et al., 2007), our objective was to examine the performance of the same YYS-supplemented diets for shorter feeding durations of 1 and 2 weeks prior to disease challenge in the current study. Sources of YYS were supplemented in diets at the manufacturer's recommended level for fish to determine their effects on immune function and survival following *E. ictaluri* infection in juvenile channel catfish. Feeding of experimental diets continued after fish were challenged. We hypothesized that the shorter feeding periods would lead to increased resistance of channel catfish to ESC not seen with longer YYS feeding durations.

Materials and methods

Experimental fish

Juvenile channel catfish (NWAC 103) raised from yolk-sac fry to juveniles on a commercial fry diet were acclimated to laboratory conditions and fed the basal experimental diet without YYS for approximately 4 weeks. At the end of the acclimation period, fish weight averaged 39.1 ± 1.5 g (mean ± SD; average of 125 fish). Thirty fish were randomly selected and stocked in each of forty 57-l aquaria. The total weight of fish in each aquarium was 1173 ± 9 g with approximately 1% variation in total weight between aquaria. The aquaria were supplied with flow-through, dechlorinated city water heated with a central water heater at an initial rate of 0.7 l/min. Water flow rates were checked and adjusted twice daily to ensure proper water exchange. The water was continuously aerated with compressed air diffused through air stones, and the photoperiod was maintained on a 12:12-h light/dark schedule. Dissolved oxygen and temperature in three randomly chosen aquaria were measured daily using an YSI model 58 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH, USA). During the trial, water temperature averaged 27.1 ± 0.1 °C (mean ± SEM), and dissolved oxygen averaged 4.5 ± 0.3 mg/ml. Care and handling of fish were in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the USDA, ARS Aquatic Animal Health Research Unit, Auburn, AL, USA and the 'Guidelines for the Use of Fishes in Research' of the American Fisheries Society, Bethesda, MD, USA (http://www.fisheries.org/afs/docs/policy_guidelines2004.pdf).

Table 1 Composition of basal diet

Ingredient	Per cent in diet
Menhaden fish meal	8.0
Soybean meal	45.0
Corn meal	25.0
Wheat middlings	14.0
Carboxymethyl cellulose	3.0
Menhaden oil	3.0
Dicalcium phosphate	1.0
Mineral premix*	0.5
Vitamin premix†	0.5

*Mineral premix (mg/kg diet unless otherwise stated): cobalt chloride hexahydrate, 0.2; zinc sulphate heptahydrate, 659.6; iron sulphate pentahydrate, 199.0; manganese sulphate monohydrate, 77.0; copper chloride, 4.7; potassium iodide, 6.5; and sodium selenite, 0.2.
†Vitamin premix (mg/kg diet unless otherwise stated): vitamin A-acetate, 4000 IU; vitamin D3, 2000 IU; vitamin K, 10; α-tocopherol acetate, 50; thiamin, 10; riboflavin, 12; pyridoxine, 10; pantothenic acid, 32; nicotinic acid, 80; folic acid, 5; biotin, 0.2; cyanocobalamine, 0.01; choline chloride, 400; L-ascorbyl acid-2-polyphosphate (15% vitamin C activity), 75.

Feed and feeding

A nutritionally complete, practical basal diet formulated to contain approximately 32% crude protein, 5.6% crude lipid, and 2877 kcal of digestible energy (DE)/kg based on feedstuff values reported in NRC (1993) (Table 1) was supplemented with commercially available YYS products[1], MacroGard® (Biotec-Mackzymal, Tromsø, Norway), Bio-Mos® Aqua Grade (Alltech, Nicholasville, KY, USA), Betagard A® (Aqua-In-Tech, Seattle, WA, USA), and Levucell SB20® *Saccharomyces cerevisiae* (Lallemand Animal Nutrition, Milwaukee, WI, USA), supplied by the manufacturers.[1] MacroGard®, and Betagard A® are

[1]Use of trade name or commercial products is solely for purpose of providing specific information and does not imply endorsement by the USDA.

polysaccharides of β-1,3/1,6-linked glucose molecules extracted from the cell wall of the yeast *S. cerevisiae*, while Bio-Mos® is a mannan oligosaccharide derived from *S. cerevisiae*. Levucell SB20® is comprised of live, whole-cell *S. cerevisiae*. Yeast or yeast subcomponents were supplemented in the basal diet at the expense of corn meal at the manufacturer recommended concentrations (MacroGard®, 1 g/kg; Bio-Mos® Aqua Grade, 2 g/kg; Betagard A®, 0.1 g/kg; Levucell SB20®, 0.1 g/kg). Each diet was mixed thoroughly and processed into 3-mm diameter pellets as described by Lim et al. (1996). Diets were dried at room temperature (23 °C) to a moisture content of approximately 10%, ground in a S-500 Disc Mill (Glen Mills, Glenmill, NJ, USA), and sieved with a No. 14 US standard sieve. The particles retained in the sieve were stored at −20 °C in sealed plastic bags until used. Determined crude protein content of the basal diet was 33.1 ± 0.3%. Two sets of 20 aquaria (each diet was randomly assigned to four aquaria) were used for the feeding trials. One set was used for the 1 week pre-challenge feeding regimen, and the second set was used for the 2 week pre-challenge feeding duration. Feeding of catfish under the 2 week regimen was initiated 1 week prior to the 1 week feeding duration so that the disease challenge for both groups was conducted simultaneously. Fish were fed to apparent satiation twice daily (between 07:30 and 08:30 hours and between 15:00 and 16:00 hours) for periods of 1 or 2 weeks, and feeding of experimental diets continued for 3 weeks after disease challenge (described below). Feed intake after challenge was only 1/4 of pre-challenge intake. Due to the short experimental feeding periods, growth parameters were not measured. At the end of the feeding trial, fish were sampled to measure haematological and immunological parameters. Thereafter, all YYS groups were tested for resistance *E. ictaluri* infection as outlined below. No feeding was done on disease challenge or sampling days. All the aquaria were cleaned thoroughly once every other week. On cleaning days, fish were fed only in the afternoon.

Haematological and immunological assays

After the termination of the feeding trial, blood was sampled from the caudal vasculature of three anaesthetized (100 mg/l tricaine methanesulfonate or MS-222; Argent Chemical Laboratories, Redmond, WA, USA) fish per aquarium with an air-dried, heparinized (500 U sodium heparinate/ml) tuberculin syringe. After blood samples were collected, fish were euthanized with 200 mg/l MS-222. Haematocrit of each fish was determined in duplicate using a microhaematocrit method (Brown, 1988). Red and white blood cell counts were performed in duplicate for each sample by diluting whole blood and counting in a Spencer Bright Line hemacytometer as described by Barros et al. (2002). Haemoglobin was analysed using a kit from Pointe Scientific (Canton, MI, USA), and values were adjusted by cyanomethaemoglobin correction factor for channel catfish described by Larsen (1964). The remaining whole blood was centrifuged at 1000 *g* for 10 min, and plasma was stored frozen at −80 °C for subsequent assays of bactericidal, lysozyme and spontaneous haemolytic complement (SH_{50}) activities. Plasma lysozyme activity was determined by the method of Litwack (1955) as modified by Sankaran and Gurnani (1972). Spontaneous haemolytic complement activity (SH_{50}) was calculated using the method reported by Sunyer and Tort (1995). Bactericidal activity was determined as described by Kampen et al. (2005) with modifications for plasma. Plasma total protein was determined by the bicinchoninic acid (BCA) method using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Detailed descriptions of the immune assay procedures are given in Welker et al. (2007). At the end of the disease challenge trial (day 22), blood was sampled from the caudal vasculature of four surviving fish in each aquarium by syringe and centrifuged at 1000 *g*. Plasma was collected and stored frozen at −80 °C for subsequent determination of post-challenge agglutinating antibody titre against *E. ictaluri*. Necropsies were performed and anterior kidney tissue from dead fish was cultured to confirm death due to *E. ictaluri* (Klesius, 1992). Plasma collected at the termination of the feeding trials for use in immune assays (n = 3 fish per aquarium) was used to determine pre-challenge antibody titres – all sampled fish were negative for *E. ictaluri*. Agglutinating antibody titre against *E. ictaluri* (AL-93-75) in pre- (from immune assay blood sampling) and post-challenge plasma was determined by modifying the method of Chen and Light (1994). See Welker et al. (2007) for a more complete description of the antibody titre assay.

Disease challenge

The *Edwardsiella ictaluri* (AL-93-75) from an outbreak of ESC was used to challenge channel catfish by immersion according to the procedure outlined in Welker et al. (2007). The number of channel catfish

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remaining in the original aquaria at the end of the 1 and 2 week feeding trials was adjusted to 20. All fish were removed from an aquarium, anaesthetized, and 20 fish were randomly netted and returned. Fish were allowed 3 days to acclimate and were then challenged in aquaria by addition of the *E. ictaluri* culture at a rate to produce approximately 1×10^7 CFU/ml. The final challenge concentration was 9.5×10^6 CFU/ml, determined using a spiral autoplater and Qcount (Spiral Biotech, Norwood, MA, USA) after challenge. As stated above, the start of the 1 and 2 week pre-challenge feeding periods were staggered by 1 week so that they ended at the same time allowing both groups to be challenged on the same day using the same challenge culture. Fish continued to be fed twice daily with the experimental diets after challenge, and mortality was recorded twice a day for 21 days.

Statistical analysis

Source of YYS (MacroGard®, Bio-Mos® Aqua Grade, Betagard A®, Levucell SB20® and control diets) and pre-challenge feeding duration (1 or 2 weeks) were fixed effects in this study. Bayesian statistical testing procedures (Rouanet, 1996) were conducted to determine the effect of YYS immunostimulants and feeding duration (main effects) on measured haematological and immunological parameters and susceptibility to *E. ictaluri* infection. A Bayesian posterior probability distribution ($|\delta|/s_{obs}$) was calculated for the standardized effect size (d/s) of each main effect using LePAC software, which is freely distributed by the Equipe Raisonnement Induction Statistique (ERIS) research group at the Centre National de la Recherche Scientifique, University de Rouen, Normandy, France. A credibility level γ ($\geq$0.90) (non-informative prior probability) and a limit to determine largeness $l_{lar} \geq 0.60$ or smallness $l_{small} < 0.40$ are declared to assert magnitude of effect – if the posterior probabilities calculated for d/s exceed the credibility level of 0.90 based on these limits, largeness or smallness is declared respectively. If neither scenario is true, the effect would be medium or inconclusive. Cohen's effect size limits for large and small effects were used (Cohen, 1988). To determine differences between individual treatment means, the Waller–Duncan k-ratio *t*-test (WD) (Waller and Duncan, 1969), which controls the Type I and Type II error rates based on Bayesian principles and minimizes the Bayes risk under additive loss assumptions (Steel and Torrie, 1980), was conducted using SAS (Statistical Analysis Software) (SAS, Cary, NC, USA). If an interaction effect was significant ($|\delta|/s_{obs} > 0.90$), then each factor was analysed within each level of the other factor using the SLICE option of the LS Means and orthogonal contrasts (simple effects analysis) in SAS, and differences between treatment means are shown in figures rather than tables. Traditional ANOVA procedures (two-way analysis of variance) were also conducted, and the results are reported for reference and comparison. Kaplan and Meier survival curves (Kaplan and Meier, 1958) were constructed for each diet × feeding duration treatment for 1 and 2 week feeding durations. Because there was a significant interaction between diet and feeding duration, the curves for diets were analysed separately for each feeding duration. The curves were compared using the log-rank test to determine differences between curves and whether the trends in survival were different between curves. Differences between individual survival curves for diet were determined by Holm-Sidak method. A significance level of $\alpha = 0.05$ was used for frequentist statistical analyses. Measured values from individual fish were averaged for each aquarium (experimental unit) for use in statistical analyses.

Results

Haematological parameters tended to be somewhat lower for the control group in relation to other dietary groups and for fish fed the experimental diets for 1 week compared to 2 weeks (Table 2). Although F-test p-values for the effect of feeding duration on total, red and white blood cell counts were <0.05, the Bayesian posterior probabilities suggest that the effects of diet and feeding duration were only small or medium on blood cell counts and other haematological parameters. The effects of diet and feeding on immune responses (SH_{50}, lysozyme, bactericidal activity and total protein) were medium or inconclusive (Bayesian analysis); however, the F-test suggests that lysozyme activity was higher in catfish fed experimental diets for 1 week pre-challenge (Table 3).

The survival curve analysis suggests that differences exist between diets ($p < 0.004$) for the 1 week pre-challenge feeding duration (Fig. 1), with the control fed fish exhibiting a survival curve significantly lower than those fed the MacroGard® or Betagard A® supplemented diets. A significant interaction existed between feeding duration and diet, and further analysis of variance components revealed that survival of fish fed Levucell® for 1 week prior to challenge was also significantly greater than in the control group (explained further

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Table 2 Bayesian and frequentist statistical analysis for channel catfish haematology. Values given for diet and time are mean ± SD*

	Haematocrit (%)	Haemoglobin (g/dl)	TCC ($10^6/\mu l$)	RBC ($10^6/\mu l$)	WBC ($10^5/\mu l$)
Pre-challenge† feeding duration					
1 week	38.8 ± 2.2	7.2 ± 1.3	3.10 ± 0.60	2.44 ± 0.43	6.58 ± 0.24
2 weeks	39.6 ± 1.8	7.7 ± 0.6	4.01 ± 0.62	3.14 ± 0.46	8.70 ± 0.34
Bayesian					
|d/s|	2.4×10^{-4}	0.34	1.14	1.16	0.77
Pr(d/s_{obs} > 0.6)	0.003	0.13	0.89	0.85	0.75
Pr(d/s_{obs} < 0.4)	0.93	0.61	0.003	0.002	0.07
Effect	Small	Medium	Medium	Medium	Medium
Frequentist					
F-value	1.24×10^{-6}	2.332	25.94	27.03	11.88
p-value	0.99	0.14	<0.001	<0.001	0.002
Diet					
Control	36.9 ± 2.0	7.0 ± 1.3	3.22 ± 0.67	2.51 ± 0.44	7.16 ± 0.26
Macrogard®	37.9 ± 3.2	7.7 ± 0.5	3.84 ± 0.80	3.02 ± 0.60	8.26 ± 0.34
Beta-gard®	40.9 ± 1.3	7.7 ± 1.3	3.80 ± 0.72	3.04 ± 0.61	7.60 ± 0.55
BioMos®	39.0 ± 2.4	7.6 ± 1.0	3.43 ± 0.77	2.65 ± 0.60	7.86 ± 0.27
Levucell®	39.5 ± 1.1	7.1 ± 1.2	3.63 ± 1.12	2.89 ± 0.76	7.35 ± 0.40
Total	38.8 ± 2.4	7.4 ± 1.1	3.58 ± 0.84	2.83 ± 0.62	7.64 ± 0.37
Bayesian					
|d/s|	0.66	0.28	0.45	0.52	0.28
Pr(d/s_{obs} > 0.6)	0.74	0.03	0.37	0.50	0.12
Pr(d/s_{obs} < 0.4)	<0.001	0.81	<0.001	<0.001	0.65
Effect	Medium	Medium	Medium	Medium	Medium
Frequentist					
F-value	3.438	0.644	1.606	2.151	0.611
p-value	0.02	0.64	0.20	0.099	0.66
Interaction					
Bayesian					
|d/s|	0.004	0.41	0.29	0.26	0.16
Pr(d/s_{obs} > 0.6)	0.025	0.51	0.37	0.34	0.002
Pr(d/s_{obs} < 0.4)	0.89	<0.001	<0.001	<0.001	0.99
Effect	Medium	Medium	Medium	Medium	Small
Frequentist					
F-value	7.81×10^{-5}	0.68	0.331	0.261	0.967
p-value	1.00	0.61	0.86	0.90	0.44

TCC, total cell count; RBC, red blood cell count; WBC, white blood cell count.

*The average of three fish sampled from four tanks per diet (experimental unit) for a total of 20 aquaria at each feeding duration were used to calculate treatment means and used in statistical analyses.

†Catfish were fed experimental diets for 1 or 2 weeks prior to challenge with *Edwardsiella ictaluri*. Feeding of diets continued for 21 days after challenge.

below). The log-rank test for trends indicates that no evidence of trends exists between the diet curves for the 2 week feeding duration (p = 0.32). Fish fed the Levucell SB20® diet for 2 weeks prior to challenge had a higher rate of survival initially until 10–11 days post-challenge when the survival rate decreased markedly, giving the treatment one of the lowest overall survival rates (Fig. 2).

Bayesian posterior probabilities show that feeding duration had a large or significant effect on survival of channel catfish to *E. ictaluri* infection with catfish fed the experimental diets for 1 week prior to challenge having higher survival than those receiving the diets for 2 weeks pre-challenge (Table 3). The effect of diet on post-challenge survival was medium or inconclusive; however, there was a significant interaction between feeding duration and diet. The interaction was further analysed by simple effects testing, which indicated that survival of channel catfish fed the Beta-gard A®, Levucell SB20®, and MacroGard® diets was significantly lower after 2 weeks pre-challenge feeding in relation to the 1 week feeding duration (Fig. 3). There was no difference in survival between 1 and 2 week pre-challenge feeding durations when fish

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Table 3 Bayesian and frequentist statistical analysis for immune function and survival to enteric septicaemia infection*. Values given for diet and time are mean ± SD†

	SH_{50}‡ (units/ml)	Lysozyme (μg/ml)	Ab titre§ (log 10)	BA¶	Protein (mg/ml)	Survival (%)**
Pre-challenge†† feeding duration						
1 week	12.7 ± 3.2	5.9 ± 0.9	2.47 ± 0.24	0.161 ± 0.019	30.5 ± 3.0	56.7 ± 9.8
2 weeks	12.9 ± 7.3	5.2 ± 0.6	2.96 ± 0.27	0.150 ± 0.028	30.4 ± 2.3	46.4 ± 7.5
Bayesian						
|d/s|	0.03	0.63	1.90	0.25	0.02	0.91
$Pr(d/s_{obs} > 0.6)$	0.005	0.55	0.99	0.20	0.004	0.91
$Pr(d/s_{obs} < 0.4)$	0.92	0.02	<0.001	0.57	0.93	0.02
Effect	Small	Medium	Large	Medium	Small	Large
Frequentist						
F-value	0.014	8.01	72.12	1.29	0.01	15.36
p-value	0.91	0.008	<0.001	0.27	0.94	<0.001
Diet						
Control	14.6 ± 8.4	5.8 ± 0.9	2.41 ± 0.29	0.165 ± 0.021	29.6 ± 2.3	42.2 ± 6.6
Macrogard®	13.3 ± 7.4	5.9 ± 1.0	2.81 ± 0.20	0.146 ± 0.022	32.3 ± 4.1	55.5 ± 10.1
Beta-gard®	11.5 ± 4.3	5.2 ± 0.7	2.80 ± 0.31	0.146 ± 0.032	31.6 ± 2.9	51.7 ± 8.0
BioMos®	11.1 ± 2.6	5.4 ± 0.9	2.78 ± 0.26	0.159 ± 0.042	29.3 ± 2.4	55.5 ± 8.8
Levucell®	13.4 ± 5.6	5.6 ± 0.7	2.77 ± 0.23	0.163 ± 0.031	29.4 ± 2.2	50.9 ± 7.5
Total	12.8 ± 5.9	5.6 ± 0.8	2.71 ± 0.25	0.156 ± 0.030	30.4 ± 3.0	51.1 ± 8.2
Bayesian						
|d/s|	0.24	0.37	0.23	0.29	0.49	0.72
$Pr(d/s_{obs} > 0.6)$	0.09	0.24	0.61	0.14	0.44	0.86
$Pr(d/s_{obs} < 0.4)$	0.82	<0.001	0.30	0.52	<0.001	<0.001
Effect	Medium	Medium	Medium	Medium	Medium	Medium
Frequentist						
F-value	0.46	1.10	3.451	0.69	1.90	4.178
p-value	0.77	0.37	0.05	0.61	0.14	0.06
Interaction						
Bayesian						
|d/s|	0.49	0.21	1.44	0.34	0.50	0.82
$Pr(d/s_{obs} > 0.6)$	0.58	0.30	0.95	0.43	0.62	0.92
$Pr(d/s_{obs} < 0.4)$	<0.001	<0.001	<0.001	0.22	<0.001	<0.001
Effect	Medium	Medium	Large	Medium	Medium	Large
Frequentist						
F-value	0.993	0.171	4.306	0.466	1.004	2.72
p-value	0.45	0.95	0.01	0.76	0.42	0.05

*Survival and antibody titre were measured 21 days post-immersion challenge.
†The average of three fish (four for post-challenge antibody titres) sampled from four tanks per diet (experimental unit) for a total of 20 tanks at each feeding duration were used to calculate treatment means and used in statistical analyses.
‡Spontaneous haemolytic complement activity. Expressed as the dilution that produced 50% lysis of sheep red blood cells.
§Agglutination antibody titre (log 10) to *Edwardsiella ictaluri*.
¶Plasma BA activity reported as absorbance units.
**Percent cumulative survival after challenge with *E. ictaluri*.
††Catfish were fed experimental diets for 1 or 2 weeks prior to challenge with *E. ictaluri*. Feeding of diets continued for 21 days after challenge.

were fed the control or Bio-Mos® diets. Among the 1 week feeding duration group, the Betagard A®, MacroGard® and Levucell SB20® but not Bio-Mos® diets produced significantly higher survival in relation to the control diet, although survival of fish fed the Bio-Mos® supplemented diets remained high after the 2 week feeding duration trial (Fig. 3). No differences in survival were seen among diets after 2 weeks of pre-challenge feeding.

The effect of feeding duration on agglutination antibody titres against *E. ictaluri* was large but inversely related to cumulative survival – titres were significantly higher for the 2 week pre-challenge feeding duration in relation to the 1 week feeding trial (Table 3). Diet had only a medium (inconclusive) effect on the antibody response, but the Bayesian posterior probability distribution suggests that there was a significant interaction between feeding

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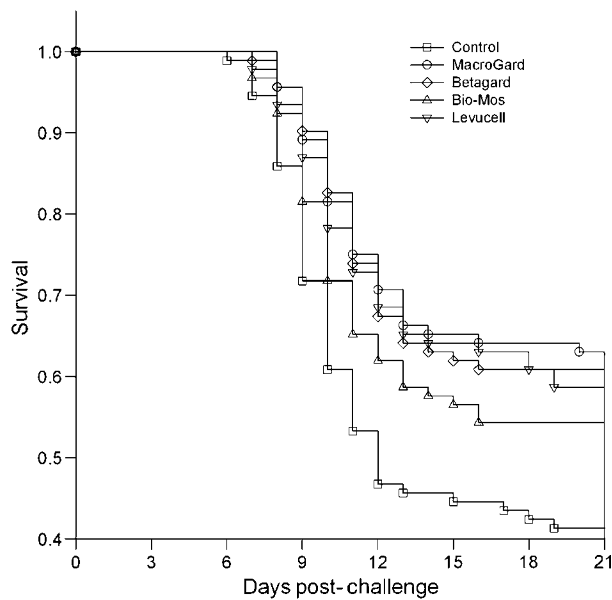

Fig. 1 Kaplan and Meier survival curves for diets fed for 1 week pre-challenge. The curves were compared using the log-rank test to determine differences between curves and whether trends in survival were different between curves. The log-rank test indicates that evidence of trends exists between the curves ($p < 0.05$). At total of 20 fish per tank (experimental unit) were challenged with *Edwardsiella ictaluri* by immersion. The average of four tanks per diet (experimental unit) was used to calculate cumulative survival (%) for treatments after 1 week of feeding.

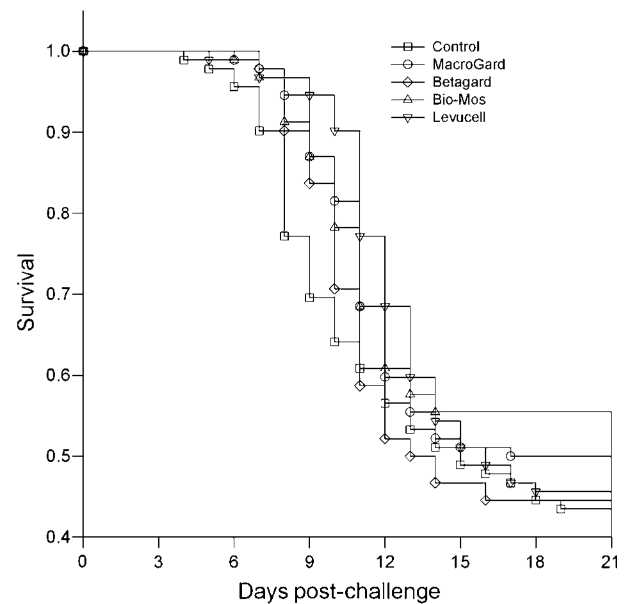

Fig. 2 Kaplan and Meier survival curves for each diet fed for 2 weeks pre-challenge. The curves were compared using the log-rank test to determine differences between curves and whether trends in survival were different between curves. The log-rank test indicates that no evidence of trends exists between the curves ($p > 0.05$). At total of 20 fish per tank (experimental unit) were challenged with *Edwardsiella ictaluri* by immersion. The average of four tanks per diet (experimental unit) was used to calculate cumulative survival (%) for treatments after 2 weeks of feeding.

duration and diet (Table 2). Tests of simple effects showed that channel catfish fed diets supplemented with Betagard A®, Levucell SB20®, and MacroGard® had significantly higher antibody responses after 2 weeks pre-challenge feeding in relation to only 1 week feeding of experimental diets (Fig. 4), which were the same diets that showed improved survival to *E. ictaluri* infection when fed for 1 week. No differences in antibody response were observed in channel catfish fed the Bio-Mos® or control diets for 1 or 2 weeks prior to challenge. A further breakdown of simple effects testing showed that in the 2 week pre-challenge feeding group, fish fed the Betagard A®, Levucell SB20®, and MacroGard® diets had significantly higher antibody titres in relation to the control but not Bio-Mos® diet (Fig. 4). No differences were seen among the diets after 1 week of pre-challenge feeding.

Discussion

Previously, we examined the effect of YYS supplementation in the diets of juvenile channel catfish on immune function and disease resistance (Welker et al., 2007). Fish were fed experimental diets containing commercial YYS products at the manufacturers' recommended concentrations for 4 weeks followed by a 2 week recovery feeding period of the basal diet (4 + 2). No differences in final post-challenge survival (positive or negative) or in immune function parameters among diets were observed. Much of the rationale behind the use of a recovery period in fish appears to have originated with Chen and Ainsworth (1992), which showed positive effects on immune function after β-glucan injection in channel catfish, but little corroborative follow-up research has occurred comparing effects of YYS administration with and without recovery periods on immunity. This practice was extended to dietary studies, where glucan supplementation was followed by a recovery period of basal diet feeding (typically 1–3 weeks) in a variety of fish species (e.g., Bagni et al., 2000; Couso et al., 2003; Bridle et al., 2005) with mixed results. It is hypothesized that a recovery period is needed to prevent 'glucan overload', a phenomenon that occurs with excessive doses of YYS, either through extended feeding duration (Sakai, 1999), high dietary concentrations, or both (Couso

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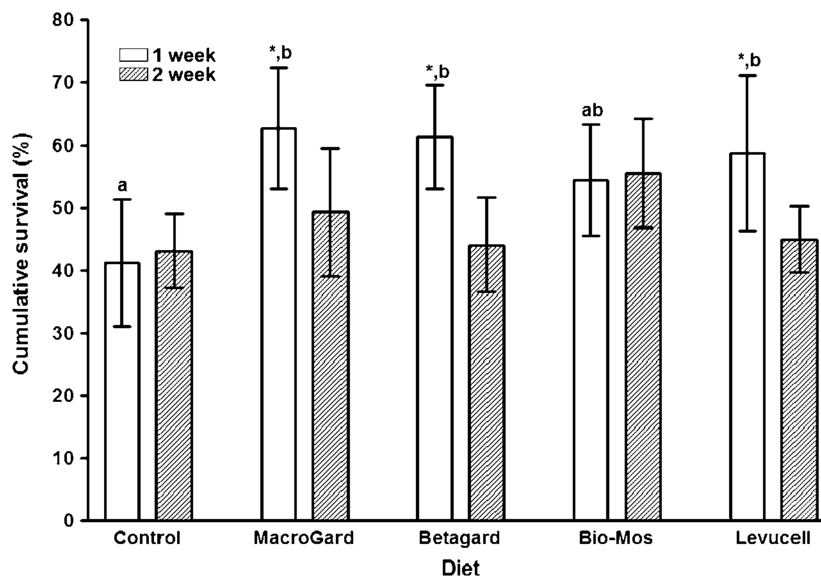

Fig. 3 Mean percent cumulative survival (±SD) 21 days after infection with enteric septicaemia (ESC) in channel catfish fed yeast or yeast subcomponents (YYS)-supplemented diets for 1 or 2 weeks prior to challenge. Bars with different letters are significantly different from one another. Survival was significantly different for 1 and 2 week feeding groups for the same diet when labelled with an asterisk. At total of 20 fish per tank (experimental unit) were challenged with *Edwardsiella ictaluri* by immersion. The average of four tanks per diet (experimental unit) at each feeding duration was used to calculate mean cumulative survival (%).

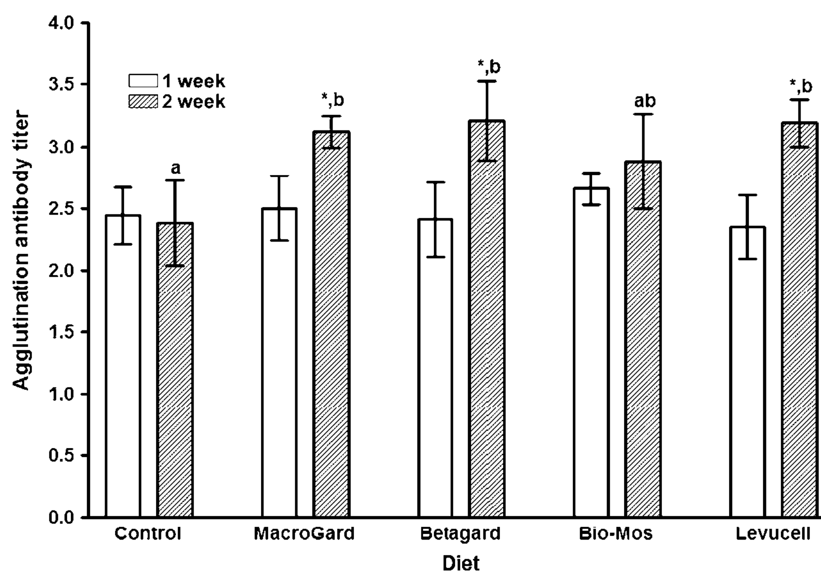

Fig. 4 Mean agglutination antibody titres (log 10) to *Edwardsiella ictaluri* (±SD) 21 days after infection with enteric septicaemia (ESC) in channel catfish fed yeast or yeast subcomponents (YYS)-supplemented diets for 1 or 2 weeks prior to challenge. Bars with different letters are significantly different from one another. Antibody titre was significantly different for 1 and 2 week feeding groups for the same diet when labelled with an asterisk. Four fish per tank (experimental unit) were sampled and averaged for antibody titres. The average of four tanks per diet (experimental unit) at each feeding duration was used to calculate mean antibody titres and in statistical analysis.

et al., 2003), which can negatively affect immunity in fish by suppressing the immune response. Based on our previous work, it would be reasonable to conclude that YYS supplementation is ineffective in positively affecting immunity in channel catfish. However, other studies not utilizing a recovery period suggest otherwise (Duncan and Klesius, 1996; Peterson et al., 2010). If we then assume that YYS supplementation in diets positively affects immunity in channel catfish and that the manufacturers' supplementation recommendations are correct, then the immunostimulatory response did not coincide with immune sampling or disease challenge under our 4 + 2 feeding regimen. The recovery period may also have been unnecessary and the immunostimulatory response was lost, or the fish may have experienced glucan overload, even with the recovery period, due to the 4 week feeding duration. To avoid the glucan overload possibility, we examined the effects of reduced feeding durations of 1 and 2 weeks prior to disease challenge without a recovery period in this study using the same YYS products supplemented at their respective manufacturer's dietary recommendations as in our previous work.

In the present study, the shorter pre-challenge feeding duration of 1 week but not 2 weeks appeared to increase resistance of channel catfish to ESC in this study. Catfish fed diets supplemented

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with MacroGard®, Betagard A®, or Levucell SB20® for 1 week had significantly higher survival to ESC infection in relation to the control diet but were not different from each other. In fish fed these same three diets, survival to ESC was significantly higher after 1 week vs. 2 weeks feeding. It appears that 2 weeks of pre-challenge YYS feeding at the recommended dietary levels was too long to be effective in stimulating immunity in channel catfish. Catfish were also fed the supplemented diets after disease challenge, and although feed intake was approximately 1/4 of intake prior to challenge, this continued feeding may have contributed to glucan overload and decreased disease resistance in the 2-week pre-challenge feeding group. Even though catfish in both feeding regimens were challenged on the same day utilizing the same bacterial culture, ESC-related mortality was first observed after four - days and six days in control groups for the 1 and 2 week pre-challenge feeding groups respectively. One aquarium in the 2 week feeding group exhibited mortalities two days post-challenge, which largely accounts for the lower average in this group. In the other three aquaria, the average time before the first mortality was observed was five and a half days, which was similar to the average for the 1 week pre-challenge feeding group.

As mentioned, YYS supplementation with a 4 + 2 feeding strategy did not influence immune function or susceptibility to *E. ictaluri* infection (Welker et al., 2007). Survival of catfish utilizing the 4 + 2 feeding regimen was 20–30% higher with the 4 + 2 feeding. Even though post-challenge survival was lowest for control groups regardless of feeding strategy, survival of catfish fed the control diet in the 4 + 2 regimen was nearly double that of the 1 and 2 week feeding duration controls in the current study. All experimental variables were generally equal between studies. The same YYS products were used at the same dietary concentrations in both studies, and fish size at the time of challenge in the 4 + 2 feeding regimen was approximately 35 g and 40 g in the present study. Water quality parameters were nearly identical (data not shown). However, even though the challenge concentrations of *E. ictaluri* were similar (9.1×10^6 CFU/ml for the 4 + 2 regimen and 9.5×10^6 CFU/ml for the present study), the challenge culture for the current study was 4×10^5 CFU/ml higher than in our previous work, which may have accounted for the lower overall survival.

Comparison of the current study and Welker et al. (2007) indicates that use of recovery feeding may prove beneficial to disease resistance in catfish after a prolonged feeding period of β-glucan depending on the concentration in the diet. In catfish fed the β-glucan diets (MacroGard® or Betagard A®), survival was highest after 1 week dietary supplementation and declined after 2 weeks pre-challenge feeding. Under the 4 + 2 feeding regimen in our previous work, survival was highest for catfish fed the Betagard A® diet, but the MacroGard® fed fish had lower survival, second only to the control group. The MacroGard® product was supplemented at a rate 10× higher (1.0 g/kg diet) than the Betagard A® product (0.1 g/kg). These results suggest feeding glucan products for 14 days or longer prior to challenge may cause a reduction in disease resistance, even at moderate dietary supplementation; however, the use of a recovery feeding period may be beneficial when β-glucan is included in the diet at the moderate level tested, but excessive dietary concentrations may not respond to recovery feeding. Fish fed the Levucell SB20® whole-cell yeast product had post-challenge survival similar to Betagard A® in catfish fed for the 1 week pre-challenge feeding duration and in our previous study. Yeast (*S. cerevisiae*) cell walls are approximately 70% β-glucan (Northcote and Horne, 1952), and therefore, the Levucell SB20® diet contains approximately 0.07 g/kg diet, similar to the Betagard A® supplementation levels, though presumably less β-glucan would be available due to losses during digestion and incomplete breakdown of the yeast cell wall. The level of β-glucan provided by the Levucell SB20® diet would certainly be closer to the Betagard A® diet than to the MacroGard® diet and may account for their similarity in survival values. This comparison between the two studies should be viewed cautiously, because they were conducted at different times, albeit under very similar conditions. It should be used as a basis for future study into the effects of glucan feeding concentration and duration in channel catfish diets.

Supplementation of Bio-Mos® did not increase channel catfish resistance to ESC after 1 week pre-challenge feeding as was observed with the other YYS supplemented diets. Fish fed the Bio-Mos® diet had similar survival after 1 and 2 weeks of pre-challenge feeding, but survival was much lower for Bio-Mos® fed fish under the 4 + 2 regimen (Welker et al., 2007). The mode of action for mannan oligosaccharides, such as that found in Bio-Mos®, is to block colonization by binding to pathogenic bacteria and rendering them unable to attach to intestinal mucosa (McCann et al., 2006). Peterson et al. (2010) showed that increased resistance of channel catfish

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to ESC occurs after feeding diets supplemented with Bio-Mos® for 4 weeks. A feeding period greater than the 2 weeks used in the present research may be needed to see increased benefits to disease resistance in channel catfish. Furthermore, we likely did not see improved disease resistance with the 4 + 2 regimen, because continual feeding of fish is recommended for Bio-Mos® supplemented diets (Peterson et al., 2010).

Even though survival to ESC infection was positively affected by short-term pre-challenge YYS feeding, we did not see any effect of diet on non-specific immune function in the present study or in our previous work (Welker et al., 2007). Addition of glucans to channel catfish diets has been shown to increase non-specific immune function. Duncan and Klesius (1996) showed that catfish fed 0.2% β-glucan but not 2.7% whole-cell *S. cerevisiae* had increased macrophage and neutrophil migration and phagocytosis. However, we did not measure these immune responses in the current study. Lysozyme activity was significantly higher (supported by ANOVA) in channel catfish fed under the 1 week but not 2 week pre-challenge feeding regimen, but the increased activity was unrelated to diet. This result was not supported by the Bayesian analysis. It is possible that this increase contributed to the higher survival in fish fed experimental diets 1 week prior to challenge, but it does not explain the differences in survival related to diet during this period. Significant increases in post-challenge antibody titres against *E. ictaluri* occurred after 2 weeks but not 1 week of YYS pre-challenge feeding in the present study, which were inversely related to survival after challenge. Typically, an antibody response in channel catfish is detected approximately 5–7 days post-exposure to *E. ictaluri* by immersion challenge, and a significant response is detected between 14 and 21 days post-challenge (Klesius and Sealey, 1995). Although the antibody titres measured at the termination of the disease challenge were lower for fish fed YYS for 1 week, it is possible that the differences in survival between the 1 and 2 week feeding durations might have been affected by the antibody response, because we only measured the antibody response 21 days post-challenge and do not know what the antibody response dynamics were from the time just after challenge up to 21 days. The effect of β-glucans on immunity can vary considerably even within the same species, and as shown, increased antibody response (Ainsworth et al., 1994) or non-specific immune response (Duncan and Klesius, 1996) in channel catfish often does not predict resistance to ESC, which is also what we observed in the present study.

Fiducial Bayesian analysis was used to analyse these data, because it offers advantages over traditional frequentist (here, ANOVA) methods. Bayesian probabilities, unlike their frequentist counterparts, provide an indication of magnitude of difference or practical significance (Wainer and Robinson, 2003). The ANOVA results were provided in this manuscript for comparison, and departures from Bayesian results were noted. The results of the fiducial Bayesian and frequentist (ANOVA) analyses in this study generally agreed in most cases, especially when the Bayesian posterior probability indicated a large (significant) or small (insignificant) effect. Some disagreement was seen when the outcome of the Bayesian analysis was medium (inconclusive) approximately 25% of the time, in these cases, the p-value was often <0.05 or 'significant' in frequentist terms.

While we may be approaching a better understanding of the effects of dietary YYS on immunity of channel catfish and many other species of fish, considerable variability in disease resistance exists with regard to type, concentration, and feeding duration of YYS, and fish age, size and species. There appears to be a balance between feeding duration and dietary YYS concentration that must be met in order to achieve increased immunity and disease resistance. For non-mannan oligosaccharide YYS immunostimulants, 1 week feeding prior to disease challenge appears to enhance disease resistance at manufacturer recommended levels, but extended feeding does not seem to be beneficial in channel catfish. Use of a recovery period may improve survival to infection after prolonged feeding, but fish fed diets with higher levels of supplementation of β-glucan may not see any benefit. Bio-Mos® mannan oligosaccharide supplemented diets may require longer feeding periods for enhancement of disease resistance, but use of recovery feeding periods will likely reduce these benefits. The variability in the effectiveness of YYS in increasing immunity in fish suggests more research is needed and that effective YYS feeding regimens may be specific to fish species and culture conditions.

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ORIGINAL PAPER

Effects of dietary dried baker's yeast on the performance, egg traits and blood parameters in laying quails

Sakine Yalçın · Fatma Oğuz · Berrin Güçlü · Suzan Yalçın

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Abstract This study was conducted to investigate the effects of dietary dried baker's yeast on laying performance, egg traits and some blood parameters of quails. In the experiment a total of 342 Japanese quails (*Coturnix coturnix japonica*) aged ten weeks were equally divided into six groups of 57 (three replicates of 19 quails each). Six levels (0, 4, 8, 12, 16 and 20%) of dried baker's yeast were included in isonitrogenous and isocaloric diets. The experimental period lasted 14 weeks. At the end of the experiment, there were no significant differences among the groups in body weight, feed intake, protein intake, egg production, feed efficiency, egg yolk index and egg haugh unit. Blood serum levels of total protein, triglyceride and cholesterol were not affected by dietary dried baker's yeast. Diets containing 4 and 8% of dried baker's yeast increased the egg weight significantly ($p<0.01$). The inclusion of dried baker's yeast at the level of 20% to the diets reduced egg shell thickness and egg albumen height. It is concluded that dried baker's yeast can be used up to 16% in the diets of laying quails without adverse effects on the measured parameters.

Keywords Baker's yeast · Blood parameters · Egg traits · Laying quail · Performance

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Introduction

The term 'single-cell proteins' refers to the dried cells of microorganisms such as algae, bacteria, yeasts, molds and higher fungi grown in large-scale culture systems for use as protein sources in foods or feeds. The first applicable single cell protein production originated in Germany during World War I when baker's yeast, *Saccharomyces cerevisiae*, was grown – with molasses as the carbon and energy source and ammonium salts as the nitrogen source – for consumption as a protein supplement (Litchfield 1983).

Yeast protein is produced by culturing different microorganisms of yeast on different substrates such as saccharide (i.e., molasses, whey) and polysaccharide

(i.e., starch, cellulose) substances, hydrocarbons and alcohols. Initially, extensive efforts were directed toward manufacturing yeast for human food, but then researchs have been directed towards using it as a protein supplement in animal feeds due to acute shortages of both soybean and fish meal in many countries (Daghir and Abdul-Baki 1977; Önol and Yalçın 1995; Şehu et al. 1997).

Yeast obtained as an agricultural byproduct or produced industrially was utilized in poultry diets as a protein source but its level of inclusion in practical diets varied widely from 5 to 30% in different studies (Waldroup et al. 1971; Önol and Yalçın 1995; Şehu et al. 1997). Some studies were carried out on yeast for broilers (Yalçın et al. 1993), quails up to 35 days of age (Şehu et al. 1997) and laying hens (Shyam Sunder et al. 1990; Önol and Yalçın 1995) but there are no published reports on the feeding value of baker's yeast for laying quails as we know. Therefore the aim of this study was to determine the effects of dried baker's yeast on laying performance, egg traits and some blood parameters of quails. Measuring blood parameters could be more useful to detect some metabolic effects of dried baker's yeast.

Materials and methods

Animals and diets

A total of 342 Japanese quails (*Coturnix coturnix japonica*) aged ten weeks were housed in cages and randomly allocated to six treatment groups each containing 57 quails. Each group was divided into 3 replicates.

Feed and water were provided *ad libitum* and the diets were offered in mash form. A photoperiod of 17 h light was maintained. The experiment was conducted for 14 weeks.

The composition of the basal diet is given in Table 1. The diets were formulated to be isocaloric and isonitrogenous. The diets were supplemented with 0,

Table 1 Ingredient and chemical composition of the diets

Ingredient (%)	Dried baker's yeast supplementation (%)					
	0	4	8	12	16	20
Maize	57.80	58.50	59.20	60.00	60.70	61.50
Soyabean meal	20.00	16.00	12.00	8.00	4.00	0.00
Full fat soyabean	8.00	7.50	7.00	6.50	6.00	5.40
Dried baker's yeast	0.00	4.00	8.00	12.00	16.00	20.00
Meat and bone meal	4.20	4.20	4.20	4.20	4.20	4.20
Vegetable fat	1.20	1.00	0.80	0.50	0.30	0.10
Dicalcium phosphate	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	7.50	7.50	7.50	7.50	7.50	7.50
Salt	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix[a]	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix[b]	0.15	0.15	0.15	0.15	0.15	0.15
Chemical composition (Analyzed)						
Metabolizable energy[c] (MJ/kg)	11.89	11.84	11.82	11.78	11.94	11.81
Crude protein (%)	19.20	19.13	19.25	19.03	19.16	19.32
Ether extract (%)	5.50	5.45	5.20	5.02	4.95	4.60
Crude fibre (%)	2.92	2.90	2.78	2.61	2.69	2.38
Crude ash (%)	11.39	11.42	11.85	11.62	11.44	11.45
Calcium (%)	3.08	2.97	2.98	3.00	2.95	2.93
Phosphorus (%)	0.60	0.63	0.63	0.70	0.68	0.69

[a] Provides (mg per kg diet): retinol 2.4, cholecalciferol 0.075, DL-α tocopherol acetate 20, menadione 2, thiamin 1.5, riboflavin 6, pyridoxol 3.5, cobalamin 0.01, niacin 25, panthotenic acid 8, folic acid 1, d-biotin 0.03, ascorbic acid 30, choline chloride 600.

[b] Provides (mg per kg diet): Mn 80, Fe 60, Zn 60, Cu 5, I 1, Co 0.2, Se 0.15.

[c] Metabolizable energy content of diets was calculated using the equation of Carpenter and Clegg (Leeson and Summers 2001).

4, 8, 12, 16 and 20% of dried baker's yeast (*Saccharomyces cerevisiae*). The dried baker's yeast has 91.00% dry matter, 44.40% crude protein, 0.52% ether extract, 0.33% crude fibre, 5.01% crude ash, 0.28% calcium, 1.21% total phosphorus and 10.79 MJ/kg metabolizable energy. The amino acid composition of dried baker's yeast is given in Table 2.

Traits measured

Nutrient composition of diets and dried baker's yeast were determined according to the AOAC (2000). The analysis of calcium (Farese et al. 1967) and total phosphorus (ADAS 1981) were done after dry combustion of diets. Metabolizable energy levels of diets and dried baker's yeast were estimated using the equation of Carpenter and Clegg: ME, kcal/kg = 53 + 38 [(crude protein,%) + (2.25 × ether extract, %) + (1.1 × starch, %) + (sugar, %)] (Leeson and Summers 2001).

Free and total amino acids of dried baker's yeast were determined with modified OPA derivatization using the HPLC system of Agilent 1100 series (Agilent Technologies, Waldbronn, Germany).

Quails were weighed individually at the beginning and at the end of the experiment. Eggs were collected daily and egg production was calculated on a bird-day basis. Mortality was recorded as it occurred.

Eggs were weighed every week individually for one day of production. Feed intake was recorded weekly and calculated as g/quail/day. Protein intake was calculated using the values of feed intake and crude protein of diets as g/quail/day. The value of feed efficiency was calculated as kg feed per kg egg and kg feed per one dozen egg.

Twelve eggs were collected from each group (4 eggs from each replicate) at the 5th, 8th, 11th and 14th week of the experiment to determine the egg traits. Individual eggs were weighed and their shell thickness was measured. Then yolk height, albumen height, yolk width, albumen width and albumen length were determined. By using these values, yolk index, albumen index and Haugh unit were calculated (Card and Nesheim 1972). Egg and shell quality analyses were completed within 24 hours of the eggs being collected.

Blood samples were collected from 15 quails randomly chosen from each group (5 from each replicate) at the slaughtering time at the end of the experiment and centrifuged. Serum was collected and stored at −20°C for determination of serum parameters. Serum concentrations of total protein, triglyceride and cholesterol were determined by a Hitachi autoanalyser (Serial Number 1238–23, Hitachi Ltd, Tokyo) using their accompanying commercial kits.

Statistical analysis

Statistical analysis were done using SPSS programme (SPSS INC., Chicago, IL, USA). One way ANOVA was used to evaluate the effects of different concentrations of dried baker's yeast on performance, egg traits and blood parameters of laying quails. The significance of mean differences between groups was tested by Duncan. The effect of dried baker's yeast on the mortality of laying quails was evaluated by the χ^2 test (Dawson and Trapp 2001).

Table 2 Amino acid composition of the dried baker's yeast (mg/100 g)

	Total amino acid	Free amino acid
Methionine	609.1	67.4
Cystine+cysteine	385.5	33.1
Lysine	4519.7	190.5
Threonine	1858.0	114.7
Arginine	2404.7	330.6
Isoleucine	1311.8	182.2
Leucine	2635.8	295.2
Valine	1691.9	246.6
Histidine	1122.1	69.8
Phenylalanine	1601.3	191.3
Glycine	1689.0	72.2
Serine	2308.8	190.8
Alanine	2417.2	466.9
Aspartic acid	4060.1	210.7
Glutamic acid	7261.3	765.2

Results and discussion

The amino acid analysis of dried baker's yeast indicates that yeast should be a source of good quality protein (Table 2). Dried baker's yeast contained high amount of lysine (4.5%), threonine (1.9%), arginine (2.4%) but low amount of methionine (0.6%). The effects of dried baker's yeast on performance and egg traits of laying quails are shown in Tables 3 and 4, respectively. The inclusion of dried baker's yeast in the diet of laying quails had no significant effect on

Table 3 The effects of dried baker's yeast on performance of laying quails

	Dried baker's yeast (%)					
	0	4	8	12	16	20
Body weight at start (g)	204±1	207±2	206±1	203±2	202±2	205±2
Body weight at end (g)	237±4	251±3	247±4	245±3	249±4	244±4
Feed intake (g/quail d)	36.8±2.4	36.1±2.0	35.3±2.2	35.9±1.9	35.0±1.5	35.5±1.8
Protein intake (g/quail d)	7.06±0.47	6.91±0.39	6.80±0.42	6.83±0.37	6.71±0.29	6.85±0.34
Egg production (eggs/quail d)	83.7±1.4	84.5±1.5	81.5±2.9	81.2±2.0	82.3±0.8	79.6±1.1
Egg weight (g)**	12.5±0.1b	13.0±0.1a	13.0±0.1a	12.6±0.1b	12.6±0.1b	12.6±0.1b
Feed efficiency (kg feed/one dosen egg)	0.53±0.04	0.51±0.04	0.52±0.05	0.53±0.04	0.51±0.03	0.54±0.03
Feed efficiency (kg feed/kg egg)	3.51±0.27	3.30±0.23	3.37±0.32	3.51±0.25	3.38±0.17	3.55±0.19

a,b: Means within a row followed by the same superscript are not significantly different ($p>0.05$); ** $p<0.01$.

the mean values of body weight. In agreement with the present study, Önol and Yalçın (1995) found that mean body weight values of laying hens were not affected by the addition of baker's yeast at the level of 5, 10 and 20%. However 15 and 20% baker's yeast in quail (up to 35 days of age) diets (Şehu et al. 1997) and 15% yeast protein produced from molasses in broiler diets (Daghir and Abdul-Baki 1977) significantly depressed growth. Waldroup et al. (1971) explained the reduction in body weight of broilers fed diets containing more than 15% yeast by the reduction in the palatability of diets rich in yeast and the reduction in feed intake due to the powdery nature of yeast.

The values for feed intake and protein intake were not significantly affected by dietary treatments over the 14 week period. Similarly some researchers (Bornstein et al. 1982; Rojas Ramirez et al. 1985; Önol and Yalçın 1995) found that diets containing different species of yeasts had no effect on the feed intake of laying hens.

No differences in bird-day egg production were observed among the groups in the present study. Similarly 5 and 10% of baker's yeast (Önol and Yalçın 1995), 10% inactive dry yeast (Shyam Sunder et al. 1990) and 7–28% dry yeast of alcohol (Gar et al. 2001) had no significant effect on egg production of laying hens. Contrary to the finding in the present study, Önol and Yalçın (1995) reported that diets containing 20% of baker's yeast reduced hen-day egg production ($p<0.01$).

Diets with 4 and 8% dried baker's yeast increased the egg weight significantly ($p<0.01$). Egg weight of groups fed diets containing 12, 16 and 20% of dried baker's yeast was similar to that of control group. Önol and Yalçın (1995) indicated that diets containing 5, 10 and 20% of baker's yeast had no effect on egg weight of laying hens. Some researchers (Bornstein et al. 1982; Shyam Sunder et al. 1990; Gar et al. 2001) also found that egg weight was not affected from the usage of different species of yeasts in laying hen diets.

Similar to the previous report of Shyam Sunder et al. (1990), inclusion of dried baker's yeast in the diets of laying quails had no effect on the feed intake per one

Table 4 The effects of dried baker's yeast on egg traits of laying quails

	Dried baker's yeast (%)					
	0	4	8	12	16	20
Egg shell thickness (μm)**	217.8±0.3a	218.4±0.2a	214.2±0.3a	211.0±0.2ab	211.2±0.2ab	205.0±0.3b
Egg albumen height (mm)*	3.61±0.08a	3.62±0.07a	3.60±0.09ab	3.76±0.09a	3.55±0.08ab	3.37±0.06b
Egg albumen index*	8.64±0.18ab	8.46±0.15b	8.42±0.26b	9.15±0.23a	8.50±0.21b	8.23±0.16b
Egg yolk index	44.8±0.5	43.9±0.5	43.8±0.4	45.2±0.5	45.3±0.5	44.5±0.5
Egg Haugh unit	83.4±0.5	82.9±0.5	82.7±0.6	84.1±0.6	82.6±0.5	81.8±0.4

a,b: Means within a row followed by the same superscript are not significantly different ($p>0.05$); * $p<0.05$, ** $p<0.01$.

Table 5 The effects of dried baker's yeast on blood serum parameters of laying quails

	Dried baker's yeast (%)					
	0	4	8	12	16	20
Total protein, g/l	49.0±1.7	48.4±2.6	47.0±2.2	46.6±2.7	45.6±1.4	44.3±2.4
Triglyceride, g/l	8.64±0.71	8.87±0.95	8.41±0.72	7.59±0.46	7.37±0.43	7.54±0.27
Cholesterol, mg/dl	178±11	166±7	161±5	160±7	172±6	172±6

No significant differences among groups ($p>0.05$).

dozen egg and per one kg egg in the present study. Contrary to the results of the present study, Önol and Yalçın (1995) reported that the group fed diets containing 20% of baker's yeast had 18% higher in feed intake than that of the control group.

During the experimental period, 4 (7.0%), 3 (5.3%), 3 (5.3%), 4 (7.0%), 4 (7.0%) and 3 (5.3%) quails died in the groups fed diets containing 0, 4, 8, 12, 16 and 20% dried baker's yeast, respectively. Mortality rate was not affected by the inclusion of dried baker's yeast ($p>0.05$). These data are consistent with the findings of studies involving laying hen (Önol and Yalçın 1995), broiler (Yalçın et al. 1993) and quail (Şehu et al. 1997) fed with diets including baker's yeast.

The group fed diets containing 20% baker's yeast had smaller values of egg shell thickness and egg albumen height than the group receiving no baker's dried yeast. The reduction in egg shell thickness in the group fed 20% of dried baker's yeast supports the results of Oguntona et al. (1983) who reported that high levels of yeast in diets reduced the availability of phosphorus. A similar explanation was given by Yoshida et al. (1974) who indicated that egg shells of hens receiving 15% yeast were 0.01 mm thinner ($p<0.05$) than those of the control hens and attributed the thinner shells to the high phosphorus content of the yeast diet. The values of egg shell thickness and egg albumen height of groups fed diets containing 4, 8, 12 and 16% of dried baker's yeast were similar to those of control group in the present study. Some researchers indicated that 5, 10 and 20% of baker's yeast (Önol and Yalçın 1995) and 10% inactive dry yeast (Shyam Sunder et al. 1990) had no significant effect on egg shell thickness.

The values for egg albumen index of the groups fed 4, 8, 12, 16 and 20% dried baker's yeast were not significantly different from the group receiving a diet without yeast. There were no significant differences in egg yolk index and egg haugh unit among the groups in the present study. Shyam Sunder et al. (1990) reported that haugh unit was not affected by 10% inactive dry yeast but the values of albumen index and yolk index were smaller in the group receiving 10% inactive dry yeast than that of the control group.

The effects of dried baker's yeast on some blood serum parameters of laying quails are shown in Table 5. It was observed in the present study that there were no significant differences among groups in serum levels of total protein, triglyceride and cholesterol. These findings are similar to some reports (Saoud and Daghir 1980; Yalçın et al. 1995). Saoud and Daghir (1980) observed that the effects of replacing soybean protein in a semisynthetic diet with yeast protein from molasses at the levels of 0, 10, 15 and 20% on serum total protein were not significant. Yalçın et al. (1995) reported that the inclusion of baker's yeast up to a level of 20% in the diets of laying hens had no effect on serum levels of total protein, triglyceride and cholesterol.

Conclusion

The inclusion of 4, 8, 12 and 16% of dried baker's yeast in the diets of laying quails gave similar performance data to that for the control diet. Egg weight was improved with the diets containing 4 and 8% of dried baker's yeast. It is therefore possible to reduce the use of protein feedstuffs such as fullfat soya and soyabean meal for laying quails by replacing part of the diet with dried baker's yeast.

It is concluded that dried baker's yeast can be used up to 16% in the diets of laying quails as a protein source without adverse effects on body weight, egg production, feed efficiency, egg weight, egg quality characteristics and blood serum parameters.

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