Oral delivery of live yeast *Debaryomyces hansenii* modulates the main innate immune parameters and the expression of immune-relevant genes in the gilthead seabream (*Sparus aurata* L.)

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**KEYWORDS**

Live yeast; *Debaryomyces hansenii*; Innate immune system; NCC; Antioxidant system; Real-time PCR; Gilthead seabream (*Sparus aurata* L.)

**Abstract** Microorganisms isolated from fish can be used as prophylactic tools for aquaculture in the form of probiotic preparations. The purpose of this study was to evaluate the effects of dietary administration of the live yeast *Debaryomyces hansenii* CBS 8339 on the gilthead seabream (*Sparus aurata* L.) innate immune responses. Seabream were fed control or *D. hansenii*-supplemented diets (10^6 colony forming units, CFU g^-1) for 4 weeks. Humoral (seric alternative complement and peroxidase activities), and cellular (peroxidase, phagocytic, respiratory burst and cytotoxic activities) innate immune parameters and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) were measured from serum, head-kidney leucocytes and liver, respectively, after 2 and 4 weeks of feeding. Expression levels of immune-associated genes, *Hep*, *IgM*, TCR-β, NCCRP-1, MHC-IIα, CSF-1R, C3, TNF-α and IL-1β, were also evaluated by real-time PCR in head-kidney, liver and intestine. Humoral immune parameters were not significantly affected by the dietary supplementation of yeast at any time of the experiment. On the other hand, *D. hansenii* administration significantly enhanced leucocyte peroxidase and respiratory burst activity at week 4. Phagocytic and cytotoxic activities had significantly increased by week 2 of feeding yeast but unchanged by week 4. A significant increase in liver SOD activity was observed at week 2 of feeding with the supplemented diet; however CAT activity was not affected by the dietary yeast supplement at any time of the experiment. Finally, the yeast supplemented diet down-regulated the expression of most seabream genes,
Introduction

The indiscriminate use of antibiotics in aquaculture has a serious environmental impact [1] and causes an increase in the bacterial resistance of pathogens [2]. Under intensive culture conditions, fish are predisposed to stress and infection [3], which results in high mortality and seriously affects the industry’s profitability. Several strategies in disease control have been proposed. Among them, the administration of probiotics (live microbial feed supplements) has appeared as a very promising biological control for aquaculture [4]. Their positive effects include the production of inhibitory compounds against pathogens, competition for nutrients and adhesion sites and the stimulation of both local and systemic immune responses [5]. They can therefore be considered as biological control agents against diseases [6]. Probiotics include bacterial and fungal microorganisms, the beneficial role of yeasts being of particular interest because they represent an important source of β-glucans [7], chitin [8], and nucleotides [9]. The use of whole yeast (wild-type or wall-modified strains) or bacterial strains (isolated from seabream or not) has shown their benefits in gilt-head seabream when used as dietary supplements since they produce a significant innate immune response [10–13]. Further studies to ascertain the role played by probiotics in fish immunomodulation and the benefits of their common use in fish farming deserves further characterization.

Debaryomyces hansenii is a polyamine-producing yeast isolated from the digestive tract of rainbow trout, which is also capable of adhering to the intestinal mucus of other teleost species [14]. It has been used as a first feed in European sea bass (Dicentrarchus labrax), often replacing live food and improving larval survival [14,15]. Natural polyamines (putrescine, spermidine and spermine) play a crucial role in cellular metabolism, and protein, RNA and DNA synthesis [16] and, therefore, growth [17]. While the role of polyamines on the immune system is well documented in mammals [18–21], limited information is available for fish, where polyamines are only known for their antioxidant properties [22].

In this study, we examined the effect of the dietary administration of the live yeast D. hansenii CBS 8339 on the humoral, cellular innate immune and antioxidant systems, as well as on the expression of several immune-relevant genes, in juvenile seabream (Sparus aurata L.), one of the most important commercial fish for Mediterranean aquaculture.

Materials and methods

Microorganism

The live yeast Debaryomyces hansenii strain CBS 8339 was isolated from the gut of rainbow trout [23] and cultured in yeast–peptone–dextrose broth (YPD medium) at 25 °C with constant aeration until the early stationary phase. The cell suspension was centrifuged for 5 min at 1000 × g at 4 °C and the pellet was recovered.

Fish and experimental design

Eighty specimens (80 ± 7 g mean body weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (Sparus aurata L.) obtained from Culmarex SA (Murcia, Spain) were randomly placed in four running seawater tanks (20 fish per tank) (flow rate 1500 l h⁻¹) at 20 °C with a 12 h dark/12 h light photoperiod. Fish in each aquarium received one of the two different diets for 4 weeks: fish in two aquaria were fed a commercial pelleted diet (ProAqua, Spain) (control group) while the fish of the other two tanks were fed the same diet supplemented with 1.1% D. hansenii strain CBS 8339 (10⁶ CFU g⁻¹). Briefly, the commercial pelleted diet was crushed, mixed with tap water and yeasts and made again into pellets. The re-made pellets were allowed to dry and stored at 4 °C until use. The control diet was similarly prepared but without the yeasts. The control diet was administered to all fish during a 1-week conditioning period. The biomass in each aquarium was measured before the experiment and the daily ration was adjusted accordingly after each sampling. The fish were fed twice daily at 2% of their biomass.

Adhesion of Debaryomyces hansenii to seabream intestine

To investigate whether the live yeast adheres to the digestive tract of seabream, an in vitro adhesion experiment was performed [14]. The yeasts were labelled with 5-[(4,6-dichlorotriazin-2-yl)amin]-fluorescein isothiocyanate (DTAF, Sigma) according to a modified method taken from Sherr et al. [24]. Briefly, the intestine from euthanized fish was dissected to obtain ~5 mm segments. The segments were re-suspended in phosphate-buffered saline (PBS, pH 7.4) with DTAF-labelled yeast and then incubated for 30 min. The gut segments were rinsed three times with sterile PBS to remove excess cells. The tissue was placed in pre-cooled cryotubes floating in liquid nitrogen before being stored at −80 °C. Cryosections (7 μm) were obtained from each sample, fixed with 4% paraformaldehyde, air-dried and mounted on cover slips with 10 μl of Vectashield Mounting Medium® with 4′, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Sections were observed under a confocal microscope Leica TCS SP2 AOBS (Leica Microsystems). DTAF emission was detected at 492–516 nm.

Sample collection

Five fish from each aquarium were randomly sampled at weeks 2 or 4 of the feeding trial. Before sampling, the fish...
were starved for 24 h and, after being euthanized, blood was collected from the caudal vein. Briefly, blood samples were collected from the caudal vasculature with a 27-gauge needle and 1 ml syringe and allowed to clot at 4 °C for 4 h. Serum was separated by centrifugation for 10 min at 2000 × g and then stored at −80 °C until used to assess innate humoral parameters.

Head-kidney leucocytes (HKLs) were isolated from each fish under sterile conditions [25]. Briefly, the head-kidney (HK) was excised, cut into fragments and transferred to 8 ml of sRPMI (RPMI-1640 culture medium (Gibco) with 0.35% sodium chloride, 100 IU ml⁻¹ penicillin (Flow), 100 mg ml⁻¹ streptomycin (Flow), 10 IU ml⁻¹ heparin (Sigma) and 5% foetal bovine serum (Gibco)). Cell suspensions were obtained by forcing fragments of the organ through a 100 mm nylon mesh. After two washes, HK leucocytes were counted with a Z2 Coulter particle counter (Beckman Coulter) and adjusted to 10⁷ cells ml⁻¹ of sRPMI. HK, liver and intestine fragments were also sampled and immediately stored at −80 °C in TRizol Reagent (Invitrogen) for RNA extraction (see below). Other liver fragments were also stored in liquid nitrogen for oxidative enzyme determination.

Natural haemolytic complement activity

The activity of the serum alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [26]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank’s buffer (HBSS) containing Mg²⁺ and EGTA were mixed with serially diluted serum to give final concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22 °C, the samples were centrifuged (400 × g, 5 min, 4 °C) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (BMG, FluoroStar 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.

The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y(1 − Y)⁻¹ against the volume of serum added (ml) on a log–log scaled graph. The volume of serum producing 50% haemolysis (ACH₅₀) was determined and the number of ACH₅₀ units ml⁻¹ was obtained for each specimen.

Phagocytic activity

The phagocytosis of Saccharomyces cerevisiae (strain S288C) by gilthead seabream HKLs was studied by flow cytometry [27]. Heat-killed and lyophilised yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5 × 10⁷ cells ml⁻¹ of sRPMI. Phagocytosis samples consisted of labelled-yeast cells and leucocytes (six yeast cells per leucocyte). Samples were mixed, centrifuged (400 × g, 5 min, 22 °C), resuspended in sRPMI and incubated at 22 °C for 30 min. At the end of the incubation time, the samples were placed on ice and 400 µl iced-cold PBS was added to each sample to stop phagocytosis. The fluorescence of the extracellular yeasts was quenched by adding 40 µl of ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled S. cerevisiae or leucocytes were included in each phagocytosis assay. All samples were analysed in a flow cytometry assay. Phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells, FL1⁺) within the phagocyte cell population.

Respiratory burst activity

The respiratory burst activity of gilthead seabream HKLs was studied by a chemiluminescence method [28]. Briefly, samples of 10⁶ leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtitre plate, to which was added 100 µl of HBSS containing 1 µg ml⁻¹ phorbol myristate acetate (PMA, Sigma) and 10⁻⁶ M luminol (Sigma). The plate was shaken and immediately read in a plate reader over a period of 1 h at 2 min intervals. The reaction kinetic was analysed and the maximum slope of each curve was calculated. Backgrounds of luminescence were calculated using reagent solutions containing luminol but not PMA.

Natural cytotoxic or NCC activity

The natural cytotoxic activity, carried out by the non-specific cytotoxic cells (NCC), of gilthead seabream HKLs was evaluated using a flow cytometry technique based on double-fluorescent labelling [29]. Briefly, tumour target cells from the L-1210 line (mouse lymphoma, ATCC CCL-219) in exponential growth were labelled with 10 µg ml⁻¹ of 3,3'-diocthleyloxacarbocyanine perchlorate (DiO, Sigma) for 1 h in darkness. After labelling, free DiO was removed by washing three times in PBS and cell-staining uniformity was examined by flow cytometry. Leucocytes in sRPMI (effectors) were mixed with DiO-labelled L-1210 cells (targets) (effector:target ratio of 50:1). The samples were centrifuged (400 × g, 1 min, 22 °C) and incubated at 22 °C for 2 h. Cytotoxic samples incubated for 0 h (control) were used to determine initial target viability. After incubation, 30 µl of propidium iodide (400 µg ml⁻¹, Sigma) were added and all the samples were analysed in a flow cytometer set to accept the positive FL1 region, which corresponds to DiO-labelled target cells (FL1⁺FL2⁻). The percentage of dead or non-viable target cells showing green and red fluorescence (FL1⁺FL2⁺) was related to the cytotoxic activity of gilthead seabream leucocytes. Cytotoxic activity, a parameter describing the percentage of non-viable target cells, was calculated using the formula

\[
\text{Cytotoxic activity} = \frac{100 \times (\% \text{sample} - \%\text{control})}{(100 - \% \text{control})}
\]

Serum and leucocyte peroxidase activity

The peroxidase activity was measured as an indicator of leucocyte activation by a colorimetric method [30]. Briefly, 5 µl of serum were diluted with 50 µl of HBSS in flat-bottomed 96-well plates. HKLs (10⁶) were dispensed into flat-bottomed 96-well plates and lysed with 50 µl of 0.02% cetyltrimethylammonium bromide (CTAB, Sigma). Serum or HKL samples were mixed with the peroxidase substrate (80 µM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and
2.5 mM H2O2). The colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes were used as blanks. The peroxidase activity was determined defining as one unit the peroxidase that produces an absorbance change of 1 OD.

**Enzymatic activities: superoxide dismutase and catalase**

Superoxide dismutase (SOD) and catalase (CAT) activities were assayed in liver tissues from seabream specimens fed the control- and yeast-supplemented diets. Briefly, 0.1 g of tissue was homogenized in cold 0.05 M PBS for 10 s and centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was removed and stored at −80°C until used.

SOD activity was measured by the method described by McCord and Fridovich [31]. Briefly, the activity was determined from the inhibition of cytochrome c reduction; that is, SOD competes with cytochrome c for the superoxide generated by the hypoxanthine and xanthine oxidase reaction. SOD activity was recorded at 550 nm. Catalase activity, however, was measured using the method described by Beauchamp and Fridovich [32], following the decrease in absorbance at 240 nm caused by the disappearance of H2O2 in 1 min. Briefly, the assay mixture consists of the sample and a solution of 0.05 M PBS (pH 7.0) and 19 mM hydrogen peroxide.

**Real-time PCR**

After 4 weeks of yeast-feeding, total RNA was extracted from 0.5 g of pooled seabream liver, head-kidney and intestine tissue, using TRIzol Reagent (Invitrogen). It was then quantified and the purity assessed by spectrophotometry; 260:280 ratios were 1.8–2.0, and treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript™ III reverse transcriptase (Invitrogen) with an oligo-dT18 primer.

The expression of nine selected immune-relevant genes was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2× SYBR Green supermix, 5 μl of primers (0.6 μM each) and 5 μl of cDNA template) were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. For each mRNA, gene expression was corrected by the ribosomal RNA 18S subunit content in each sample. The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice. The results are expressed as the fold change in the yeast-fed group compared with the control group.

**Statistical analysis**

All bioassays were made in duplicate and all measurements were performed on three replicates; the mean + SD for each dietary group was calculated. One-way ANOVA was performed to determine the effects of dietary live yeast on different parameters using SPSS v.15.0 software. Differences were considered significant at *P* < 0.05.

**Results**

**Adhesion of live yeast to seabream intestine**

Labelled cells of Debaryomyces hansenii (CBS 8339) showed high adhesion to the intestinal mucus of seabream

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**Table 1 Primers used for real-time PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene abbreviation</th>
<th>GenBank number</th>
<th>Primer sequences (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal RNA 18S subunit</td>
<td>18S</td>
<td>AY587263</td>
<td>CGAAAGGATTTGCAGAAT</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Hep</td>
<td>EF625900</td>
<td>AGTTGAGCAGTATATATGC</td>
</tr>
<tr>
<td>Immunoglobulin M</td>
<td>IgM</td>
<td>AM493677</td>
<td>GCCATCGTGCTCACCTTTAT</td>
</tr>
<tr>
<td>T-cell receptor β</td>
<td>TCR β</td>
<td>AM261210</td>
<td>CTGGACCAAGAACGGAAAGA</td>
</tr>
<tr>
<td>Non-specific cytotoxic cell receptor Protein 1</td>
<td>NCCRP-1</td>
<td>AY651258</td>
<td>TTAGAGGTGGTTGGTGG</td>
</tr>
<tr>
<td>Major histocompatibility complex class Ia</td>
<td>MHCIIa</td>
<td>DQ019401</td>
<td>CATGCCAAGAAGCCGAAAA</td>
</tr>
<tr>
<td>Colony-stimulating factor receptor-1</td>
<td>CSF-1R</td>
<td>AM050293</td>
<td>AGCATCGTGCTCAGTTCGAGT</td>
</tr>
<tr>
<td>Complement 3</td>
<td>C3</td>
<td>CX734936</td>
<td>ATAGAACAAAGGCGGTGCAT</td>
</tr>
<tr>
<td>Tumour necrosis factor α</td>
<td>TNFa</td>
<td>AJ413189</td>
<td>TGGCGACCAAGAAGCCGAAAA</td>
</tr>
<tr>
<td>Interleukin-1β</td>
<td>IL-1β</td>
<td>AJ277166</td>
<td>GGCGCGTCATCAAGTCGAGT</td>
</tr>
</tbody>
</table>

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juveniles, as seen by confocal microscopy of the fluorescently-labelled cells which remained on the intestinal segments after thorough rinsing (not shown).

Peroxidase activity

Serum peroxidase activity from individuals fed the yeast-supplemented diets did not differ from the activity of fish fed the control diet at any time during the experiment (data not shown), although there was a slight non-significant stimulation of this parameter after 2 and 4 weeks of feeding D. hansenii. In contrast, the peroxidase activity in leucocytes was significantly enhanced in fish receiving D. hansenii in the diet at 4 weeks (Fig. 1).

Natural haemolytic complement activity

The dietary intake of yeast resulted in a higher natural haemolytic complement activity than the control diet at both sampling times (Table 2) but not to a significant extent.

Phagocytic activity

The percentage of phagocytic cells (phagocytic ability) in head-kidney leucocytes was significantly enhanced after the administration of Debaryomyces hansenii for 2 weeks. However, no significant differences were found at week 4 (Fig. 2).

Respiratory burst activity

The respiratory burst activity, measured as production of reactive oxygen species (ROS), of head-kidney leucocytes

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Weeks of treatment</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>71.58 ± 6.24</td>
<td>42.36 ± 7.49</td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td>102.92 ± 16.05</td>
<td>61.91 ± 12.18</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD (n = 10).

SOD and CAT activities

A significant increase in liver SOD activity was observed at week 2 in fish fed the experimental diet compared with fish fed control diet (Table 3); however, this activity had a non-significant decrease by the end of the experiment (week 4). Catalase activity from fish fed the yeast-supplemented diets was not different from the activity of fish fed the control diet at any time of the experiment.

Real time PCR

In this study, the mRNA transcript of nine selected immune-related genes was always significantly higher in head-kidney leucocytes isolated from fish fed the yeast-supplemented diet was significantly higher at week 4 but not at week 2 compared with the control group (Fig. 3).
Figure 4 Natural cytotoxic (NCC) activity of head-kidney leucocytes from gilthead seabream specimens fed non-supplemented (control) and Debaryomyces hansenii-supplemented diets (10^6 CFU g^-1). Data represent the mean ± SD (n = 10). Asterisk denotes statistically significant differences between control and yeast-fed groups (P ≤ 0.05).

from fish fed the yeast-supplemented diet (Fig. 5). In liver, only C3 gene expression was significantly up-regulated by the experimental diet whilst the other genes were significantly down-regulated. Intestine showed increased expression of TCR-β, C3 and TNF-α genes when yeast was administered and the level of C3 mRNA increased up to 365-fold in the yeast-fed group compared to fish fed the control diet, a much higher increase than in HK and liver. However, the other genes were also down-regulated by the yeast feeding.

Discussion

The use of yeast as a source of the most popular immunostimulants (e.g. β-glucan) has increased in the last decades. The yeast used in the present work was chosen on the basis of previous studies that identified its potential use as a probiotic [14,33]. The principal characteristic of a potential probiotic should be its ability to colonize and persist in the host or in its environment. In our study, D. hansenii CBS 8339 adhered to seabream Sparus aurata intestine, as a means of remaining in situ. These results agree with some previous studies [14,15] which also reported a significant adhesion of D. hansenii to gut of Dicentrarchus labrax larvae and Paralabrax maculatofasciatus juveniles. The adhesion capacity of D. hansenii is probably related to its common presence in the intestines of fish being part of the microbiota and displaying its benefits as potential probiotics [34].

The effects of probiotics have been studied in cultured aquatic species, particularly as a strategy for pathogen control [35], or because of their capacity to enhance the fish innate immune system [12,13,36]. In the present work seabream were fed the live yeast Debaryomyces hansenii CBS 8339 for 4 weeks and the main humoral and cellular innate immune parameters were evaluated after 2 and 4 weeks. The humoral innate immune parameters did not seem to be significantly affected by yeast administration, although non-significant enhancement of the alternative complement activity (ACH50) compared with the control group, was observed at week 2. Previous reports have revealed a significant positive effect of different probiotic bacteria in the complement activity [37]. In contrast to this and in agreement with our results, Kumari and Sahoo [38] found no modulation of this activity in Asian catfish, Clarias batrachus (L.) fed diets containing β-1,3 glucan from the yeast Saccharomyces cerevisiae. Similarly, seabream fed with bacterial probiotics [13] or whole S. cerevisiae yeast [10,11] failed to significantly modulate the complement activity. More studies are needed to understand the role played by probiotics in this important humoral parameter.

By contrast, cellular innate immune parameters were positively modulated by the D. hansenii-supplemented diet. Enhanced ROS production and peroxidase activity in HK leucocytes during oxidative burst were concomitantly increased at week 4, which suggests that an enhanced leucocyte microbe-killing capacity is a key factor in increased resistance to disease. Positive effects of yeast D. hansenii CBS 8339 on the immune system have also been demonstrated in leopard grouper Mycteroperca roxacei [33]. On the other hand, the NCC and phagocytic activities of head-kidney leucocytes were significantly enhanced at week two in fish that received the yeast-supplemented diet but had become non-significant by week 4 of the experiment. The present results are in accordance with those performed in rainbow trout [39] and gilthead seabream [10,11] where phagocytic and/or NCC activities were enhanced by dietary supplementation with S. cerevisiae. In vertebrates, phagocytic process is followed by the production of reactive oxygen species, such as superoxide anion (O2^-), hydrogen peroxide (H2O2) and hydroxyl radical (OH^-), all of which are highly microbicidal [40,41]. The main enzymes which detoxify reactive oxygen species in all organisms are superoxide dismutase, catalase and glutathione peroxidase, all of them abundant in fish tissues [42]. Unfortunately, the measurements of SOD and CAT enzymes in liver during ROS production in HKLs do not correlate well. However, it is the first time that these enzymes have been determined after feeding fish with live yeast. SOD catalyses the dismutation of the highly reactive O2^- to the less reactive H2O2 [43] and belongs to the main antioxidant defence pathways in response to oxidative stress [44]. Ochoa et al. [45] proposed the use of the yeast Debaryomyces hansenii strain C-11 as a potential source of superoxide dismutase because it produces considerable amounts of this antioxidant enzyme. Other biological compounds considered to have antioxidant properties in fish are the polyamines
putrescine, spermidine and spermine [22], which are produced abundantly by Debaryomyces Hansenii strain CBS 8339 [14]. However, more studies are needed to demonstrate the effects of polyamines produced by the dietary-administered yeast cells on the immune system.

Real-time PCR was performed to analyse the expression of Hep, IgM, TCR-β, NCCRP-1, MHC-IIα, CSF-1R, C3, TNF-α and IL-1β genes in different tissues, including head-kidney, liver and intestine. The present work demonstrates for the first time in fish that yeast-supplemented diets up-regulated the expression of these nine genes in the head-kidney of seabream, one of the main haemopoietic organs. In contrast, only the expression of the C3 gene was enhanced in the liver, whereas TCR-β, TNF-α and C3 gene expression was up-regulated in the intestine, the up-regulation of C3 being the most pronounced. However, the reasons underlying the great differences in the yeast-mediated gene regulation in the three tissues are not understood and further studies would help in this regard. Antimicrobial peptides are important molecules in the innate immune system which acts against bacteria and yeast [46]. Thus, the antimicrobial peptide hepcidin was up-regulated in seabream fed with D. hansenii in head-kidney and in turbot (Scophthalmus maximus) head-kidney, spleen, skin and gill following bacterial challenge [47]. Strikingly, seabream hep gene expression was not changed by intraperitoneal injection of S. cerevisiae but increased by bacteria and virus [48]. Several genes have been used as potential markers to establish differences in leukocyte numbers and distribution. Thus, the expression of IgM and TCR-β genes for B and T lymphocytes in head-kidney were greatly enhanced in those fish fed a yeast-supplemented diet. However, it remains to be clarified whether this effect is produced by greater gene expression per cell or by stimulation of lymphocyte proliferation. There also seems to be an increase in IgM production since serum levels of IgM were enhanced by several dietary immunostimulants in seabream, including S. cerevisiae [49]. Although it has not been previously evaluated, fish would be producing an adaptive immune response against probiotics that are not present in their environment or natural conditions. This could help to explain why these non-common microorganisms are better probiotics for fish than the normal or common strains. Other genes such as NCCRP-1, MHC-IIα and CSF-1R encode leucocyte receptors that in some instances might be considered cell markers for NCC, antigen-presenting cells and macrophages, respectively, and several studies have concluded that they are very good candidates for this end [50–53]. However, the regulation mechanisms by which these genes are down-regulated in liver and gut, but up-regulated in head-kidney, due to the presence of yeast in the microbiota, remain to be investigated. Further research into this field is recommended in order to uncover the pathways by which probiotics are able to modulate fish health.

In conclusion, our results provide new evidence that live yeast D. Hansenii strain CBS 8339 may be considered an interesting probiotic for farmed gilthead seabream. Not only does it stimulate the seabream innate immune and antioxidant system, but also strongly regulates the mRNA expression of immune-associated genes, especially in haemopoietic organs like the head-kidney.

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Oral administration of *D. hansenii* regulates immune responses in seabream