Modulation of the Intestinal Microbiota and Immune System of Farmed *Sparus aurata* by the Administration of the Yeast *Debaryomyces hansenii* L2 in Conjunction with Inulin

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**Abstract**

Fish gastrointestinal tract is one of the most important sites of interaction with the external world. Interactions between the intestinal microbiota and the host modulate the functionality of the intestinal mucosa and gene expression. Application of probiotics, prebiotics and symbiotics for aquatic animals is increasing to improve welfare and promote growth. However, data about the use of probiotics in conjunction with prebiotics, in farmed marine organisms are scarce. The objective of this work is to study the modulation ability of the intestinal microbiota and the immune system of gilthead seabream exerted by the probiotic yeast *Debaryomyces hansenii* L2 in conjunction with the prebiotic inulin. Fish were fed a commercial diet (control diet I), and with the same diet supplemented with 1.1% *D. hansenii* strain L2 (10⁶ CFU g⁻¹) plus inulin (3%) (diet II) for 4 weeks. The whole intestines of healthy fish from each group were aseptically removed at 2 and 4 weeks after starting the experiment and they were analysed by PCR-Denaturing Gradient Gel Electrophoresis (DGGE). Samples of blood and head kidney were obtained for humoral and cellular immune parameters determination. The expression of 12 selected genes related to the immune response (IgM, MHCIIa, MHCIIc, C3, IL-1β, TLR, TNFa, CSF-1R, NCCRP-1, Hep, TCRβ y CD8) were analyzed by real-time PCR from skin, intestine, liver and head-kidney tissue.

In this study, relevant changes in the intestinal microbiota of gilthead seabream specimens fed the diets assayed has been demonstrated. An important effect on the intestinal microbiota by the dietary administration of a symbiotic mixture has also been detected, especially in fish receiving the supplemented diet for 4 weeks. These changes coincided in the same time with an up-regulation of the expression of immunological genes in skin and head kidney of gilthead seabream at 2 and 4 weeks, respectively.

**Introduction**

According to the FAO, at present, 52% of the wild 600 fish species with economic value are heavily depleted, 17% over fished and 7% fully exploited. Supply from capture fisheries will be static over the next 30 years. Growing percentages of world aquatic production derives from aquaculture, whose importance is increasing dramatically as a result of overfishing of the world waters and increasing demands for seafood [1].

In large scale production facilities, where farmed animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often result in economic losses [2,3]. Control of pathogens in fish farms has been routinely achieved by the administration of antimicrobial agents, but the excessive use of these agents has produced the emergence of resistant bacteria making the treatments less successful [4]. One of the main challenges to achieve productive, feasible and sustainable aquaculture is to develop alternative preventive practises that may help to maintain high animal welfare standards as well as healthy environment, resulting in a better production and higher profits. In view of the increasing interest in the environmental and nutritional control of diseases [5], application of probiotics, prebiotics and symbiotics for aquatic animals is increasing to improve welfare and promote growth [6-9].

Probiotics are often defined for aquatic organisms as applications of entire or components of a microorganism which are beneficial to the health of the host [10]. Yeasts are commonly used in aquaculture [11], either alive as a feed ingredient [12,13], or as source of immunostimulants [14-16]. In this context, *Debaryomyces hansenii* has been proposed as probiotic due to its beneficial effects on the enzymatic antioxidative status of farmed fish such as sea bass *Dicentrarchus labrax* larvae [17] and juvenile leopard grouper (Mycteroperca rosacea) [18] and in the immune response of *Sparus aurata* [19, 20].

Prebiotics are defined as food ingredients non-digestible by the host which beneficially affects by selectively stimulating the growth and the activity of bacterial groups that improve the host health [21]. Several probiotics have been assayed in farmed fish [17,22-24], the inulin being one of them [25]. This fructooligosaccharide has shown some prebiotic properties for fish, such as an improvement of the feed efficiency and energy retention [26], and weight [22,27]. However, it does not seem to exert an immunostimulant effect for gilthead seabream [28], and this prebiotic is not appropriate for supplementation of the diet of beluga (*Huso huso*, Linnaeus 1758), due to negative effects with some performance indices [29].

Fish gastrointestinal tract is one of the most important sites of interaction
with the external world [9], and it is considered one of the major portals for pathogenic invasion in fish [30]. Interactions between the intestinal microbiota and the host modulate the functionality of the intestinal mucosa and gene expression [31-33]. An imbalanced microbiota may contribute to the development of diseases or reduced functionality [34]. Establishment of a healthy microbiota plays an important role in the generation of immune-physiologic regulation by providing crucial signals for the development and maintenance of the immune system [35]. Some yeasts have activities, such as the production of siderophores and proteases [36], which can have antibacterial properties [9], and which can affect on the bacteria comprising the fish intestinal microbiota. In addition, effects of inulin on the intestinal microbiota of artichoke char (Salvelinus alpinus, L.) and hybrid striped bass (Morone chrysops x Morone saxatilis) have been demonstrated [37-39]. However, data about the use of probiotics in conjunction with prebiotics, in farmed marine organisms [40] are scarce, although this possibility could have a high priority in future studies [25].

In short, the objective of this work is to study the modulation ability of the intestinal microbiota and the immune system of gilthead seabream exerted by the probiotic yeast Debaryomyces hansenii L2 in conjunction with the prebiotic inulin.

Materials and Methods

Debaryomyces hansenii strain L2

The live yeast Debaryomyces hansenii strains L2 was provided by Centro de Investigaciones Biológicas del Noroeste (CIBNOR, México) and belongs to the yeast collection of CIBNOR. Briefly, the yeast was isolated from the surface of lemon fruit and cultures by across-streak on YPD-agar (containing 2.0% glucose, 2.0% peptone, 1.0% yeast extract and 1.5% agar prepared with distilled water and supplemented with 0.05% chloramphenicol) at 30°C. Yeast cells were removed with a bacteriological loop and suspended them in YPD-medium (1000 ml or 1,000-ml in Erlenmeyer flasks) following a new incubation on a rotary shaker at 30°C for 48 h with constant aeration until the early stationary phase. Yeast cells were then removed from the growth medium by centrifugation (5 min, 1000g, 4°C) and the pellet was recovered the final cell concentration being adjusted accordingly.

Fish and experimental design

Sixty specimens (80g mean weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (Sparus aurata L.) obtained from Culmarex S.A. (Murcia, Spain) were randomly placed in four running seawater tanks (10 fish per tank) (flow rate 1500 l/h-1) at 20°C with a 12 h dark/12 h light photoperiod. Seabream were confirmed bacterial-free by standard microbiological techniques [41] and by a PCR technique using the primers combination strategy described by Vazquez-Juarez et al. [42] to determine the presence or absence of allochthonous A. hydrophila. Fish in three. Fish in two aquaria were fed a non-supplemented commercial diet (ProAqua, Spain) (control diet I), while the fish of the other three tanks were fed the same diet supplemented with 1.1% D. hansenii strain L2 (10⁶ CFU g⁻¹) plus inulin (3%) (diet II) for four weeks. Briefly, the commercial pelleted diet was crushed, mixed with tap water (to which the yeast suspensions were added inulin (3%) and made again into pellets. The re-made pellets were allowed to dry and stored at 4°C until use. The control diet was put under the same process that the experimental diet but without yeast. The viability of the yeast after incorporation in the feed was determined by means of colony counts on YPDagar. Briefly, one gram quantities of food were homogenized in 9 ml distilled water. The homogenate was serial dilutions prepared to 10⁻⁵ the colony counts being determined after incubation for 24 h at 30°C.

The control diet was administered to all fish during two weeks 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.

Fish sample collection

The studies presented in this manuscript were approved by the Bioethical Committee of the University of Murcia. Five fish from each aquarium (15 fish/treatment) were randomly sampled at weeks 2 or 4 of the feeding assay. Before sampling, the fish were starved for 24 h. The whole intestines from sampled fish were aseptically removed and stored at -80°C intestinal microbiota analysis. Tissue fragments from skin, intestine, liver and head-kidney were obtained and immediately stored at -80°C in TRIzol reagent (Invitrogen) for RNA extraction. Samples of blood and head kidney were obtained for immunological assays as described below.

Intestinal microbiota analysis

The intestinal contents of four fish from each treatment were sampled on days 14 and 28 after starting the experiment. The whole intestines were aseptically removed and stored at -80°C until further analysis.

Total DNA was extracted from samples as described by Martinez et al. [43]. Agarose gel (1% [wt/vol]) electrophoresis in the presence of ethidium bromide was used to visually check for DNA quality and yield.

In order to compare DGGE patterns of the intestinal microbiota of gilthead seabream specimens receiving the different diets, the DNA was amplified using the 16S rDNA bacterial domain-specific primers 677R-GC (5’ CGGCGCAGGCGCAGGCGGATCTACGTTTTCACCCTGCACTAC-3’) and 309-F (5’ACTCTCTACGGGAGGCAGCAGCGCAG-3’). PCR mixtures (50µl) contained 1U Robust Taq polymerase (KapaBiosystem, Spain), 20mM Tris-HCl (pH 8.5), 50mM KCl, 3mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 5 pmol of the primers, 1 µl of DNA template, and UV-sterilized water. The PCR was performed in a T1 thermocycler (Whatman Biometra, Göttingen, Germany) using one cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 56°C for 30 s and 68°C for 30 min, followed by one cycle of 68°C for 5 min. Aliquots (5µl) were analyzed by electrophoresis on 1% (wt/vol) agarose gels containing ethidium bromide to check for product size and quantity.

The amplicons obtained from the intestinal lumen-extracted DNA and the probiotic strain were separated by Denaturing Gradient Gel Electrophoresis (DGGE) [44] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed in 8% polyacrylamide gels (37:5:1 acrylamide-bisacrylamide; dimensions; 20 X 20cm x 1mm). The gels contained a 30 to 55% gradient of urea and formamide increasing in the direction of the electrophoresis. A 100% denaturing solution contained 7M urea and 40% (vol/vol) deionized formamide. PCR samples were applied to gels in aliquots of 13µl per lane. The gels were electrophoresed for 16 h at 85 V in 0.5 X TAE (20mM Tris acetate [pH 7.4], 10mM sodium acetate, 0.5mM Na₂-EDTA) buffer at a constant temperature of 60°C [45] and subsequently stained with AgNO₃ [46]. The number of DGGE bands and similarity indices were calculated from the densitometric curves of the scanned DGGE profiles with the software FSPquest 4.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity between DGGE profiles was determined by calculating a band similarity coefficient Pearson. The position tolerance used in the band comparison was 1.00%. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method using Arithmetic Averages (UPGMA).

In order to determine the structural diversity of the microbial community corresponding to the DGGE banding patterns several parameters were calculated: (1) Range-weighted richness (Rr) [47] calculated as the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analysed, according to the following formula: Rr = (N’ x D’), where N represents the total number of bands in the pattern, and D the denaturing gradient comprised between the first and the last band of the pattern. The Rr defines the carrying capacity of the system, and it is related to the amount and localisation of the DGGE bands in the gel [47]; (2) the Shannon
index (H') was calculated following the function: $H' = -\sum Pi \ln Pi$, where Pi is defined as (ni/N), ni is the peak size of each band, and N is the sum of all the peak sizes of all bands, and its value ranges from 1 to 5 indicating a low and high diversity, respectively; (3) the specific richness (R) was calculated based on the total number of bands; and (4) the Simpson's index was calculated in base on the function $D = 2p^2$, and its value ranges from 0 to 1 indicating a low and high dominance, respectively.

**Cloning of the PCR-amplified products**

16S rRNA gene-targeted PCR amplicons (500 bp) were generated with the same set of primers used for DGGE and were purified with NucleoSpin Extract II (Macherey-Nagel, The Netherlands) according to the manufacturer's instructions. PCR products were cloned into Escherichia coli XL1-Blue competent cells (Stratagene) using the Promega pGEM-T easy vector system (Promega, Madison, WI). Ligation and transformation reactions were performed according to the protocol described by the manufacturer. PCR was performed on cell lysates of ampicillin-resistant transformants using vector specific primers T7 (TAATACGACTCCTATAGG) and Sp6 (GATTTAGGTGACACTATAG) to confirm the size of the inserts. A total of 96 ampiclons of the correct size (per sample) were subjected to amplified ribosomal DNA restriction analysis (ARDRA) using the restriction enzymesMspI, CfoI, and AluI. From each sample, clones corresponding to a unique RFLP pattern were used to amplify V6–V8 regions of 16S rRNA genes with the primers 309-F and 677-CG as described previously, and they were selected for subsequent sequence analysis according to their migration position in the DGGE gel compared to the amplicons of the original DGGE profile of the sample.

**Sequence analysis**

PCR amplicons (0.5 kb) of transformants selected by the above-described ARDRA/DGGE screening procedure were purified with NucleoSpin Extract II (Macherey-Nagel, The Netherlands) according to the manufacturer's instructions.

The samples were subjected to DNA sequence analysis with the primers SP6 and T7. Sequences were analyzed for similarity with sequences deposited in public databases using the BLAST tool at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST).

**Blood serum and head kidney leucocytes**

Blood was collected from the caudal vein with a 27-gauge needle and 1 ml syringe and allowed to clot at 4°C for 4 h. Serum was obtained by centrifugation (10 min, 200 g, 4°C) and stored at -80°C until used for humoral immune parameters determination (peroxidase level, natural haemolytic complement activity and Ig M level). Head kidney (HK) was dissected out sterilely by a ventral incision, cut into small fragments and transferred to 8 ml of sRPMI (RPMI-1640, Gibco, culture medium containing P/S, 2% FCS and 0.35% NaCl). Head–kidney leucocytes (HKLs) were isolated from each fish under sterile conditions [48]. Briefly, leucocytes were washed, counted in a Z2 Coulter Particle Counter (Beckman Coulter, Spain) and adjusted to 107 cells ml-1 for subsequent sequence analysis according to their migration position in the DGGE gel compared to the amplicons of the original DGGE profile of the sample.

**Immunological assays**

**Peroxidase:** The peroxidase activity was measured as an indicator of leucocyte activation by a colorimetric method [49]. Briefly, 5 µl of serum were diluted with 50 µl of HBSS in flat-bottomed 96-well plates. HKLs (105) were dispensed into flat-bottomed 96-well plates and lysed with 50 µl of 0.02% cetyltrimethylammonium bromide (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 2M sulphuric acid and the optical density was read at 450 nm in a plate reader (BMG, Fluoro Star Galaxy). 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.

**Phagocytic activity:** The phagocytosis was studied by flow cytometry [50]. Heat-killed yeast cells were labeled 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. Samples were mixed, centrifuged (5 min, 400g, 22°C), resuspended in sRPMI and incubated (22°C, 30 min). At the end of the incubation time, the samples were placed on ice and 400 µl ice-cold PBS was added to each sample to stop phagocytosis. The fluorescence of the extracellular yeasts was quenched by adding 40 µl 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 cytometer set to analyse the phagocytic cells. Phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells, FL1') within the phagocyte cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells.

**Respiratory burst activity:** The respiratory burst activity of gilthead seabream HKLs, measured as the production of reactive oxygen intermediates, was studied by a chemiluminescence [51]. Briefly, samples of 105 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 mg ml-1 of phorbol myristate acetate (PMA, Sigma) and 10–4M 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 analyzed and the maximum slope of each curve was calculated. Backgrounds of luminescence were calculated using reagent solutions containing luminol but not PMA.

**Natural haemolytic complement activity:** The activity of the serum alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [52]. 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 (400g, 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. 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) 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.

**Serum IgM level:** Total serum IgM levels were analyzed according using the enzyme-linked immunosorbent assay (ELISA) [53], serial dilutions of gilthead seabream serum (from 1/1 to 1/1000) and the commercial monoclonal antibody as indicated by the manufacturer's instructions. The 1/100 serum dilution gave an OD in the linear range of the serum dilution versus absorbance curve and was chosen to compare the total IgM level in different serum samples. Thus, 20 µl per well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4°C with 200 µl of carbonate-bicarbonate buffer (35mM NaHCO3 and 15mM Na2CO3, pH 9.6). After three rinses with PBS (20mM Tris-HCl, 150mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA) in PBS, followed by three rinses with PBS. The plates were then incubated for 1 h with 100µl
per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer). After exhaustive rinsing with PBST the plates were developed using 100 ml of a 0.42mM TMB solution, prepared daily in a 100mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H2O2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 ml of 2M H2SO4, and the plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose OD values were subtracted for each sample value.

RNA purification: Total RNA was extracted from 0.5g of gilthead seabream skin, intestine, liver and head-kidney tissue, using Trizol RNA isolation reagent (Invitrogen). It was then quantified and the purity assessed by spectrophotometry; 260:280 ratios were 1.8–2.0, and treated with DNase (Promega, USA) to remove DNA contamination. Complementary DNA (cDNA) was synthesized from total RNA using the SuperScript™ III reverse transcriptase (Invitrogen). It was then quantified and the purity assessed by spectrophotometry; 260:280 ratios were 1.8–2.0, and treated with DNase (Promega, USA) to remove DNA contamination. Complementary DNA (cDNA) was synthesized from total RNA using the SuperScript™ III reverse transcriptase (Invitrogen) and using 1μg of total RNA with oligo-dT18 primer.

Real-time PCR: The expression of 12 selected genes (IgM, MHCⅠa, MHCⅡa, C3, IL-1β, TLR, TNFa, CSF-1R, NCCRP-1, Hep, TCRβ and CD8) were analysed by real-time PCR using the SYBR Green PCR Core Reagents dye detection (Applied Biosystems). The amplification was performed in a 96-well plate in a 20 μl reaction volume containing 10 μl of 2xSYBR Green supermix, 5 μl of concentration primers, and 5 μl of cDNA template. The thermal profile for SYBR Green real-time PCR was 95°C for 10 min followed by 40 cycles of denaturation (95°C for 15 S), annealing (60°C for 60 S) and extension (95°C for 15 S). For each mRNA, gene expression was corrected against the endogenous level of 18S content in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality [19]. No amplification product was observed in negative controls and no primer-dimer formations were observed in the control templates. In all cases, each PCR was performed with triplicate samples and repeated at least twice. The primers used are detailed in Table 1. The results are expressed as the fold change in the yeast-group compared with the control group.

Statistical analysis

All bioassays were made in triplicate and all measurements were performed on three replicates; the mean ± standard error (SE) for each immune parameter was calculated. One-way ANOVA was performed to determine the effects of dietary live yeast plus inulin on different parameters after checking for normality and homogeneity of variance by the Kolgomorov-Smirnoff and Levene tests (P < 0.05), respectively. All statistical tests were conducted using the computerized software Statistical Package for Social Sciences (SPSS) v15.0 software. Differences were considered significant at P < 0.05. The intestinal microbiota analysis data were processed by analysis of variance using the program STATGRAFICS Plus 5.0 (Statgraphics Corporation, Rockville, MD, USA). For real time PCR, gene expression was normalized to the ribosomal protein S18 content in each sample using the comparative Ct method (2^{-ΔΔCt}) (fold), where the ΔCt value is determined by subtracting the average 18S Ct value from the average of each gene Ct. The results are expressed as the fold change in the yeast group compared with the control group.

Results

The number of bands in the PCR-DGGE patterns ranging from 4-6 until 20-25 depending on the diet and the weeks supplied is summarized in Table 2. DGGE patterns from fish fed the diet supplemented with Debaryomyces hansenii strain L2 and inulin (diet II) for 4 weeks showed a significant (p<0.05) lower number of bands compared to the DGGE profiles obtained from the specimens receiving diet I for 2 and 4 weeks, and diet II for 4 weeks. A clustering analysis was applied to the PCR-DGGE patterns from the intestinal microbiota of the specimens to analyse the variability of the intestinal microbiota of fish receiving the different diets assayed. Figure 1 shows the dendrogram obtained, and it revealed that fish fed diet I for 2 and 4 weeks and diet II for 2 weeks showed a similarity index about 20%. On the contrary, fish receiving the diet II for 4

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Table 1: Primers used for real-time PCR.
weeks drastically increased their similarity index (about 100%), indicating a lower intragroup variability of the intestinal microbiota of the specimens in comparison with the intestinal microbiota of fish fed with control diet. The Shannon index obtained for fish receiving diet I and II for 2 weeks were not statistically different (2.07 and 2.23, respectively). On the contrary, values of the Shannon's index significantly (p<0.05) decreased when the diets were supplied for 4 weeks, 1.88 and 0.86, respectively. The values of the Simpson's index only showed some grade of dominance (value near to 1) from the intestinal samples of fish fed diet II for 4 weeks.

At 2 and 4 weeks from the beginning of the experiment, the values of Rr obtained from fish fed with diet I were 40.5 and 38.4, respectively whilst this value was 35.2 for specimens receiving diet II for 2 weeks (Table 2), and they corresponded to environments classified as high range-weighted richness (Rr>30) [47]. On the contrary, this value drastically was reduced from fish receiving the diet II for 4 weeks (13.4). These results correspond to environments with a medium range-weighted richness (Rr between 10 and 30) [47].

Tables 3 and 4 summarize the bacterial species identified from the cloned sequences from intestines of fish fed the diets I and II for two and four weeks. The phylogenetic affiliations of those sequences which were not clearly related to members of bacterial genera were determined (Figure 2), being the majority of these cloned sequences related to *Pseudomonas* genus. Majority of cloned sequences from intestines of fish fed with diet I for 2 days were identified or phylogenetically related to *Pseudomonas* and *Serratia* genera. The cloned sequences related to <i>Serratia</i> sp GIST-WP4w1 (control 1), *Pseudomonas fluorescens* strain XXPFMDU1 (control 6), *Pseudomonas sp* VS05_112 (control 12) and γ-proteobacterium 6.1.12_1 (control 14), phylogenetically related to *Pseudomonas fluorescens*, corresponded to one of the most predominant bands in the PCR-DGGE profiles. Other cloned sequences related to uncultured bacterium clone 2B (control 17) and clone nb36b09 (control 18) also corresponded to predominant bands of the PCR-DGGE patterns. The last cloned sequence was phylogenetically related to species of *Serratia* genus. Remaining cloned sequences also corresponded to one of the bands detected in the PCR-DGGE profiles, although the intensity of these bands was lower than those previously mentioned. Other clones sequences showed a high similarity to *Stenotrophomonas*, *Ewingella*, *Yersinia* and *Vibrio* genera, whilst other were phylogenetically related to *Acromonas* and *Chlorobacterium* genera. When the diet I was supplied for 4 weeks, 15 different cloned sequences were characterized, and sequences control 24, 26 and 32 showed a very high similarity with members of *Pseudomonas* genus. Other sequences such as control 23 and 29 were phylogenetically related to *Pseudomonas*. Other cloned sequences showed a high similarity to *Lactobacillus* (control 22 and 35) and *Ralstonia* genera. Most of these cloned sequences related to uncultured bacterium clone 2B (control 17) and clone nb36b09 (control 18) also corresponded to predominant bands of the PCR-DGGE patterns. Other sequences such as control 3 and 20 showed a high similarity to *Stenotrophomonas*, *Ewingella*, *Yersinia* and *Vibrio* genera.

Twenty two different cloned sequences were characterized from intestines of fish fed diet II for 2 weeks. Thirteen of them showed a very high similarity (L2+I_1, L2+I_11, L2+I_18 and L2+I_19), or were phylogenetically related (L2+I_1, L2+I_5 L2+I_7, L2+I_9, L2+I_12, L2+I_15, L2+I_16, L2+I_17 and L2+I_21) to *Pseudomonas* genus. All these sequences corresponded to some of the predominant bands detected in the PCR-DGGE profiles, and the cloned sequence L2+I_1 corresponded to one of the most intensive bands. Remaining cloned sequences showed a high similarity or were phylogenetically related to *Serratia*, *Pseudoalteromonas*, *Vibrio* and *Enterobacteriaceae* genera. Some of the cloned sequences did not correspond to some of the predominant bands of the PCR-DGGE profiles. After 4 weeks receiving the diet II, sixteen cloned sequences were characterized, but the majority of these cloned sequences did not correspond to some of the predominant bands detected in the PCR-DGGE profiles. Two sequences (L2+I_23 and L2+I_35) showed high similarity or were phylogenetically related (L2+I_34) with species of *Pseudomonas* genus. Other sequences were identified or related to *Vibrio harveyi*, *Shewanella alga*, *Enterobacter*, *Ralstonia* and *Psychrobacter* genera.
genera which were not detected from intestinal samples from fish fed diet II for 2 weeks. The majority of cloned sequences were related to γ-Proteobacteria, but three cloned sequences corresponded to β-Proteobacteria, two of them related to Ralstonia (L2+I_29) genus. β-Proteobacteria were not detected from cloned sequences identified as members of Pseudomonas genus.

The phylogenetic analysis based on 16S rRNA gene showed that, P. fluorescens was in the majority of cases the species more frequently characterized corresponding from the cloned sequences identified as members of Pseudomonas genus.

On the other hand, about the humoral and cellular immunological parameters, this study show a significant effect on peroxidase activity in serum in fish fed diet II at week 4. The highest values in the haematological parameters were recorded from the groups fed diet II (Figure 3), although they were not significant.

A quantitative RT-PCR was used to estimate the regulation of immune gene expression in gilthead seabream fed diet II, and in this study the number of mRNA transcripts of 12 selected immune-related genes was generally higher at week 2 in skin and intestine (Figure 4). The maximum transcript levels were found in the intestine for the major histocompatibility complex (MHC) genes, MHC-I and MHC-II, which were significantly up-regulated more than 241- and 127-fold respectively. On the other hand, after 4 weeks (Figure 5), a relatively lower expression of genes was recorded in the skin, intestine and liver but not in the head-kidney, where they were up-regulated, except MHC I, TLR and CD8. Interestingly, the C3 transcript was significantly increased more than 244-fold in the intestine.
Table 4: 16S rRNA sequence similarities to closest relatives obtained from BLAST search, of DNA corresponding to Sparus aurata specimens fed the different diets as assayed for four weeks. Diet I is a commercial diet, and diet II, is a commercial diet supplemented with the symbiotic mixture.
Discussion

The demonstration that the gut microbiota is an important component of the mucosal barrier has resulted in the promotion of its manipulation through the use of probiotics [54] and synbiotic [40]. Results obtained in this study suggest the ability of the yeast *Debaryomyces hansenii* strain L2 in conjunction with the prebiotic inulin to modify the status of the intestinal microbiota of gilthead seabream. In this context, the evaluation of the results derived from the analyses of the PCR DGGE patterns suggests that the intragroup variability in the intestinal bacterial communities of *Sparus aurata* specimens was higher than in those fish receiving the diet supplemented with the synbiotic. Similar results have been reported for the intestinal microbiota of other farmed fish such as Senegalese sole (*Solea senegalensis*) [55]. This intragroup variability also was observed for the intestinal microbiota of *Sparus aurata* specimens fed the diet II for 2 weeks. On the contrary, the PCR-DGGE profiles corresponding to fish receiving for 4 weeks the diet supplemented with the synbiotic mixture showed
a very low intragroup variability and a very important reduction of the number of bands. These results suggest that the addition of the symbiotic mixture to the feed could exert an important influence on bacterial intestinal groups and yield a faster homogenization of the intestinal microbial community. This ability to modulate the intestinal microbiota by the symbiotics has also been reported in humans and other animals [13,40].

The interpretation of the molecular fingerprints in terms of mean Rr values obtained fish fed with diet I for 2 and 4 weeks and 2 weeks for fish receiving diet II showed that these were higher than 30. They are associated with very habitable environments characterized by a high microbial diversity with a high range-weighted richness, and they can host a lot of different microorganisms and genetic variability [47]. Bacterial diversity plays an important role in the functioning of microbial ecosystems [56]. Biodiversity protects ecosystems against declines in their functionality and allows for adaptation to changing conditions providing a greater guarantee that some will back up a given function when other fail [57,58]. On the contrary, the fish received the diet II for 4 weeks showed mean values of Rr ranging from 10 to 30, and they are considered as a medium range-weighted richness [47], which is a less habitable environment than the intestine of fish fed with the diets previously mentioned.

The intestinal microbiota of fish play an important role as a defensive barrier against pathogens [25], and it can regulate the expression of great numbers of genes in the digestive tract implied in the epithelial proliferation, promotion of nutrient, metabolism and immune response [31]. In a previous study, a stimulatory effect on the immunological system induced by the supplementation of feed with has Debaryomyces hansenii strain L2 been detected [20]. However, the different immunostimulatory effect on the expression of genes in different organs was different depending on the time that fish were receiving the diet supplemented with the microorganism.

In this study, a reduction of the number of predominant PCR-DGGE bands was detected from fish fed diet II for 2 and especially 4 weeks, in comparison with those detected from fish receiving the diet I. It could indicate a reduction of the diversity of the predominant microbial species of the intestinal microbiota. It is assumed that the reduction of the diversity could suggest a negative effect because the diversity protects ecosystem against declines in their functionality and allows for adaptation to changing conditions [47,56,57]. However, in this study an important number of cloned sequences identified from intestinal samples from fish fed diet II for 4 weeks did not correspond to visible bands in the PCR-DGGE patterns. Similar results have been reported by other authors studying complex bacterial communities [59] and the effect of fermentable carbohydrates on piglet faecal bacterial communities [60]. It could indicate a higher diversity of that detected only from the number of visible bands of PCR-DGGE profiles. The absence of these bands in fish fed with the other diets may not exclude the presence of the same bacterial species in the samples, since they could be below the detection limit of the DGGE technique which has an abundance limit of 1% [61], although some of them can be selected using a cloning approach [60,62]. However, some authors suggest that those less abundant taxa could be less relevant for the functioning of the microbial community [63], but this point must be demonstrated.

Different microbial species were detected in fish fed for 2 and 4 weeks, and sequences related to Serratia genus were frequently detected from intestinal samples from fish fed both diets for 2 weeks, but they were not detected from samples recovered at 4 weeks. On the other hand, cloned sequences related to ß-Proteobacteria were not detected from fish fed with the diets for 2 weeks although they were detected at 4 weeks. Pseudomonas was the only one genus detected from all intestinal samples of fish fed both diets for 2 and 4 weeks, although its frequency of detection was lower from fish receiving the diet II for 4 weeks. Ps. fluorescens was the most frequent species identified from cloned sequences. Several species of Pseudomonas genus have been described as pathogenic for different farmed fish [64-69]. Ps. fluorescens being one of them [70]. However Pseudomonas species have been identified as members of autochtonous microbiota of farmed fish [71-74], and several authors have reported the potential probiotic role play for strains of Pseudomonas genus [8,71,75,76].

Hematological parameters are typically used to assess the health status and detect physiological changes in a number of fish species [77]. It is known that inulin, particularly long chain molecules thereof, stimulates the human immune system by binding to specific lectin-like receptors on leucocytes and increasing macrophage proliferation [78]. In this work, cellular immunological parameters did not differ from the activity of fish fed the control diet at any time during the experiment, although there was a slight non-significant stimulation in these parameters in gilthead seabream. About the humoral parameters, serum peroxidase activity from individuals fed diet II was significantly enhanced at week 4 compared with the control group, which suggests that an enhanced leucocyte microbe-killing capacity is a key factor in increased resistance to disease. It has become readily evident that the microbiota affected by prebiotics plays integral roles in numerous processes including growth, digestion, immunity and disease resistance of the hot organism [21,79]. However, the relationship between the changes produced in the intestinal microbiota of fish receiving the symbiotic diet and the enhanced immunological response needs to be more studied. Cereza et al. [28] have studied the effect of inulin in seabream; however, immunostimulant effect of inulin on the innate immune system of gilthead seabream was not detected [28]. On the other hand, Reyes et al. [20] have observed that D. hansenii L2 is an effective immunostimulant in juveniles seabream.

Real-time PCR was performed to analyse the expression of IgM, MHCIIa, MHCIIc, CI, IL-1ß, TLR, TNFa, CSF-1R, NCORP-1, Hep, TCRγ and CD8 in different tissues, including skin, intestine, liver and head-kidney. The present work demonstrates for the first time in fish that yeast-supplemented diets plus inulin (3%) up-regulated the expression of genes in skin and head-kidney of gilthead seabream, at week 2 and 4, respectively. In contrast, only the expression of MHCIIa was up-regulated in skin at week 4, the up-regulation of C3 being the most pronounced at week 4. Similar effect were observed in our laboratory where yeast D. hansenii L2 diets up-regulated the expression of several genes principally in skin and head-kidney [20]. Fish have a well-developed complex system that plays an important role in their innate immune response, including membrane attack complex (which lyses bacteria), opsonization and phagocytosis [80]. On the other hand, the mRNA transcript levels of MHC I, MHC II and TNF-a showed a tendency to increase in the intestine at 2 weeks, suggesting the importance of the intestinal tract as a site of immune activity. However, immunostimulant effect of inulin on the innate immune system of gilthead seabream was not detected [28]. These results suggest that Debaryomyces hansenii L2 could stimulate the immune system by itself. TNF-α is a key cytokine in acquired cell-mediated immunity to intracellular bacteria and tissue injury and it can stimulate immune responses by activating lymphocytes, or by inducing the release of other cytokines capable of triggering macrophages, NK cells and lymphocytes [81]. Genes of the MHC encode two classes of structurally similar, but functionally distinct, glycoproteins, MHC class I and class II, both of which are involved in the immune response at various levels [82]. These results suggest that D. hansenii L2 in conjunction with inulin could stimulate the expression of genes related of immune system. Reyes et al. [20] observed that this strain L2 by itself can induce immune responses in gilthead seabream Sparus aurata.

In this study, relevant changes in the intestinal microbiota of gilthead seabream specimens fed with the diets assayed have been demonstrated, and an important effect on the intestinal microbiota by the dietary administration of a probiotic mixture has also been detected, especially in fish receiving the diet supplemented with a symbiotic mixture for 4 weeks. These microbial
changes coincided in the same time with a up-regulation the expression of immunological genes in skin and head kidney of gilthead seabream at 2 and 4 weeks, respectively. It would be very suggestive to think that this up-regulation expression of genes could be related with the changes produced on the fish intestinal microbiota. Looking at the molecular level, it is clear from our study that the yeast D. hansenii L2 in conjunction withulin enhanced the immune response in terms of immune gene expressions in seabream. However, more studies are necessary to understand the potential effects of the intestinal microorganisms on the immune system of fish.

Acknowledgments

Tapia-Paniagua wishes to thank the Ministerio Español de Educación y Ciencia for a F.P.U. scholarship. M.R.L. received postdoctoral scholarships from the Ministerio de Ciencia e Innovación (AGL2008-05119-C02-01 and AGL2008-05119-C02-02, Spain) and Fundación Séneca de la Región de Murcia (06232/GERM/07).

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