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Changes in intestinal microbiota, immune- and stress-related transcript levels in Senegalese sole (*Solea senegalensis*) fed plant ingredients diets intercropped with probiotics or immunostimulants

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Abstract

Senegalese sole (*Solea senegalensis*) is a highly valued flatfish that grows well with diets containing plant ingredients but their effects on immune competence is still a matter of debate. The current study aimed to examine changes in innate immune parameters and gut microbiota in Senegalese sole fed with 35% or 72% of plant ingredients with or without probiotic or yeast supplementation. Overall, fish fed diets with 72% of plant ingredients showed lower transcript levels of key immune- and stress-related genes in distal intestine, rectum and head-kidney than the 35% diets. In particular, *hsp90b* mRNA levels in distal intestine were down-regulated by 70% and 60% with the use of high content of plant ingredients in the diet containing the multispecies probiotic and autolyzed yeast, respectively. Denaturing gradient gel electrophoresis showed lower similarity values for distal intestine than rectum. Also fish fed high content of plant ingredients displayed lower similarity values, pointing to a difference in the microbial populations between fish fed different plant ingredients content on the diet. Our data revealed that inclusion of plant ingredients was associated with differences in gene expression and a more diverse microbiota profile but without a significant effect on growth performance. Moreover, probiotic supplementation resulted in up-regulation of *hsp90b*, *gpx*, *cat* and *apoa1* transcript levels in distal intestine concomitantly with a growth rate reduction compared to non-supplemented fish.

1. Introduction

Significant advances have been made in alternative protein diet formulations, effectively replacing a large proportion of fishmeal in aquafeeds. Formulated commercial feeds are composed of several ingredients, mixed in various proportions to complement each other, and form a nutritionally complete compounded diet. In Europe, the commercial aquafeeds are largely dependent on soybean products and corn or wheat products (Rana, et al., 2009). A number of different plant ingredients have been successfully used as fishmeal replacement in fish feed (Kaushik, et al., 1995) without reducing growth performance. The ability of Senegalese sole (*Solea senegalensis*) to efficiently use diets with high levels of plant protein (PP) sources has been widely reported (Cabral, et al., 2011; Silva, et al., 2009; Valente, et al., 2011) and is an important asset towards the intensification and commercialization of this species. Recent studies showed that sole could effectively use practical diets containing up to 75% of PP sources, without affecting feed intake, growth performance or nutrient utilization whilst preserving the nutritional fillet value (Cabral, et al., 2013). Nevertheless, the extent to which high levels of plant ingredients may affect health conditions of fish, including the anti-oxidant and immune capacity remains controversial. The suppression of the innate immune response upon feeding high amounts of plant protein ingredients has been reported in rainbow trout (Burrells, et al., 1999) and in gilthead seabream, *Sparus aurata* (Sitjà-Bobadilla, et al., 2005). Conversely, another study using soybean in rainbow trout (Rumsey, et al., 1994) showed an increase in the innate immune parameters examined. There are limited studies focused on the immune responses of the Senegalese sole, particularly using a multifactorial approach; i.e. interactions between biotic and abiotic stressors (reviewed by Morais, et al. (2014)).

Prebiotics and probiotics are increasingly used as preventive therapy, with high success rates in reducing the incidence of diseases and increasing fish resistance to infection (Gaggìa, et al., 2010). The use of probiotics has increased in the aquaculture sector due to its success in livestock production (Fulton, et al., 2002) and in human health (Gill, 2003). The probiotic genera commonly used in animal feeding are *Saccharomyces*, *Lactobacillus*, *Enterococcus* and *Bacillus* (Gaggìa, et al., 2010). *Lactobacillus* are common bacteria in the gut microbiota of humans and animals, whereas their occurrence and number are host dependent. *Enterococcus* as well as *Lactobacillus* belong to the lactic acid bacteria (LAB) group and are found naturally in food products.

Bacillus are Gram-positive spore-forming microorganisms and are normally allochthonous microbes to the intestinal tract as a result of ingestion (Gaggìa, et al., 2010).

The use of a multispecies probiotic, from one or preferentially more genera, has provided superior results over monostrain probiotic preparations (Timmerman, et al., 2004). Paubert-Braquet, et al. (1995) tested mono and multistrain *Lactobacillus* in mice challenged with *Salmonella typhimurium* and observed a clear protective effect of the multistrain probiotic preparation. Modulation of the innate humoral and cellular defences is one of the benefits of probiotic treatment described in fish (Nayak, 2010), since the innate immune system is the first line of host defense against pathogenic organisms (Sinyakov, et al., 2002). The inter-relationship between gut mucosal epithelial cells, mucus, antimicrobial products, and organisms resident in the gut and immune cells in the mucosa/sub-mucosa are vital for the health and well-being of the fish (Merrifield, et al., 2010b). Several studies have demonstrated the immunological and haematological stimulation of fish defence mechanisms by probiotic bacteria (Brunt, et al., 2008; Pieters, et al., 2008). In fish most of the beneficial effects, have been recorded within a dietary probiotics feeding regime of 1-10 weeks (Nayak, 2010). The time course for optimum induction of immune response differs with respect to probiotic strain and also with the type of immune parameter (Choi and Yoon, 2008). Batista, et al. (2014) studied the effects of dietary probiotic supplementation (mono-species and multi-species) in Senegalese sole (*Solea senegalensis*) juveniles during a 1-month trial and this time duration was not able to produce any effect on growth or humoral innate immune response. The short-time probiotic feeding may lead to failure of the probiotic strains to establish themselves in the fish gut and therefore long-time administration should be investigated.

As the use of plant protein sources to replace fish meal is a major trend in aquafeeds, it is important to gather health-related information to confirm the suitability of the newly developed plant-based diets in farmed fish. Therefore, the aim of this study is to evaluate the effects of two dietary plant protein levels intercropped with probiotic supplementation on the growth performance, innate immune response and intestine microbiota diversity in sole (*Solea senegalensis*).

2. Materials and methods

The current study was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.1. Feeding experiment

The Senegalese sole feeding experiment took place at the CIIMAR facilities (University of Porto, Porto, Portugal). Fish were fed for 73 days, in triplicate, with six isoproteic (55% crude protein) and isolipidic (8% lipid) diets with similar amino-acid composition (Table 1 and supplementary table S1). Two practical diets were formulated to contain either low or high plant protein (PP) inclusion levels (35% and 72%, respectively). Each of these diets were supplemented with either PROB or YEAST, and compared with the unsupplemented ones (UN). The duration of the trial was selected as the best option to ensure the establishment and multiplication of probiotic strains in the fish gut while allowing diets to impact on fish growth. Diets were extruded (3 mm pellet) by the use of a pilot-scale twin-screw extruder (Clextral BC45). PROB was a multispecies probiotic bacteria (AquaStar® Growout: *Bacillus* sp, *Pediococcus* sp, *Enterococcus* sp, *Lactobacillus* sp) incorporated at 1.34×10^{10} CFU kg^{-1} diet (CFU – colony forming unit) in the extruded pellets by means of vacuum coating (Dinnisen Pegasus vacuum mixer, PG-10VCLAB) using fish oil as a carrier. YEAST was autolyzed yeast (Levabon® Aquagrow E: *Saccharomyces cerevisiae*) supplemented in the mixture at 4g kg^{-1} diet. Diets were prepared according to the normatives ISO (6887-1:1999, 7218:2007, 6498:2012) for the microbiological examinations. Thereafter, the isolation and enumeration of bacteria in the diets followed the European standards for *Enterococcus* spp. (EN 15788:2009), *Lactobacillus* spp. (EN 15787:2009), *Pediococcus* spp. (EN 15786:2009) and *Bacillus* spp. (EN 15784:2009). Quantification of viable bacteria in the formulated diet following blending was performed by Biomin (Austria) using the plate count method. Bacterial viability was examined immediately prior to the feeding trial and the diets were kept at 4 °C for the duration of the experiment.

Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and transported to the rearing facilities of CIIMAR. After 5-week acclimation, fish of similar weight and length were distributed into 18 fibreglass tanks of 50 L, supplied by a recirculation system. Fish were at a density of 5.8 kg m^{-2} (31 fish per tank with a mean

initial body weight of 33.1 ± 0.2 g). Of the 18 tanks, nine received 35% PP (PP35) the PP35 diet and the other nine 72% PP (PP72) diet. Of the nine from each diet, three received the PROB supplementation, three YEAST and three were unsupplemented. Rearing conditions, such as salinity (32.0 ± 0.1 ppm), temperature (19.7 ± 0.5 ºC), ammonia (0.33 ± 0.27 mg L^{-1} NH_3), nitrite (0.14 ± 0.07 mg L^{-1} NO_2) and flow rate of 1.5 L min^{-1}, were monitored during the feeding entire trial. The recirculation system was equipped with UV lights and ozone generator to hamper bacteria growth in the rearing water. Fish were fed to apparent satiety based on visual observation of acceptance and refusal of feed using temporized automatic feeders. To prevent stressful conditions, high stocking densities and repeated handling stress were avoided.

2.2. Sampling procedures

Prior to sampling, fish were fasted for 24 h and then sacrificed with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg L^{-1}). All fish were individually weighed and measured at the beginning and at the end of the experiment in order to determine growth performance. Ten fish from the initial stock and two fish from each tank at the end of the trial (6 fish per treatment) were sampled and stored at -20 ºC for body composition analyses. For the evaluation of humoral innate immune parameters (lysozyme, peroxidase and alternative complement pathway – ACH50) blood was withdrawn from the caudal vein of 9 fish per treatment using heparinised syringes and centrifuged at 5000 × g for 10 min at 4 ºC to collect the plasma. The liver and viscera weights were recorded to calculate HSI (hepatosomatic index) and VSI (viscerosomatic index). The posterior intestine was aseptically sampled and divided in two sections (distal intestine and rectum). The intestinal microbiota, including the probiotic bacteria tested, were determined in both intestine sections, separately. The expression of immune-related genes, were quantified (RT-qPCR) in the head-kidney, distal intestine and rectum. All samples were stored at -80 ºC for further analyses.

2.3. Chemical analyses of diets and body composition

All chemical analyses were carried in duplicate according to Aoac (2006). Fish were minced without thawing using a meat mincer, pooled and used to determine their dry matter content (105 °C for 24 h). Fish were then freeze-dried and analysed for dry matter, ash (Nabertherm L9/11/B170; Germany; 550ºC for 6 h), crude protein (N×6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid

(petroleum ether extraction, 40–60ºC, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

2.4. Humoral innate immune parameters

All measurements were done in triplicate on a Power-Wave™ microplate spectrophotometer (BioTek Synergy HT, USA). Plasma lysozyme activity (EU ml^{-1}) was determined using a turbidimetric assay adapted to microtitre, as described by Hutchinson and Manning (1996). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in absorbance of levels per min. Plasma peroxidase activity (EU ml^{-1}) was measured following the procedure adapted to *Solea senegalensis* by Costas, et al. (2011), defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL-1; RaRBC), as reported by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of RaRBC.

2.5. Immune- and stress-related gene expression

Except for lysozyme c (*lyzc*), target and reference genes used in this study were based on published information (Table 2). The immune-related genes selected are: lysozymes (*lyzc* and *lyzg*), heat shock proteins (*hsp90a*, *hsp90b* and *hsp90b1*), iron chelating proteins (*ftm* and *apoa1*), complement factors (*c3a1* and *c3a2*), the cysteine protease *casp3* and oxidative enzymes (*cat* and *gpx*).

2.5.1. RNA extraction and cDNA synthesis

Senegalese sole tissues (head-kidney, distal intestine or rectum) were homogenised with Lysing matrix D beads for 20 s at 5500 rpm on a MagNA Lyser instrument (Roche) and used for RNA extraction following the QIAzol protocol (Qiagen). Assessment of RNA quality, RNA quantification and cDNA synthesis followed the methodology described by Campos, et al. (2010).

2.5.2. Quantitative real-time PCR (qPCR)

Quantification of mRNA levels was done by qPCR in LightCycler 480 (Roche) using SYBR green (Qiagen), as detailed elsewhere (Campos, et al., 2010; Fernandes, et al., 2008). Briefly, 10 µL reactions were prepared in 96-well plates and included 4 µL of 25

×-diluted cDNA template, 1 µL of each primer pair at 5 µM and 5 µL of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). Samples were denatured (15 min at 95 ºC) and then amplified (45 cycles), according to the following thermocycling profile: denaturation for 10 s at 95 ºC, annealing for 20s at 60 ºC and extension for 20 s at 72 ºC. Six-point standard curves of a 2-fold dilution series (1:1–1:32) from pooled cDNA were used for PCR efficiency calculation. Specificity of the qPCR reactions was determined by melting curve analysis and further confirmed by Sanger sequencing of the PCR product, as reported by Campos, et al. (2010). Data were analysed by the relative quantification method after normalization using the geometric mean of the best reference genes (Fernandes, et al., 2008).

2.6. *PCR amplification and denaturing gradient gel electrophoresis (PCR + DGGE)*

The DGGE analysis of PCR-amplified genes coding for 16S rRNA was carried out in the distal intestine and rectum (2 fish / treatment) to make a qualitative study of the microbial community in the intestine. DNA was extracted using cetyltrimethylammoniumbromide (Zhou, et al., 1996). For the detection of the multispecies bacteria PROB, the PCR amplification of DNA was carried out using the primer combination of 341F with GC clamp, CCTACGGGAGGCAGCAG and 907R, CCGTCAATTCMTTTGAGTTT (Muyzer, et al., 1995). Yeast was detected in animals by PCR amplification using *Saccharomyces cerevisiae* specific primers combination of SCIF, GTGCTTTTGTTATAGGACAATT and SI5R, AGAGAAACCTCTCTTTGGA (Chang, et al., 2007). PCR amplification by a modified touchdown protocol was performed as described Dhanasiri, et al. (2011), except for the temperature of the initial denaturation step (96^{o}C). DGGE was performed on the PCR products from DNA samples using a 16 × 16 cm gel on the Bio-Rad DCode ™ system (Bio-Rad, USA), as described by Dhanasiri, et al. (2011) but using a 30 to 55% (w/v) urea and formamide denaturing gradient. PCR products from distal intestine and rectum sections were loaded into two separated gels (6 fish/ treatment) and electrophoresis was performed with 0.5% TAE buffer, at 20 V for 15 minutes and then at a constant voltage of 75 V for 17 hours. DGGE profile analysis (Quantity One® version 4.6.3 software, Bio-Rad) was performed for gels A and B (Fig. 1) to determine similarity matrices (Dhanasiri, et al., 2011). Bands of interest were excised, purified and sequenced. The results were subjected to BLAST sequence similarity search using BLASTN from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

2.7. Calculations of growth performance

Feed conversion ratio was calculated as: dry feed intake (g) / wet weight gain (g), and the daily growth index (% BW day^{-1}) as: $100 \times [(W_1)^{1/3} - (W_0)^{1/3}]$ / days, where W_0 and W_1 are the initial and the final fish mean weights in grams and days is the trial duration. Voluntary feed intake (% BW day^{-1}) was calculated as: 100 × (dry feed intake (g) / average BW (g) / days), where average BW was calculated as: $(W_1+W_0) / 2$. The protein efficiency ratio was calculated as weight gain (g) / protein ingested (g). The hepatosomatic index was calculated as: 100 × [liver weight (g) / whole body weight (g)] and the viscerosomatic index as 100 × [viscera weight (g) / whole body weight (g)].

2.8. Statistical analysis

Statistical analyses were performed with the software SPSS (IBM SPSS STATISTICS, 17.0 package, IBM Corporation, New York, USA). Results are expressed as mean ± standard deviation (SD_{pooled} as weighted average of each group's standard deviation) and the level of significance used was $P \leq 0.05$. Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were log-transformed whenever necessary. Data were analysed by a two-way ANOVA with diet and probiotic as main factors. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were carried out to identify significantly different groups. When data did not meet the ANOVA assumptions, a non-parametric Kruskal–Wallis test was performed for each factor. Evaluation of expression stability of reference genes was done using the statistical application geNorm (Vandesompele, et al., 2002). Expression of target genes was evaluated by the relative quantification method as reported in Fernandes, et al. (2008). Heat maps of transcript levels were produced using PermutMatrix software, with the Euclidean distance clustering algorithm and gene expression normalized for rows.

3. Results

3.1. Growth performance

Data from growth performance are presented in table 3. Fish grew from 33.1 ± 0.20 g to 50.6 ± 1.2g (PP72_UN). Growth performance did not differ between PP35 and PP72 groups. PROB groups had significantly lower final body weight (45.0 ± 1.9) and daily growth index (0.5 ± 0.1) compared to UN groups (50.5 ± 2.0 and 0.7 ± 0.1,

respectively). Additionally, UN groups had significantly better feed conversion ratio (1.5 ± 0.1) and higher protein efficient (1.3 ± 0.1) than the probiotic supplemented groups. Voluntary feed intake was also lower in UN groups (0.8 ± 0.0), and differed significantly from YEAST groups (0.9 ± 0.1). Visceral somatic index and hepatosomatic index did not differ between treatments.

3.2. Humoral innate immune parameters

After the 73-day feeding trial, humoral immune parameters did not present any significant differences between treatments (supplementary table S2). Lysozyme, peroxidase and ACH50 varied between 1225.9 ± 251.0 and 2061.7 ± 366.2 EU mL^{-1}, 22.5 ± 9.0 and 50.3 ± 14.1 EU mL^{-1} and 88.2 ± 14.0 and 110.3 ± 14.9 U mL^{-1}, respectively.

3.3. Immune- and stress-related gene expression

Expression of immune-related genes is presented in Fig. 2 and supplementary tables S3, S4 and S5. Distal intestine transcript levels were significantly affected by plant ingredients content (*hsp90b* and *apoa1*) and by probiotic supplementation (*hsp90b1* and *gpx*). PP72 groups have lower values for *hsp90b* (1.4 ± 0.8) and *apoa1* (9.6 ± 5.4) genes, compared to PP35 groups (3.2 ± 1.9 and 14.9 ± 7.3, respectively). YEAST groups presented higher values for *hsp90b* (3.4 ± 1.9) compared to PROB (2.1 ± 1.8) and UN (1.5 ± 0.8) groups. However for *hsp90b1* and *gpx* expression, PROB groups (4.1 ± 2.4 and 1.1 ± 0.4 respectively) had higher values compared to YEAST (1.8 ± 0.5 and 0.6 ± 0.3 respectively) and UN (2.2 ± 1.0 and 0.7 ± 0.3 respectively) groups. Considering the distal intestine *cat* expression, PP35_PROB (21.3 ± 12.1) was significantly higher than PP72_YEAST (7.0 ± 2.3) treatment. No significant differences were detected in distal intestine gene expression for lysozymes (*lyzc* and *lyzg*), *c3* complement components (*c3-1* and *c3-2*), *hsp90a*, *ftm* and *casp3* genes.

Similarly to distal intestine, some rectum genes transcript levels were affected by the use of plant ingredients. PP72 groups (*casp3*: 2.0 ± 0.6, *gpx*: 0.1 ± 0.0 and *cat*: 2.3 ± 0.6) showed significantly lower values compared to PP35 groups (*casp3*: 2.6 ± 0.6, *gpx*: 0.2 ± 0.1 and *cat*: 2.9 ± 0.8). Rectum *hsp90a* expression was two-fold higher for PROB (0.04 ± 0.02) groups compared to YEAST (0.02 ± 0.01) groups. Rectum *ftm* expression was higher for UN (3.3 ± 0.6) groups compared to PROB (2.5 ± 0.8) groups but not different to YEAST (2.7 ± 0.7) groups. *apoa1* expression was significantly higher in

PP35_YEAST (1.3 ± 1.1) in rectum, compared to PP35_UN (0.0 ± 0.1), PP35_PROB (0.1 ± 0.2) and PP72_YEAST (0.2 ± 0.2). No significant differences in transcript levels for lysozymes (*lyzc* and *lyzg*), heat shock proteins (*hsp90b* and *hsp90b1*), complement components (both *c3* analysed) were detected in rectum.

In head-kidney, only mRNA levels of *hsp90b1* were significantly lower in PP72 (0.4 ± 0.2) than in PP35 (0.6 ± 0.2) groups.

3.4. Probiotic detection and gut microbiota profiles

The marker bands (PROB bacteria profile) are present in samples of fish that have been fed the multispecies bacteria probiotic (Fig. 1A). A comparison of the DGGE profiles (Table 4) between distal intestine and rectum tissues revealed lower similarity values for distal intestine (34.4 – 54.9%) than rectum (47.9 – 72.4%), showing higher microbiota diversity in distal intestine. We also observed that fish fed PP72 diets have a tendency to display a lower similarity value compared with fish fed PP35 diets (34.4 – 54.9% for distal intestine and 47.9 – 72.4% for rectum).

In distal intestine, comparison of the profiles between PP35_UN *vs* PP72_UN revealed similarity values less than 34.4%, pointing to a difference in the microbial populations between fish fed different plant ingredients content on the diet. However, when adding the probiotic to the diet, similarity values, in distal intestine, increase (54.9% for PROB and 46.3% for YEAST).

4. Discussion

It is well recognised that plant ingredients have to be increasingly employed in aquafeeds to cater to the demand of the industry. However, the application of these ingredients at relatively high levels in the diets of some carnivorous fish species may cause nutritional imbalances and influence the immune response as they may contain anti-nutritional factors (Hardy, 2010). In order to cope with the need to depend on the plant materials in fish feeds, efforts have to be made to alleviate the negative influence of these components may have on fish. Our approach in this direction has been to exploit the potential of probiotic organisms to counter any negative influence that may arise upon increasing the levels of plant components in fish diets. Senegalese sole that received plant diets supplemented with the selected probiotics seems to have altered immune and stress responses compared to fish receiving plant diets that lacked the probiotics. Our findings are discussed mainly based on the expression of the immune

and stress-related genes in the intestinal segment which is considered as an immunologically relevant region in fish (Rombout, et al., 2011) where the applied diets can have a direct impact on the elicited responses.

In fish, the induction of heat shock proteins (*hsp*) is a component of the cellular stress response against a diversity of stressors, such as osmotic stress, heat shock or infections (Basu, et al., 2002). *hsp90b1* (also known as *gp96* or *grp94*) is the primary chaperone of the endoplasmic reticulum and has crucial immunological functions, serving as a natural adjuvant for priming innate and adaptive immunity (Strbo and Podack, 2008). No differences in *hsp90a* expression were detected between treatments in the present study, regardless of the tissues examined. Distal intestine *hsp90b* and head-kidney *hsp90b1* mRNA levels were significantly affected by diets, with lower expression in fish fed higher plant ingredients inclusion level. In contrast, high levels of plant protein in the feeds did not affect the expression of *hsp70* and *hsp90* in Atlantic cod (*Gadus morhua* L.) (Hansen, et al., 2006). Interestingly, considering the effect of the probiotic, Senegalese sole fed yeast supplemented diets had higher *hsp90b* expression in the distal intestine, while fish fed multispecies probiotic displayed higher *hsp90b1* and *hsp90a* transcript levels in distal intestine and rectum, respectively.

In addition to their well-known role in reverse cholesterol transport and lipid metabolism, apolipoproteins display anti-inflammatory, antimicrobial and antioxidant activities (Barter, et al., 2004; Tada, et al., 1993). The main high density lipoprotein, Apoa1, has antioxidant properties (Barter, et al., 2004) and is involved in regulation of fish complement (Magnadóttir, 2006).

Rectum *apoa1* expression, from fish fed PP35 diet, was up-regulated by the yeast supplementation (PP35_YEAST). However, the same gene *apoa1* was down-regulated in the distal intestine from fish fed PP72 diet compared with PP35. Borges, et al. (2013) reported that *apoa1* expression in sole is not regulated by dietary lipid levels and our data indicate that the use of different protein sources may affect *apoa1* expression.

Some probiotics are known to be effective in enhancing the natural complement activity in fish (Choi and Yoon, 2008; Panigrahi, et al., 2007; Salinas, et al., 2008). In our study, no significant differences were observed in transcript levels of two *c3* paralogues in distal intestine, rectum or head-kidney, related to the dietary treatment. Expression of the key effector caspase *casp3* did not change with treatment in distal intestine and head-kidney, but in rectum, the transcript level were significantly lower for PP72 groups compared to PP35 groups, suggesting that plant ingredients could be associated

with a reduction in apoptotic activity. Nevertheless, in mice, Van Breda, et al. (2005) observed that 7 genes involved in apoptosis were up-regulated by consumption of a 40% plant protein diet.

Certain nutrients or immunostimulants, including probiotics, can be supplemented in the feed to modulate serum lysozyme activity in fish (Kim and Austin, 2006). In our study, none of tissues analysed presented treatment-related differences for *lyzc* and *lyzg* transcript levels. Synthesis of ferritin is repressed under conditions of iron deprivation (Torti and Torti, 2002) and the inclusion of probiotic PROB in the diet down-regulated *ftm* in Senegalese sole rectum, compared to unsupplemented diets. It is plausible that oxidative stress in the rectum, which may have indirectly mobilised iron (Pantopoulos and Hentze, 1995), accounting for the observed *ftm* down-regulation. Further the up-regulation of *gpx1* and *cat* transcript levels in the distal intestine could also be indicating an antioxidative effect of dietary probiotic supplementation. Catalases are a class of enzymes that facilitates the dismutation of hydrogen peroxide to oxygen and water (Nicholls, 2012) and *gpx1* is as an enzyme counteracting oxidative stress due to its hydroperoxide-reducing capacity (Brigelius-Flohe and Maiorino, 2013). Rueda-Jasso, et al. (2004) suggested a relationship between *cat* activity and diet composition (lipid level and starch type). Nutritional imbalances and diet composition may play a role in oxidation processes and antioxidation defense mechanisms (Rueda-Jasso, et al., 2004). In Senegalese sole, dietary plant ingredients were associated with a decrease in *cat* and *gpx1* transcript levels in rectum, compared to control groups, thus indicating that plant ingredients may have an impact on antioxidation defense mechanisms.

The stimulatory effect of probiotics on the innate immune system of fish has been reported (Nayak, 2010). However, complement, lysozyme and peroxidase activities did not differ between probiotic and control groups in the present study. These results suggest that the immunostimulatory effect of probiotics varies greatly among fish species and probiotic strains, and is in agreement with previous reports in Senegalese sole (Batista, et al., 2014) and rainbow trout (Merrifield, et al., 2010a). The use of probiotics or dietary raw materials that could modulate the microbiota to prevent pathogen colonisation has the added advantage of enhancing animal health (Tuohy, et al., 2005) but this is still poorly understood in flatfish, including Senegalese sole. In the present study, the presence of the bacterial probiotic consortium and the yeast *Saccharomyces cerevisiae* in the intestine was confirmed intestine of animals fed PROB and YEAST treatments, respectively. It should be noted that a band for *Lactobacillus* sp

was found in all gut samples regardless of probiotic supplementation, corroborating a previous report that lactobacilli are part of the natural gut flora in Senegalese sole (Tapia-Paniagua, et al., 2015).

It was observed that fish fed PP72 diets had a higher number and diversity of bacteria in their gut compared to the PP35 diets. Microbial diversity was also affected by soybean meal in gilthead sea bream (*Sparus aurata*) (Dimitroglou, et al., 2010) and Atlantic salmon (*Salmo salar*) (Bakke-Mckellep, et al., 2007) where fish fed the soybean meal diet had higher total number as well as a more diverse population composition of adherent bacteria in the distal intestine. Intestinal microbiota protects against infections and actively exchanges regulatory signals with the host to prime mucosal immunity (Gaggìa, et al., 2010). A recent report on Atlantic salmon, demonstrated the ameliorating effect of a microbial additive on combating intestinal inflammation and establishing intestinal homeostasis (Vasanth, et al., 2015). In Senegalese sole, the distal intestine contained a higher microbiota diversity compared to rectum, which may indicate variable immune properties across the different parts of the intestine (Inami, et al., 2009). In addition to ascertaining microbial diversity by molecular methods, we used a conventional microbiological approach to identify culturable bacteria in the gastrointestinal tract. Dietary probiotic supplementation caused an increase in the proportion of *Bacillus* sp, concomitantly with a reduction in *Vibrio* sp content (Batista, et al., unpublished).

Growth performance did not differ between from PP35 and PP72 groups, showing that Senegalese sole copes well with diets in which animal protein is replaced by plant source, in accordance to the literature (Silva, et al., 2009). It is noteworthy that the use of dietary probiotic supplementation produced a significant decrease in growth performance. In fact, there are contradictory reports on the effect of probiotics and prebiotics on growth performance in fish. For example, the use of lyophilized probiotic positively influenced the growth performance, promoting the feed efficiency and growth performance in Atlantic cod (Lauzon, et al., 2010), rainbow trout (Merrifield, et al., 2010a; Ramos, et al., 2015), Japanese flounder (Taoka, et al., 2006) and sea bream (Dawood, et al., 2015). In contrast, Gunther and Jimenez-Montealegre (2004) and García De La Banda, et al. (2012) showed that lyophilized probiotic supplementation did not improve growth performance in Nile tilapia and in Senegalese sole, respectively. Moreover, Ridha and Azad (2015) have recently reported that dietary supplementation with a commercial *Lactobacillus acidophilus* probiotic resulted in growth depression in

juvenile Nile tilapia. In the present study, this intriguing effect cannot be explained by a reduction of appetite, since there were no differences in voluntary feed intake. Lower feed conversion ratios in fish fed the unsupplemented diet suggest that the observed reduction in growth performance with probiotic supplementation may be due to diminished digestibility. It is plausible that probiotic bacteria may interfere with absorption efficiency by altering the microbial ecology and even morphology of the intestine (e.g., length, width, surface area of the villi, number of goblet cells and muscle layer thickness).

5. Conclusion

Our data revealed that inclusion of probiotics and plant ingredients in the diet was associated with differences in immune- and stress-related gene expression. Overall, fish fed PP72 diets showed lower transcript levels than the PP35 diets. In particular, multispecies bacteria supplementation resulted in up-regulation of genes involved in the antioxidative stress response (*gpx* and *cat*) in distal intestine, concomitantly with the down-regulation of *ftm* mRNA in rectum. Moreover, the distal intestine of *S. sole*, showed higher microbiota variability than rectum. Inclusion of plant ingredients was associated with a more diverse microbiota profile with no effect on growth performance.

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Figure Legends

Figure 1 – DGGE images showing bands corresponding to the intestinal bacteria present in the distal intestine wall (A) and rectum wall (B) samples of Senegalese sole juveniles that were fed for 73 days with diets with or without probiotic/ yeast supplementation. Lanes: 1 and 14 – probiotic bacteria marker; 2 and 3 – PP35_UN; 4 and 5 – PP35_PROB; 6 and 7 – PP35_ YEAST; 8 and 9 – PP72_UN; 10 and 11 – PP72_PROB; 12 and 13 – PP72_ YEAST. Arrowheads with numbers indicate the bands that were excised and sequenced for molecular identification (1 - *Enterococcus* sp; 2 - *Lactobacillus* sp; 3 - *Bacillus* sp; 4 - *Enterococcus* sp; 5 – *Pediococcus* sp), n=2 fish per treatment group.

Figure 2 – Heat maps showing the normalized mRNA levels of selected genes in the distal intestine, rectum and head kidney tissues of Senegalese sole juveniles after the 73-day feeding trial. Each block represents the mean mRNA level of 6 fish quantified by qPCR. Letters indicate significant differences ($P<0.05$).

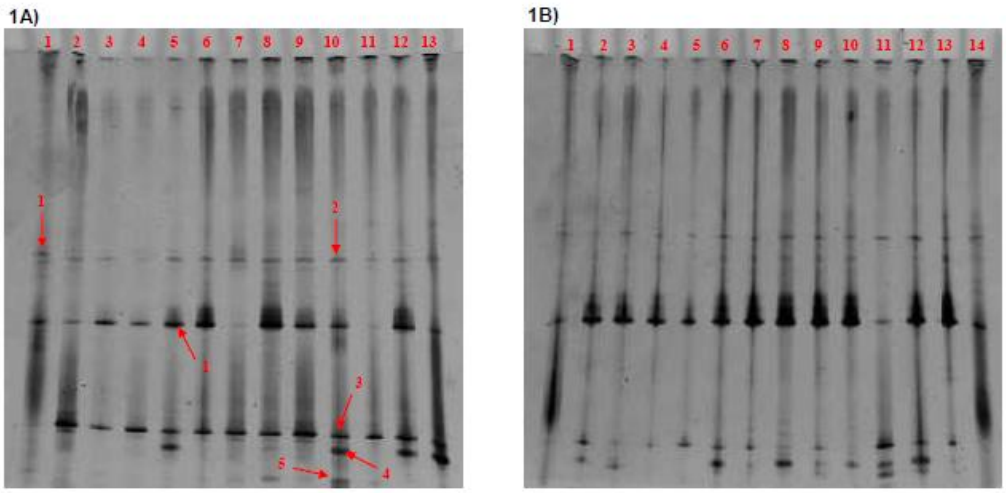

Figure 1

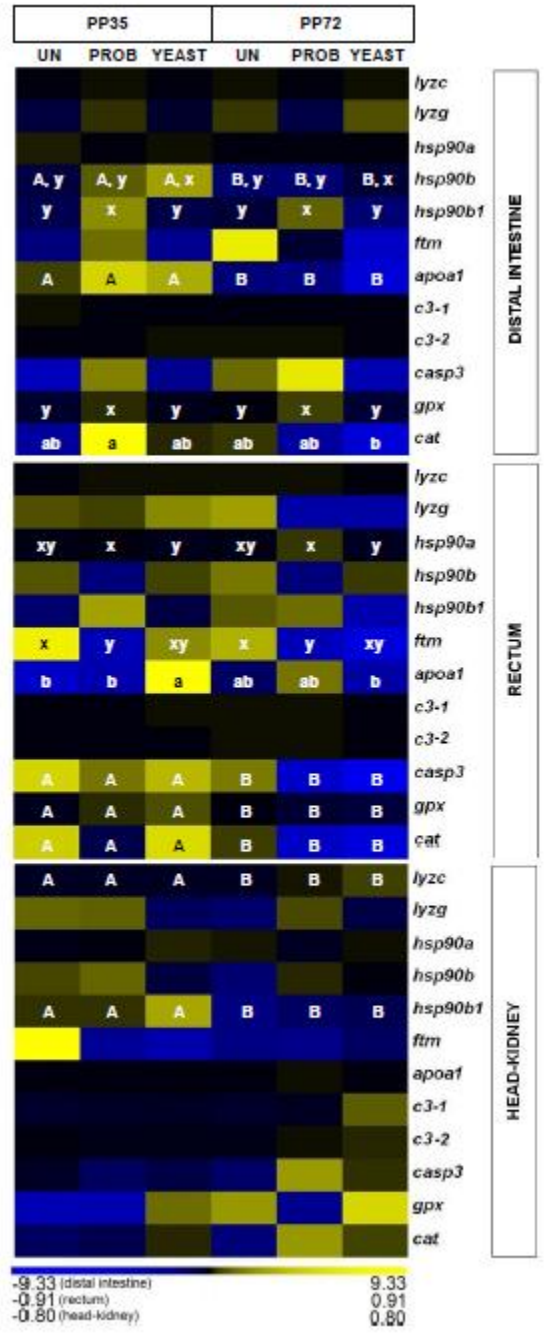

Figure 2

Table 1 - Feed ingredients and proximate composition of the experimental basal diet

	Diets	
	PP35	***PP72***
Feed ingredients (%)		
Fishmeal 70 L	24.5	5.5
Fishmeal 60	27.0	0.0
CPSP[a]	5.0	5.0
Squid meal	5.0	5.0
Pea (Lysamine GP)	0.0	14.0
Soycomil PC	0.0	6.0
Soybean meal	12.5	9.0
Potato concentrate	0.0	6.0
Wheat gluten	0.0	7.0
Corn gluten	0.0	9.0
Extruded peas (Aquatex G2000)	11.0	11.5
Wheat meal	11.0	9.6
Fish oil	2.0	5.7
Vitamin[b] and Mineral Premix[c]	1.0	1.0
Di-calcium phosphate	0.0	4.0
L-Lysine	0.0	0.5
DL-Methionine	0.0	0.2
Binder (Alginate)	1.0	1.0
Proximate composition		
Crude protein (%DM)	54.43	53.31
Crude fat (%DM)	11.75	11.98
Gross Energy (kj g−1DM)	21.04	21.98

DM, dry matter

[a] Soluble fish protein hydrolysate (75% crude protein, Sopropêche, France)

[b] Vitamins (mg or IU kg^{-1}diet): Vitamin A (retinyl acetate), 20000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg;

[c] Minerals (% or mg kg^{-1}diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulfate), 9 mg; Co (cobalt sulfate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulfate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18,6%; NaCl (sodium), 4%.

Table 2 – List of the reference and target gene primers used for real-time PCR

Gene	Accession	Reference	Forward sequence (5'→ 3') Reverse sequence (5'→ 3')	Product size (bp)	Ta (ºC)	Efficiency
Reference genes						
eef1a1	AB326302	(Campos, et al., 2010)	ATTGGCGGCATTGGAACA CATCTCCACAGACTTGACCTC	117	60	2.03
rps4	AB291557	(Campos, et al., 2010)	CTGCTGGATTCATGGATGTG GGCAGTGATGCGGTGGAC	103	60	2.10
Target genes						
lyzc	EU380795	N/A	ATCAGAGCCTGGGTTGCC TTAAACTCCACATCCTCTCACG	42	60	1.92
lyzg	AB428773	(Salas-Leiton, et al., 2010)	ACTGCTCGCGGTGAATGGGACA CCTGAAAATTTATTACGGATTCGGCCAATG	96	60	1.99
hsp90a	AB367526	(Manchado, et al., 2008)	GACCAAGCCTATCTGGACCCGCAAC TTGACAGCCAGGTGGTCCTCCCAGT	79	70	2.18
hsp90b	AB367527	(Manchado, et al., 2008)	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA GCCAAGGGGCTCACCTGTGTCG	148	60	1.88
hsp90b1	FJ263549	(Osuna-Jiménez, et al., 2009)	GAGTCTTCTCCCTTTGTTGAGCGGCTG TGATGCCTTCCTTTGCCACGTTCTG	142	70	2.00
ftm	FF682434	(Prieto-Alamo, et al., 2009)	ATGGAGTCTCAAGTGCGTCGGAACTACCAC CATGCTCCTTCTCCTCCTCGCTGTTCTC	171	60	1.80
apoa1	FF283994	(Tingaud-Sequeira, et al., 2009)	TTGAGGCTAATCGTGCCAAA CCTGCGTGCTTGTCCTTGTA	76	60	1.97
c3-1	FJ345403	(Makridis, et al., 2009)	TATAAGAACAAGGATCACGATG GGTAGATGATCAATGAACCTC	106	54	2.01
c3-2	FF682240	(Prieto-Alamo, et al., 2009)	ACCTTAGACTGCCCTACTCTGCTGTCCGTG GCACTGCACACATCATCCGTCTCAGAC	147	60	2.26
casp3	HQ115741	(Costa, et al., 2011)	CATCATCAACAACAAGAACTTTGACG ATGGTCTTCCTCCGAGGCTT	182	60	1.83
gpx	HM068301	(Costa, et al., 2011)	ATGAACGAGCTGCACTGTCG AGATAGACAAACAAGGGGTGTG	208	60	2.10
cat	GU946411	(Costa, et al., 2011)	TGAGCAGGCTGAAAAGTTCC GGCATGTTACTTGGGTCAAAG	163	60	1.90

Table 3 – Growth performance of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	Diet	Supplementation	Diet*Supplementation
	UN	PROB	YEAST	UN	PROB	YEAST				
IBW (g)	33.03	33.16	33.02	33.16	33.04	33.25	0.21	0.44	0.95	0.35
FBW (g)	50.49^{x}	45.73^{y}	45.82^{xy}	50.58^{x}	44.21^{y}	47.73^{xy}	2.91	0.91	0.02	0.60
DGI (g kg^{-1} BW day^{-1})c	0.67^{x}	0.50^{y}	0.51^{xy}	0.67^{x}	0.45^{y}	0.56^{xy}	0.10	0.98	0.02	0.68
FCR (g g^{-1})	1.46^{y}	2.04^{x}	2.11^{x}	1.49^{y}	2.42^{x}	2.02^{x}	0.34	0.53	0.01	0.51
VFI (g kg^{-1} BW day^{-1})	0.83^{y}	0.88^{xy}	0.90^{x}	0.85^{y}	0.94^{xy}	0.94^{x}	0.33	0.13	0.02	0.80
PER (g g^{-1})	1.27^{x}	0.91^{y}	0.89^{y}	1.27^{x}	0.79^{y}	0.97^{y}	0.16	0.87	0.00	0.62
Somatic indexes (g Kg^{-1})										
VSI	2.34	2.04	2.08	2.18	2.48	2.06	0.19	0.35	0.18	0.05
HSI	0.89^{AB}	0.82^{B}	0.84^{A}	0.89^{AB}	0.78^{B}	1.05^{A}	0.08	0.18	0.03	0.05

In each line, different superscript letters indicate significant differences ($P<0.05$): for a particular diet, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among the diets are shown using A, B. Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. The other abbreviations are: IBW = Initial body weight; FBW = Final body weight; DGI = Daily growth index; FCR = Feed conversion ratio; VFI = Voluntary feed intake; PER = Protein efficiency ratio; VSI = Viscerosomatic index; HIS = Hepatosomatic index. SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=3.

Table 4 – Average percentage of similarity obtained for the DGGE profiles of the distal intestine wall (A) and rectum wall (B) samples of Senegalese sole juveniles after the 73-day feeding trial

A)

		Percentage of similarity (%)[a]					
		PP35			PP72		
		UN	PROB	YEAST	UN	PROB	YEAST
PP35	UN	48.2					
	PROB	**55.7**	**55.6**				
	YEAST	43.6	**54.0**	**52.5**			
PP72	UN	34.4	45.2	**56.9**	43.0		
	PROB	47.2	**54.9**	**54.9**	42.4	42.6	
	YEAST	35.8	44.2	46.3	40.4	40.3	20.6

B)

		Percentage of similarity (%)[a]					
		PP35			PP72		
		UN	PROB	YEAST	UN	PROB	YEAST
PP35	UN	**61.4**					
	PROB	**63.4**	**68.5**				
	YEAST	**58.6**	**62.4**	48.4			
PP72	UN	**72.4**	**63.9**	**67.9**	**81.4**		
	PROB	**52.8**	47.9	49.5	**61.3**	25.5	
	YEAST	**62.3**	47.1	**51.3**	**65.5**	**51.2**	**51.3**

[a] Percentage of similarity computed using Quantity One® program. If the lanes are identical to each other, the percentage of similarity is 100. Similarity values higher than 50% are presented in bold. Dietary treatments (n=2) are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast.

Statement of relevance

There is an increasing trend in the aquaculture industry to replace fishmeal for plant ingredients, as a means of promoting sustainability of the industry. This paper contributes significantly to our limited knowledge of how plant ingredients and supplements affect gut microbiota and immunocompetence.

Highlights

- Increased inclusion of plant ingredients is associated with a more diverse gut microbiota profile.
- Senegalese sole fed higher concentrations of plant ingredients displayed lower immune- and stress-related transcript levels than their control counterparts.
- Multispecies bacteria supplementation resulted in up-regulation of genes involved in the antioxidative stress response.
- Dietary probiotic supplementation had a negative impact on growth performance.