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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Innate immune response, intestinal morphology and microbiota changes in Senegalese sole fed plant protein diets with probiotics or autolysed yeast

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Abstract The effects of using plant ingredients in Senegalese sole (*Solea senegalensis*) diet on immune competence and intestine morphology and microbial ecology are still controversial. Probiotics or immunostimulants can potentially alter the intestinal microbiota in a way that protects fish against pathogens. The current study aimed to examine the intestine histology and microbiota and humoral innate immune response in juvenile sole fed diets with low (35 %) or high (72 %) content of plant protein (PP) ingredients supplemented with a multispecies probiotic bacteria or autolysed yeast. Fish fed the probiotic diet had lower growth performance. Lysozyme and complement activities were significantly higher in fish fed PP72 diets than in their counterparts fed PP35 diets after 17 and 38 days of feeding. At 2 days of feeding, fish fed unsupplemented PP72 showed larger intestine section area and longer villus than fish fed unsupplemented PP35. At 17 days of feeding, fish fed unsupplemented PP72 showed more goblet cells than the other dietary groups, except the group fed yeast supplemented PP35 diet. High dietary PP level, acutely stimulate fish innate immune defence of the fish after 2 and 17 days of feeding. However, this effect does not occur after 73 days of feeding, suggesting a habituation to dietary treatments and/or immunosuppression, with a reduction in the number of the goblet cells. Fish fed for 38 days with diets supplemented with autolysed yeast showed longer intestinal villus. The predominant bacteria found in sole intestine were *Vibrio* sp. and dietary probiotic supplementation caused a reduction in *Vibrio* content, regardless of the PP level.

Keywords Probiotics · Immune status · Plant protein ingredients · Senegalese sole · Intestinal morphology · Intestinal microbiota

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Introduction

Aquaculture is one of the fastest growing food-producing sectorsin the world (FAO), and during the last years, much effort have been done to improve fish growth by manipulation of dietary formulations. Senegalese sole (*Solea senegalensis*) is a promising flatfish species in the European aquaculture, due to its high commercial value and nutritional properties. The ability of Senegalese sole to efficiently use novel plant-based diets (Silva et al. 2009) is an important asset towards the intensification and commercialization of this species. Currently, diseases outbreaks remain a primary constraint to aquaculture expansion. One of the newest scientific areas of high interest to improve fish growth efficiency and to prevent and/or control fish diseases is the application of probiotics and microbial feed additives as alternatives to antibiotics (Dimitroglou et al. 2011; Magnadottir 2010). These feed additives or dietary raw materials favourably affect animal

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performance and welfare, particularly through the modulation of the intestinal microbiota which plays a critical role in maintaining host health (Tuohy et al. 2005) and through the modulation of the immune system, influencing various humoral and cellular defences (Nayak 2010).

Among promising probiotic candidates, the *Bacilli* class has been widely assayed in fish and numerous studies have demonstrated that its administration enhances immune responses and disease resistance (Aly et al. 2008; Kumar et al. 2008; Newaj-Fyzul et al. 2007; Salinas et al. 2005). On the other hand, the use of some selectively fermented ingredients like yeasts allows specific changes in the composition and/or activity of the gastrointestinal microflora that confers benefits upon the host's wellbeing and health (Marcel 2008). Natural immunostimulants are valuable for activating the fish immune system and protecting fish against adverse conditions (Anderson 1992; Sakai 1999). Among them, the yeast *Saccharomyces cerevisiae* has been found to be a good enhancer of the trout immune system (Siwicki et al. 1994) and it can also add nutritional value in fish diets as a possible alternative to commonly used fish meal protein (Rumsey et al. 1992).

The microbial flora present in fish intestine plays an important role on breaking down ingested food or inhibiting the colonization of the intestine by pathogens (Manzano et al. 2012). The composition of bacterial communities in fish can be affected by several factors, including the environment and their interaction with diet. A balanced intestinal microbiota constitutes as an efficient barrier against pathogen colonization, producing metabolic substrates (e.g. vitamins and short-chain fatty acids) and stimulating the immune system (Gaggìa et al. 2010). In this context, probiotics and some immunostimulants may play a significant role to improve the resistance to pathogenic bacteria colonization and enhancement of host mucosa immunity, thus reducing pathogen load and an enhancing health status of the animals (Choct 2009). Bacterial diversity has an important role in the function of ecosystems (Bell et al. 2005). Biodiversity protects ecosystems against a decline in their functionality and allows for adaptation to changing conditions, as the coexistence of many bacterial species provides effective fail-safe measures to help keep the pathogen bacteria from spreading (Yachi and Loreau 1999). The complex microbial ecology of the intestinal tract (GI) provides nutritional benefit and protection against pathogens. Moreover, microbial ecology may play an important role in modulating interactions between environment and the immune system (Balcázar et al. 2007). The potential pathogens present within the GI tract should be reduced in order to avoid mucosal damage and improve absorptive surface area (Merrifield et al. 2010). Rawls et al. (2004) observed that microbiota stimulated intestinal epithelial proliferation and influenced enterocyte morphology.

The aim of the current study was to evaluate the effects of dietary plant protein levels intercropped with a multispecies probiotic (from the *Bacilli* class) or an autolysed yeast supplementation on growth performance, innate immune response, intestine morphology and microbiota diversity in Senegalese sole (*S. senegalensis*).

Materials and methods

The feeding experiment took place at the CIIMAR facilities (University of Porto, Portugal). 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) and according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE. The current study is an extension of a submitted work by Batista et al. (2016).

Feed and fish

Nowadays, marine fish diets are composed of at least 30 % plant ingredients. Six isoproteic (55 %) and isolipidic (8 %) diets with similar amino acid composition were formulated to contain 35 % (PP35) or 72 % (PP72) of plant ingredients (Table 1) each supplemented with PROB and YEAST (commercial products) or unsupplemented (UN) in a 2×3 factorial design. PROB (AquaStar® Growout - Biomin® Holding GmbH, Herzogenburg, Austria) was a multispecies probiotic (*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 (Levabon® Aquagrow - Biomin®) was autolysed yeast (*S. cerevisiae*) supplemented in the mixture at 4 g 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).

Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and transported to the rearing facilities of CIIMAR. After a 5-week acclimation period, fish were distributed into 18 fibreglass tanks of 50 L each, at a density of 5.8 kg m^{-2} (31 fish per tank, 33.1 ± 0.2 g each) and fed the experimental diets in triplicate. The tanks were supplied with seawater (32.0 ± 0.1 ppm NaCl, 19.7 ± 0.5 °C, 0.33 ± 0.27 mg L^{-1} NH$_3$, 0.14 ± 0.07 mg L^{-1} NO$_2$) at a flow rate of 1.5 L min^{-1}, in a controlled semi-closed recirculation system under a constant photoperiod (12 h light:12 h darkness). Rearing conditions were monitored during the entire trial. The recirculation system was equipped with UV lights and ozone generator to hamper bacteria growth in the rearing

Table 1 Feed ingredients, proximate composition and amino acid (g 16 g^{-1}N) of the experimental diets

	PP35			PP72		
	UN	PROB	YEAST	UN	PROB	YEAST
Feed ingredients (%)						
Fishmeal 70 L[a]	24.5	24.5	24.5	5.5	5.5	5.5
Fishmeal 60[b]	27.0	27.0	27.0	0.0	0.0	0.0
CPSP[c]	5.0	5.0	5.0	5.0	5.0	5.0
Squid meal	5.0	5.0	5.0	5.0	5.0	5.0
Pea (Lysamine GP)	0.0	0.0	0.0	14.0	14.0	14.0
Soycomil PC[d]	0.0	0.0	0.0	6.0	6.0	6.0
Soybean meal[e]	12.5	12.5	12.5	9.0	9.0	9.0
Potato concentrate	0.0	0.0	0.0	6.0	6.0	6.0
Wheat gluten	0.0	0.0	0.0	7.0	7.0	7.0
Corn gluten	0.0	0.0	0.0	9.0	9.0	9.0
Extruded peas (Aquatex G2000)[f]	11.0	11.0	11.0	11.5	11.5	11.5
Wheat meal	11.0	11.0	11.0	9.6	9.6	9.6
Fish oil	2.0	2.0	2.0	5.7	5.7	5.7
Vitamin[g] and mineral premix[h]	1.0	1.0	1.0	1.0	1.0	1.0
Di-calcium phosphate	0.0	0.0	0.0	4.0	4.0	4.0
L-Lysine	0.0	0.0	0.0	0.5	0.5	0.5
DL-Methionine	0.0	0.0	0.0	0.2	0.2	0.2
Binder (alginate)[i]	1.0	1.0	1.0	1.0	1.0	1.0
Proximate composition						
Crude protein (%DM)	54.26	54.37	54.66	53.16	53.31	53.46
Crude fat (%DM)	11.90	11.41	11.93	12.21	11.45	12.27
Gross energy (kj g^{-1} DM)	20.84	21.18	21.12	21.84	22.05	22.04

[a] Peruvian fishmeal LT (71 % crude protein, 11 % crude fat, EXALMAR, Peru)

[b] Fair Average Quality (FAQ) fishmeal (62 % crude protein, 12 % crude fat, COFACO, Portugal)

[c] Soluble fish protein hydrolysate (87 % crude protein, 6.5 % crude fat, Sopropêche, France)

[d] Soycomil-P (soy protein concentrate, 65 % crude protein, 0.7 % crude fat, ADM, The Netherlands)

[e] Dehulled solvent extracted soybean meal (micronized)

[f] Aquatex G2000 (dehulled, grinded pea grits, 24 % crude protein, 0.4 % crude fat, SOTEXPRO, France)

[g] Vitamins (mg or IU kg^{-1} diet): vitamin A (retinyl acetate), 20,000 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), 3 mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg

[h] Minerals (% or mg kg^{-1} diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulphate), 9 mg; Co (cobalt sulphate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulphate), 6 mg; Cl (sodium chloride), 2.41 %; Ca (calcium carbonate), 18.6 %; NaCl (sodium), 4 %

[i] Diatomaceous earth: Kielseguhr: LIGRANA GmbH, Germany

water. The trial lasted 73 days and fish were fed to apparent satiety based on visual observation of acceptance and refusal of feed using temporized automatic feeders.

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 °C for body composition analyses. Total feed consumption was registered during the entire feeding trial. The innate immune response and intestine histology were analysed at days 3, 17, 38 and 73 of the feeding trial.

For the evaluation of humoral innate immune parameters (lysozyme, peroxidase and alternative complement pathway—ACH50), blood was withdrawn from the caudal vein of nine fish per treatment using heparinized syringes and centrifuged at 5000×g for 10 min at 4 °C. Liver and intestine of each fish was sampled, cut and fixed in buffered formalin to histology analysis. To examine intestinal microbiota, the whole intestine of other nine fish per treatment was aseptically removed at 17, 38 and 73 days of feeding the experimental diets.

For microbial ecology determination, the intestinal contents of three non-fasted fish per tank were sampled by stripping and pooled prior homogenized in 1 mL sterile phosphate-buffered saline (PBS, pH 7.2).

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; 550 °C for 6 h), crude protein (N × 6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid (petroleum ether extraction, 40–60 °C, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

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 per minute. Plasma peroxidase activity (EU mL^{-1}) was measured following the procedure adapted to *S. senegalensis* by Costas et al. (2011), defining that 1 unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) activity was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL^{-1}), as reported by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50 % lysis of rabbit red blood cells.

Histological evaluation

The samples (liver and a segment with 1.5 cm length from the proximal intestine) were fixed in phosphate-buffered formalin (4 %, pH 7, VWR, Portugal) for 24 h. They were subsequently dehydrated and embedded in paraffin according to standard histological procedures. Thereafter, 3 micra transversal sections were obtained and stained with haematoxylin and eosin (H&E, Merck, Portugal) and periodic acid-Schiff (PAS, Merck, Portugal) before examination under a light microscope (Olympus BX51, cell^B software, GmbH, Hamburg, Germany).

Three different intestine sections of each animal were used for quantitative measurements. The intestine section area (mm^2), villus height (μm), muscular layer thickness (from serosa to submucosa, μm) and goblet cells (number per section) positive to PAS staining of the intestinal wall were measured according to Batista et al. (2014). The villus width (μm) was measured across the base of the villus at the luminal surface of ten selected *villi* per section.

In the liver, the occurrence of possible pathological damage (presence or absence of defined vacuoles indicator of fat degeneration) was evaluated. For each sampled fish, the cytoplasm vacuolization degree (H&E) and glycogen content (PAS) of the hepatocytes were analysed (Fig. 1). Observations were consistently made using a combination of low magnification (objective of 10×) for noticing the general aspects and then with higher magnification (objective of 40×) for categorizing. Ten randomly sampled fields were analysed per section. Evaluation of the hepatocyte vacuolization degree was made using a semi-quantitative approach, according to the following three grades and criteria: 0 (none)—less than 1/3 of the hepatocyte cytoplasm shows vacuoles, 1 (moderate)—between 1/3 and less than 2/3 of the hepatocyte cytoplasm shows vacuolization, 2 (high)—more than 2/3 of the hepatocyte cytoplasm shows vacuolization. Assessment of hepatocyte carbohydrates (namely glycogen) content also followed a semi-quantitative approach, with three degrees: 0 (none)—when hepatocytes in PAS sections did not stain positively or had a very weak colour, 1 (moderate)—PAS sections presented a median staining, 2 (high)—PAS sections present hepatocytes with a very strong staining.

Intestinal microbiota

Microbial populations were isolated from the intestinal content as described by Merrifield et al. (2009). Intestinal content samples were serially diluted to 10^{-2} with PBS and 100 μL were spread onto duplicate tryptone soy agar (TSA, 0.9 % NaCl) plates. Plates were incubated at 25 °C for 48 h and colony-forming units (CFU) g^{-1} was calculated from statistically viable plates (i.e. plates containing 30–300 colonies). One hundred colonies of one of these plates were randomly collected from each dietary treatment assayed and cultured on TSA to obtain pure cultures. Then the colonies were identified by the amplification and sequencing of a fragment of 16S rDNA. Briefly, the colonies isolated were suspended in 100 μL of sterile Milli-Q water. Samples were boiled at 100 °C for 15 min and adjusted to 1 mL. These suspensions were centrifuged at 12,000 rpm for 5 min and 1–5 μL of the
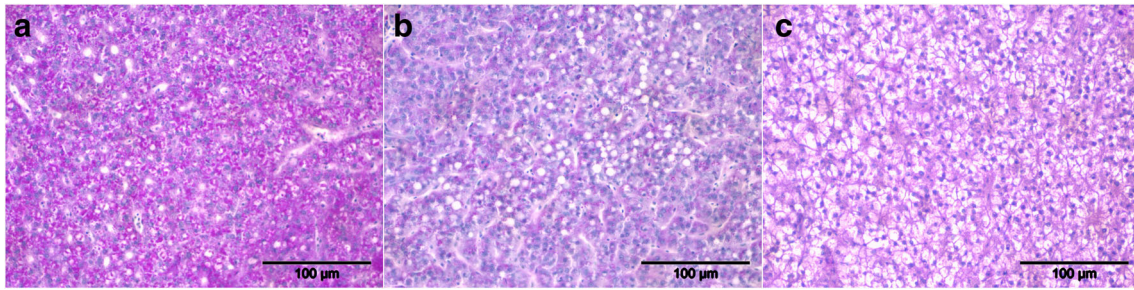
Fig. 1 Liver histology of Senegalese sole for quantification of vacuoles and glycogen content. **a** High content of glycogen and reduce vacuolization. **b** Moderate content of glycogen and vacuoles. **c** High content in vacuoles and small amounts of glycogen (PAS stained, *bar* = 100 μm)

supernatant was used to carry out the PCR reactions. The fragment 16S rDNA was amplified using the universal primers SD-Bact-0008-a-S20 (5′ AGAGTTTGATCCTGGCTCAG 3′) and SD-Bact-1492-a-A-19 (5′ GGTTACCTT GTTACGACTT 3′) (Kim and Austin 2006). Polymerase chain reactions were carried out in a 50 μL reaction mixture that included 5 pmol of each primer, 200 μM dNTPs, 1× PCR buffer, 2 mM MgCl$_2$, 1 U BIOTAQ DNA Polymerase (Bioline, London, UK) and 1 μL of a boiled colony suspension. Thermal cycling consisted of an initial step of 2 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 40 s at 72 °C and a final extension step 5 min at 72 °C. Polymerase chain reaction products were electrophoresed on 1 % agarose gel, visualized via ultraviolet transillumination and then they were purified with a QiaQuick PCR purification kit (QIAGEN, Hilden, Germany). The PCR products were sequenced by cycling sequencing using SD-Bact-0008-a-S20 and SD-Bact-1492-a-A-19 by Macrogen (Corea). The resulting sequences (~500 bp) were compared with the sequences from the National Center for Biotechnology Information (NCBI) or Greengenes DNA sequence database using the BLAST sequence algorithm (Altschul et al. 1990). Chimaeric sequences were identified by using the CHECK CHIMERA program of the Ribosomal Database Project (Maidak et al. 1999), and the sequences reported in this study have been deposited in the GenBank database under the following accession numbers: KU725824 to KU725849.

Calculations of growth performance

Feed conversion ratio (FCR) was calculated as dry feed intake (g)/wet weight gain (g) and the specific growth rate (SGR, % BW day^{-1}) as $100 \times (\ln W_0 - \ln W_1)$/days, where W_0 and W_1 are the initial and the final fish mean weights in grammes and days is the trial duration. Voluntary feed intake (VFI, % BW day^{-1}) was calculated as $100 \times$ (dry feed intake (g)/ ABW (g)/days), where ABW (average body weight) was calculated as $(W_1 + W_0)/2$. The net protein utilization (NPU) was calculated as (PBF − PBI)/protein fed, where PBF is the final protein content of fish and PBI is the initial protein content of fish.

Statistical analysis

Statistical analyses (except for intestinal microbiota) 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 nonparametric Kruskal-Wallis test was performed. For intestinal microbiota, Kruskal-Wallis test was used to test the significance of the differences among dietary treatments.

Results

Growth performance

Fish grew from 33.1 ± 0.20 to 50.6 ± 1.2 g after feeding the experimental diets for 73 days (Table 2). Growth performance did not differ between PP35 and PP72 groups. PROB groups had significantly lower final body weight (FBW, 45.0 ± 1.9) and daily growth index (SGR, 0.42 ± 0.1) compared to UN groups (50.5 ± 2.0 and 0.58 ± 0.0, respectively). Additionally, UN groups had significantly better feed conversion ratio (FCR, 1.5 ± 0.1) and net protein utilization (NPU, 19.07 ± 2.3) than the PROB (2.2 ± 0.4 and 10.4 ± 1.9, respectively) and YEAST (2.1 ± 0.4 and 13.0 ± 4.6, respectively) supplemented groups. Voluntary feed intake (VFI) was also lower in the UN groups (0.8 ± 0.0) and differed significantly from the YEAST groups (0.9 ± 0.1).

Table 2 Growth performance of Senegalese sole juveniles after the 73 days of feeding the experimental diets

	Dietary treatments							P value		
	PP35			PP72			SD_{pooled}	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Growth performance										
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
SGR	0.58^x	0.44^y	0.45^{xy}	0.58^x	0.40^y	0.49^{xy}	0.08	0.99	0.02	0.68
FCR	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	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
NPU	20.11^x	10.48^y	10.83^y	18.03^x	10.31^y	15.26^y	3.05	0.62	0.00	0.21

In each line, different superscript letters indicate significant differences ($P < 0.05$): for a particular PP level, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among PP level 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. Values represent mean ± SD_{pooled}, $n = 3$

PP plant protein level factor, *S* supplementation factor, *FBW* final body weight, *SGR* specific growth rate, *FCR* feed conversion ratio, *VFI* voluntary feed intake, *NPU* net protein utilization, SD_{pooled} pooled standard deviation

Humoral innate immune parameters

The values of humoral innate parameters analysed are present in Table 3. Peroxidase activity (EU mL^{-1}) was not significantly different ($P > 0.05$) among dietary groups for any sampling time. Lysozyme activity and ACH50 were not affected by probiotic administration, but were significantly changed by the dietary PP level at 17 and 38 days of feeding. At 17 days, fish from PP72 groups had higher lysozyme (359 ± 201 EU mL^{-1}) and ACH50 (204 ± 55 units mL^{-1})

Table 3 Effects on humoral innate immune parameters of Senegalese sole juveniles after the 2, 17, 38 and 73 days of feeding the different experimental diets

	Dietary treatments							P value		
	PP35			PP72			SD_{pooled}	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Lysozyme (EU mL^{-1})										
2 days	190.98	290.74	440.12	379.32	628.70	457.64	288.07	0.07	0.06	0.43
17 days	124.21^B	225.35^B	175.79^B	410.32^A	338.09^A	287.04^A	156.58	0.00	0.76	0.27
38 days	612.50^B	695.99^B	628.39^B	761.11^A	1047.62^A	950.69^A	439.55	0.02	0.70	0.69
73 days	2061.73	1511.73	1630.25	1612.96	1225.93	1295.15	787.12	0.23	0.25	0.51
Peroxidase (EU mL^{-1})										
2 days	122.11	97.44	101.56	105.12	59.09	92.82	48.68	0.28	0.06	0.15
17 days	141.65	111.80	110.51	100.71	71.51	122.82	61.59	0.25	0.19	0.16
38 days	59.90	80.03	48.99	54.39	78.59	31.21	45.32	0.86	0.06	0.77
73 days	26.16	22.48	33.17	30.38	50.32	33.97	26.97	0.15	0.61	0.30
ACH50 (units mL^{-1})										
2 days	112.29	113.85	103.17	97.97	119.27	113.59	47.47	0.98	0.88	0.86
17 days	161.34^B	177.01^B	167.67^B	221.55^A	195.88^A	193.10^A	51.21	0.02	0.82	0.45
38 days	174.86^B	197.38^B	177.52^B	270.95^A	247.59^A	253.50^A	58.78	0.00	0.92	0.52
73 days	110.34	108.18	100.79	88.15	104.29	98.04	43.32	0.42	0.86	0.76

In each line, different superscript letters indicate significant differences ($P < 0.05$): for a particular probiotic inclusion, differences among PP level 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 the immunostimulant yeast. Values represent mean ± SD_{pooled}, $n = 9$

PP plant protein level factor, *S* supplementation factor, *EU* enzyme unit, *ACH50* alternative complement pathway activity, SD_{pooled} pooled standard deviation

compared to fish from PP35 groups (177 ± 106 EU mL^{-1} and 169 ± 19 units mL^{-1}, respectively). At 38 days of feeding, fish from PP72 groups showed higher lysozyme (908 ± 399 EU mL^{-1}) and ACH50 (258 ± 64 units mL^{-1}) activities, compared to fish from PP35 groups (647 ± 457 EU mL^{-1} and 183 ± 49 units mL^{-1}, respectively).

Histological evaluation

Histological measurements of the intestinal mucosa of fish are present in Table 4 Muscle layer thickness did not vary significantly, regardless of the dietary treatment and sampling time. Intestine section area was significantly different at 2 days of feeding trial, showing the PP72_UN group (2.75 ± 0.66 mm^2) with higher area compared to PP35_YEAST (1.93 ± 0.30 mm^2) and PP35_UN (1.99 ± 0.42 mm^2) groups. Villus was significantly wider ($P < 0.01$) at 73 days in fish fed PP35_YEAST group (91.01 ± 1.49 μm) than fish fed PP72_YEAST (79.45 ± 7.28 μm) (Fig. 2). Villus length was significantly different ($P < 0.05$) at 2, 38 and 73 days of feeding the experimental diets (Fig. 2). At 2 days of feeding, fish fed PP72_UN diet had the longest villus (418 ± 30 μm) when compared to fish fed PP35_UN (357 ± 41 μm) and PP72_YEAST (361 ± 13 μm) diets. At 38 days of feeding, fish from YEAST groups had a significant longer villus (413 ± 45 μm) than fish from the UN groups (372 ± 44 μm). The number of goblet cells was significantly

Table 4 Intestinal morphology of Senegalese sole after 2, 17, 38 and 73 days of feeding the different experimental diets
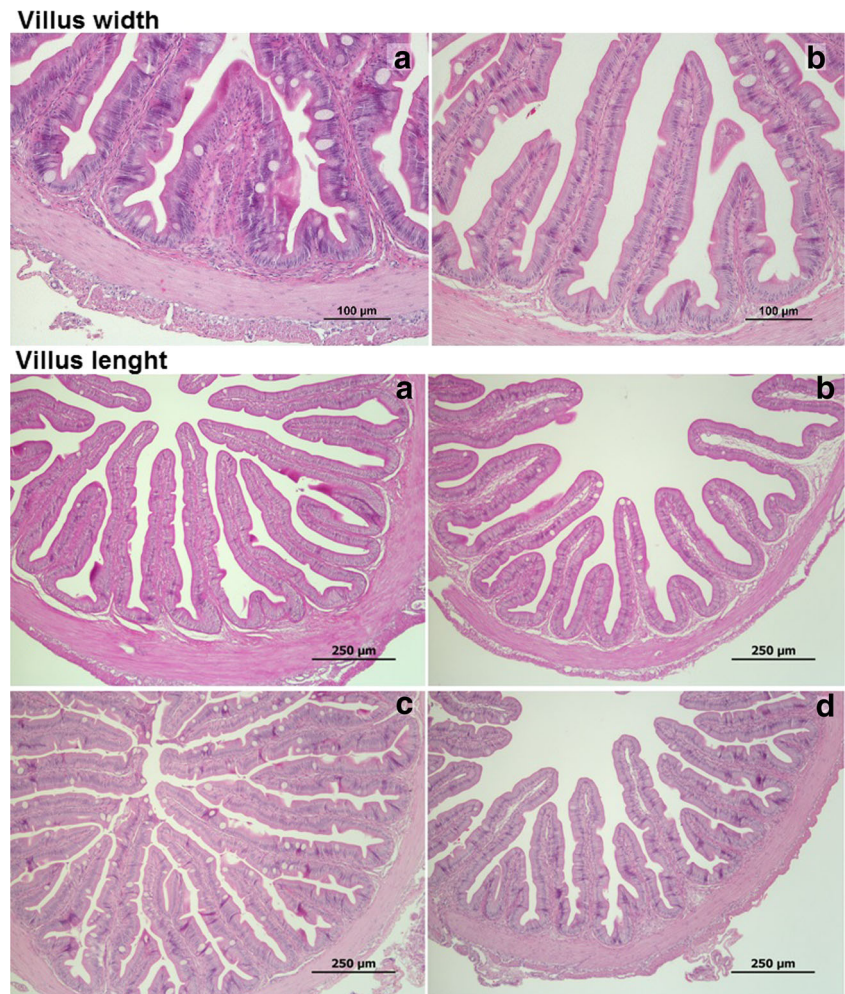
	Dietary treatments							P value		
	PP35			PP72			SD$_{pooled}$	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Intestine section area (mm^2)										
2 days	1.99^b	2.34^{ab}	1.93^b	2.75^a	2.05^{ab}	2.15^{ab}	0.44	0.09	0.14	0.01
17 days	1.88	1.89	1.76	1.82	1.75	2.01	0.55	0.92	0.94	0.55
38 days	1.78	1.79	2.18	1.85	2.00	2.12	0.42	0.56	0.10	0.67
73 days	2.80^A	2.85^A	2.47^A	2.25^B	2.16^B	2.13^B	0.55	0.00	0.47	0.02*
Muscular layer thickness (μm)										
2 days	77.83	93.53	81.05	92.65	95.77	84.93	16	0.14	0.11	0.50
17 days	87.46	81.10	82.65	80.91	88.13	79.62	20	0.88	0.86	0.60
38 days	84.12	89.09	108.18	79.48	88.08	80.48	19	0.13	0.62	0.28
73 days	85.22	103.11	93.57	96.80	99.43	96.43	21	0.54	0.37	0.58
Villus length (μm)										
2 days	356.92^b	384.17^{ab}	375.21^{ab}	418.14^a	371.91^{ab}	361.00^b	35	0.27	0.33	0.01
17 days	340.42	341.58	351.17	345.19	311.72	351.50	56	0.60	0.41	0.61
38 days	384.80^y	366.11^{xy}	414.20^x	352.39^y	400.39^{xy}	411.84^x	42	0.99	0.02	0.10
73 days	461.08^A	476.89^A	431.90^A	409.32^B	391.20^B	385.70^B	55	0.00	0.28	0.53
Villus Width (μm)										
2 days	82.33	86.00	82.87	85.41	82.00	80.78	6	0.36	0.24	0.35
17 days	76.64	75.02	76.95	75.42	76.18	78.90	9	0.81	0.72	0.88
38 days	79.34	80.46	84.61	75.45	79.33	81.37	6	0.15	0.07	0.81
73 days	90.18^{ab}	89.17^{ab}	91.01^a	82.21^{ab}	82.61^{ab}	79.45^b	6	0.00	0.90	0.01
Goblet cells (number per section)										
2 days	148.60	221.33	230.40	214.62	187.60	170.29	95	0.74	0.78	0.17
17 days	192.93^b	231.83^b	278.28^{ab}	378.81^a	173.29^b	179.63^b	90	0.71	0.04	0.00
38 days	277.06	195.56	245.56	158.58	277.74	336.63	172	0.34	0.78	0.62
73 days	383.24^A	326.04^A	384.56^A	242.43^B	256.42^B	280.04^B	179	0.04	0.80	0.85

In each line, different superscript letters indicate significant differences ($P < 0.05$): for a particular PP level, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among PP level are shown using A, B; and among treatments (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 the immunostimulant yeast. Values represent mean ± SD$_{pooled}$, $n = 9$

PP plant protein level factor, *S* supplementation factor, *SD$_{pooled}$* pooled standard deviation

*Without significant differences after post hoc analysis

Fig. 2 Histological sections of anterior intestines of Senegalese sole for villus width (VW) and for villus length (VL). VW: (*a*) PP35_YEAST diet showing higher VW than (*b*) that corresponds to PP72_YEAST diet (H&E, *bar* = 100 μm). VL: (*a, b*) 2 days samples time. (*c, d*) 73 days sampling time. Note the difference between the villus dimensions in (*a*) and (*c*) showing higher villus length when compared with (*b*) and (*d*). (**a**) PP35 diet. (**b**) PP72_YEAST diet. (**c**) PP35 diet. (**d**) PP72_YEAST diet (H&E, *bar* = 250 μm)

different ($P < 0.05$) at 17 and 73 days of feeding dietary treatments (Fig. 3). At 17 days of feeding, fish fed

PP72_UN diet had a significantly ($P < 0.05$) higher number of goblet cells (379 ± 119) compared to the other dietary

Fig. 3 Histological sections of anterior intestines of Senegalese sole for number counting of goblet cells (GC). (**a**) PP72 diet and (**b**) PP72_PROB diet, both at 17 days sampling time. (**c**) PP35 diet and (**d**) PP72_YEAST diet at 73 days sampling time. Note the difference observed between the (*a*) and (*c*) showing higher number of GC, compared with (*b*) and (*d*) (PAS stained, *bar* = 100 μm)
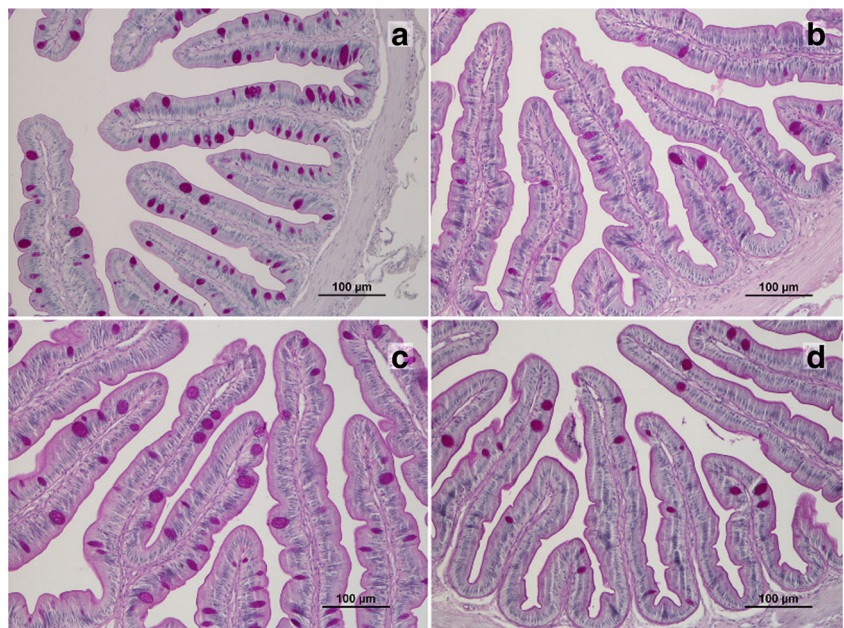
treatments, except fish fed PP35_YEAST diet. At 73 days of feeding, both villus length and number of goblet cells were affected by dietary PP level, with fish from PP35 groups having larger villus (455 ± 52 μm) and higher number of goblet cells (363 ± 210) than fish from PP72 groups (395 ± 57 μm and 260 ± 129, respectively).

Liver histology revealed different degrees of vacuolization at 2 and 73 days of feeding. Fish from the PP72 groups showed a higher vacuolization (grade 3) compared to fish from PP35 groups (grade 1).

At 2 days of feeding, fish fed diets supplemented with YEAST showed higher hepatocytes glycogen content (more than 2/3 of the liver positively stained) when compared with the fish fed the diets supplemented with PROB. At 17 days of feeding, fish fed PP72 presented hepatocytes with a weak PAS staining, indicating a very low content of carbohydrates (namely glycogen).

Intestinal microbiota

The intestinal bacteria counts varied from 8.2×10^5 to 2.4×10^7 CFU mL^{-1}, as an effect of the diet and sampling time (Table 5). The inclusion of 35 or 72 % of plant ingredients in the diet induced a significant reduction of the microbial counts in fish intestine after 38 or 73 days of feeding. In Table 5, it is possible to observe that the supplementation with the autolysed yeast in PP35 and PP72 diets induced significant differences in the level of intestinal microorganisms in comparison with the microbial recounts obtained in fish fed the non-supplemented diets. On the contrary, these significant differences were not observed when PP35 and PP72 diets were supplemented with the multispecies probiotic bacteria.

In general, the highest relative percentage corresponded to *Vibrio* sp. (24 to 99 %). Probiotic supplementation greatly reduced the concentration of intestinal *Vibrio* sp. (Table 5), especially in fish fed low dietary PP level (PP35_PROB). The duration of the dietary treatment had a clear effect on the reduction of intestinal *Vibrio* sp. concentration in fish fed PP35_PROB. *Vibrio harveyi* and *Vibrio owensii* were identified in fish fed PP72_PROB and PP72_YEAST and not identified in fish fed PP72_UN (Table 6). In addition, *Staphylococcus saprophyticus* was observed only in the intestine of fish fed PROB diets (Tables 6 and 7).

Discussion

Probiotics confer beneficial actions to the host or to their environment through different modes of action. Their application in aquaculture has been regarded as a sustainable and promising strategy not only in the context of disease control but also in nutrition, growth and immunity (Lazado and Caipang 2014).

The capacity of Senegalese sole to cope with diets in which the marine-derived protein was replaced by plant ingredients has been previously reported (Cabral et al. 2011, 2013; Silva et al. 2009). Similarly, to previous studies, the current study demonstrated that growth performance of Senegalese sole was not affected by high content of plant ingredients. The final body weight and FCR were, however, negatively affected by dietary probiotic supplementation (PROB diet). In fact, the effects of probiotics and prebiotics on fish growth performance are often contradictory. Probiotic improved the feed efficiency and growth performance in Senegalese sole (García de la Banda et al. 2012), Japanese flounder (Taoka et al. 2006) and Atlantic cod (Lauzon et al. 2010). Nevertheless, Ferguson et al. (2010) showed no clear effect on growth performance in Nile tilapia fed with *Pediococcus acidilactici* supplementation, whereas Gunther and Jimenez-Montealegre (2004) observed a growth depression in tilapia fed *Bacillus subtilis*.

Humoral immune parameters as well as some intestinal morphology parameters showed a high standard deviation, indicating that the data points are spread out over a wider range of values. As readers are generally interested in knowing variability within the sample, descriptive data should be precisely summarized with SD (Barde and Barde 2012). However, for many biological variables the standard deviation can differ significantly across groups in some data sets and the most important is that the data meet the assumptions of the normal distribution model that we used.

All humoral immune parameters analysed, plasma lysozyme, peroxidase and alternative complement (ACH50) activities were not significantly altered by PROB or YEAST. This trend was also observed by Batista et al. (2014) working with sole fed the same multispecies bacteria and Díaz-Rosales et al. (2009) working with sole fed with two different *Shewanella* sp. However, ACH50 was enhanced when rainbow trout were fed for 4 weeks with diets supplemented with heat-inactivated multispecies bacteria (Choi and Yoon 2008) and gilthead seabream fed for 3 weeks (Salinas et al. 2008). In addition, serum lysozyme activity increased significantly from 438 ± 75 U mL^{-1} (control) to 1269 ± 134 U mL^{-1} in rainbow trout fed for 14 days with diets supplemented with a autochthonous intestinal *B. subtilis* (Newaj-Fyzul et al. 2007). These changes in the innate immune responses were associated to the inhibitory effect of that probiotic against the pathogenic *Aeromonas* sp.

Previous studies have demonstrated that oral administration of fungal carbohydrates enhances fish innate immune response and protection against infections (Efthimiou 1996; Esteban et al. 2001; Siwicki et al. 1994), although this effect seems to be temporary (reversible) and dependent on its inclusion level and feeding duration (Ortuño et al. 2002). In the current study, innate immune parameters were affected by the increase of dietary plant ingredients content. At 17 and

Table 5 Concentration of microorganisms (CFU/mL) and relative percentage of *Vibrio*, *Staphylococcus* and other bacteria identified from the bacterial isolates characterized from group of specimens of *Solea senegalensis* at 17, 38 and 73 days of feeding the different experimental diets. The values of standard deviation were never higher than 20 %

Dietary treatments						
PP35			**PP72**		
UN	PROB	YEAST	UN	PROB	YEAST
CFU/mL					
17 days	$7.7 \times 10^6 \pm 1.2 \times 10^{6c}$	$6.9 \times 10^6 \pm 1.3 \times 10^6$	$3.4 \times 10^6 \pm 5.8 \times 10^5$	$1.7 \times 10^7 \pm 3 \times 10^{6a}$	$5.5 \times 10^6 \pm 5.1 \times 10^5$	$1.1 \times 10^6 \pm 1.7 \times 10^{5b,c}$
38 days	$2.1 \times 10^6 \pm 2.8 \times 10^{5d}$	$1.5 \times 10^6 \pm 1.9 \times 10^{5a}$	$6.3 \times 10^6 \pm 1.2 \times 10^{6b,c}$	$2.4 \times 10^7 \pm 4.8 \times 10^{6c}$	$3.4 \times 10^6 \pm 6 \times 10^5$	$4.0 \times 10^6 \pm 7.6 \times 10^5$
73 days	$3.1 \times 10^6 \pm 4.5 \times 10^5$	$2.5 \times 10^6 \pm 5.3 \times 10^5$	$8.2 \times 10^5 \pm 1.3 \times 10^{5d,e}$	$1 \times 10^6 \pm 1.6 \times 10^{5a,d}$	$1.8 \times 10^6 \pm 1.6 \times 10^5$	$4.2 \times 10^6 \pm 8.3 \times 10^{5b,d,f}$
***Vibrio* sp. (%)**					
17 days	95	42	92	99	24	99
38 days	99	36	63	83	59	62
73 days	98	16	97	97	50	94
***Staphylococcus* sp. (%)**					
17 days	0	30	0	0	14	0
38 days	0	12	0	0	0	0
73 days	0	24	0	0	0	0
Others (%)					
17 days	5	28	8	1	62	1
38 days	1	52	37	17	41	38
73 days	2	60	3	3	50	6

Values represent mean ± SD. Different superscript in the same row (a, b), column (c, d) and dietary treatment (e and f) indicate significant differences ($P < 0.05$). 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 the immunostimulant yeast

CFU colony-forming unit

Table 6 Closest matches of GenBank database sequences to isolates from intestinal samples of *Solea senegelensis* fed PP72 diets for 17, 38 and 73 days

Closest relative	Similarity (%)	Accession number	PP72_UN			PP72_PROB			PP72_YEAST		
			17 days	38 days	73 days	17 days	38 days	73 days	17 days	38 days	73 days
γ-Proteobacteria											
Vibrio alfacsensis	100	KU725824	+	+	+	−	+	+	+	−	−
Vibrio alginolyticus	99	KU725825	+	−	−	−	−	−	−	+	−
Vibrio communis	100	KU725826	−	−	+	+	−	+	+	+	+
Vibrio harveyi	98	KU725827	−	−	−	+	−	+	+	+	+
Vibrio ichthyoenteri	100	KU725828	+	−	+	+	+	+	−	−	+
Vibrio owensii	100	KU725830	−	−	−	+	−	−	+	−	−
Vibrio scophthalmi	98	KU725831	−	−	+	+	+	+	−	+	−
Vibrio tapetis	99	KU725833	+	+	−	+	−	−	−	−	−
Vibrio sp.	99	KU725834	+	+	+	+	+	−	−	−	−
Vibrio sp.	97	KU725837	−	−	−	−	−	−	−	+	−
Vibrio sp.	*98*	KU725838	+	−	−	−	−	−	−	−	−
Vibrio sp.	100	KU725836	+	−	−	−	−	−	−	−	−
Firmicutes											
Staphylococcus saprophyticus	100	KU725839	−	−	−	+	−	−	−	−	−

Presence (+), absence (−)

38 days of feeding trial, fish from PP72 groups had higher values of lysozyme and ACH50 activities than fish from PP35 groups, suggesting a stimulation of the innate immune activity by dietary high PP content. Also, Geay et al. (2011) observed an increase in the ACH50, while the lysozyme was lower, in fish fed a PP diet when compared to fish fed fishmeal-based diet. On the other hand, Sitjà-Bobadilla et al. (2005) reported that high level of fishmeal replacement by plant protein sources had no significant effect on lysozyme activity. Jalili et al. (2013) showed that rainbow trout fed diets with high PP levels (70 and 100 %) resulted in a decrease on the ACH50, as reported by Sitjà-Bobadilla et al. (2005) in gilthead seabream. Hepatocytes provide the major source of complement factors (Abelseth et al. 2003), and the progressive fat degeneration of liver with the PP inclusion could decrease complement proteins synthesis (Sitjà-Bobadilla et al. 2005). Liver from fish fed PP75 diets showed a higher level of vacuolization compared to fish fed PP35 diets. The use of high levels of plant ingredients is recognized to have several disadvantages, particularly related to the amino acid profiles and unsaturated fatty acid imbalances but especially due to their levels of antinutritional factors (Geay et al. 2011). In the current study, high PP level seems to result in an acute stimulation of lysozyme and ACH50, since at 17 and 38 days, fish fed PP72 diets showed higher activities than fish fed PP35 diets. Nevertheless, at 73 days of feeding trial, such effects disappeared. Food legumes and cereals may have adverse effects on some fish species after intake, resulting in morphological

problems in liver and intestine that could lead to metabolic failure (Russell et al. 2001). PP72 diet, when compared to PP35, was formulated to contain a low amount of soybean meal, but high content of mixed plant ingredients, including insoluble pea protein, as protein source replacing fishmeal. Thus, the dietary incorporation level of plant protein sources used in the current study may have provided a high level of antinutritional factors, especially after 73 days of feeding.

Intestinal microbiota is confined within a highly specialized barrier defences, composed by the stratified mucous layer, an epithelium and a lamina propria with several innate and adaptive immune cells (Maynard et al. 2012). An increase in intestine section area, villus length and villus width are directly related to an increase of surface area, which may indicate improvement of intestinal absorptive capacity nutrients (Caspary 1992). At 2 days of feeding, fish fed PP72_UN showed larger intestine section area and longer villus than fish fed PP35_UN. At 17 days of feeding, fish fed PP72_UN showed more goblet cells than the other dietary groups, except the group fed PP35_YEAST. At 73 days, however, villus length and width and goblet cells were significantly increased in fish fed low PP diets compared to high PP diets. The latter may indicate that at 2 and 17 days, fish fed the diets formulated with high levels of plant ingredient level had an enhancement on the immune defence and an improvement in the intestinal surface area in contact with nutrients. However, after 73 days of feeding, these effects were reversed, probably due to habituation of fish and/or to a negative effect of chronic

Table 7 Closest matches of GenBank database sequences to isolates from intestinal samples of *Solea senegelensis* fed PP35 diets for 17, 38 and 73 days

Closest relative	Similarity (%)	Accession number	PP35_UN			PP35_PROB			PP35_YEAST		
			17 days	38 days	73 days	17 days	38 days	73 days	17 days	38 days	73 days
γ-Proteobacteria											
Vibrio alfacsensis	100	KU725824	+	+	+	−	+	+	−	+	−
Vibrio alginolyticus	98	KU725825	−	−	−	−	−	−	−	−	+
Vibrio communis	99	KU725826	+	−	−	−	−	−	−	−	−
Vibrio harveyi	98	KU725827	−	−	−	−	+	−	+	−	−
Vibrio ichthyoenteri	98	KU725828	−	−	+	−	−	+	−	−	+
Vibrio mytilili	100	KU725829	−	−	−	−	−	−	−	−	−
Vibrio owensii	100	KU725830	+	−	−	−	−	−	−	−	−
Vibrio scophthalmi	98	KU725831	+	+	−	+	−	−	−	−	−
Vibrio sinaloensis	100	KU725832	−	−	−	−	−	−	+	+	+
Vibrio tapetis	99	KU725833	−	+	−	−	−	−	−	−	−
Vibrio sp.	99	KU725834	+	+	+	+	+	−	+	−	+
Vibrio sp.	99	KU725846	−	−	+	−	−	−	−	−	−
Vibrio sp.	100	KU725835	−	+	−	−	−	−	−	+	−
Vibrio sp.	97	KU725836	−	−	+	−	−	−	−	−	−
Vibrio sp.	98	KU725840	+	−	−	−	−	−	−	−	−
Vibrio sp.	99	KU725841	−	−	−	−	−	−	+	+	−
Vibrio sp.	98	KU725842	−	−	−	−	−	−	+	−	−
Vibrio sp.	99	KU725843	−	+	−	−	−	−	−	−	−
Firmicutes											
Staphylococcus saprophyticus	100	KU725839	−	−	−	+	+	+	−	−	−

Presence (+), absence (−)

ingestion of high PP diets and presence of antinutritional factors. Intestinal epithelium is covered by a layer of mucus continuously produced by goblet cells and being the first line of defence against microbes (Maynard et al. 2012). So, the reduced number of goblet cells, after 73 days of feeding fish with the PP72 diets, causes a decrease in mucous production, thus probably reducing intestinal protection capacity of those fish.

After 38 days of feeding the experimental diets, villus length was significantly increased in YEAST-supplemented groups, compared to unsupplemented (UN) groups. Yeast cells have been reported to be a source of nucleotides, which contribute for the intestinal maintenance in aquatic animals by improving mucosal flora and mucosal surfaces with relative elongation of the intestinal tract (Li et al. 2007). Also, (Abu-Elala et al. 2013) observed that fish treated with *S. cerevisiae* showed yeast colonization in the intestine, accompanied by an increase in the length and density of the intestinal villus. Such morphological alterations are often associated with an improvement in food digestion and absorption. After 2 days feeding the YEAST diets, fish had a higher hepatocyte glycogen content compared with fish fed diets supplemented with

PROB, showing a stimulation of the glycogen liver storage, the first line of energy source when blood glucose concentration falls during stress.

Fish intestinal microbiota changes with the diet, and this effect is of particular interest considering that the effects of high fishmeal replacement by plant ingredients are still poorly understood, especially in flatfish. Molecular techniques have facilitated culture-independent studies, becoming a valid support to traditional techniques (Ercolini 2004). In the current study, from all bacteria isolated from sole intestine, approximately 20 % were not identified. The intestinal tract of fish harbours a high density of non-culturable bacteria and the composition of which has not yet been reported, leading to differences between viable and total microbial counts (Shiina et al. 2006). In this study, it has been possible to demonstrate the ability to modulate the intestinal microbiota of Senegalese sole by feeding diets supplemented either with probiotic or autolysed *S. cerevisiae*. The results obtained are in accordance with those reported by Standen et al. (2015), who observed in tilapia (*Oreochromis niloticus*) the modulation of the intestinal microbiota and morphology caused by feeding the fish with the same multispecies probiotic used in the current study. The

modulation exerted on the intestinal microbiota by viable and inactivated cells of *S. cerevisiae* has been reported in tilapia and sturgeon (*Huso huso*) (Hoseinifar et al. 2011; Waché et al. 2006). In addition, the modulation of intestinal microbiota has also been demonstrated in fish treated with *S. cerevisiae* fermentation products (He et al. 2011) and glucans obtained from yeast (Kuhlwein et al. 2013). In the current study, the predominant bacteria found in *S. senegalensis* intestine were *Vibrio* sp., which is in agreement with other studies carried out with the same flatfish cultured under extensive, semi-extensive or intensive production systems (Martin-Antonio et al. 2007). This trend was also detected in farmed Senegalese sole fed fresh or lyophilized *Shewanella putrefaciens* (Tapia-Paniagua et al. 2010, 2015).

Fish fed dietary probiotic supplementation (PP35_PROB and PP72_PROB) had reduced intestinal bacteria related to *Vibrio* species. Previous studies observed that strains of probiotic *Bacillus* (Liu et al. 2015; Luis-Villasenor et al. 2015; Wu et al. 2014), *Enterococcus* (Lin et al. 2013) and *Pediococcus* and *Lactobacillus* (Munoz-Atienza et al. 2013) showed an antibacterial activity against fish pathogens, including *Vibrio*.

V. harveyi was detected in sole fed probiotic supplemented and especially in fish fed yeast-supplemented diets, and not detected in fish fed unsupplemented diets. These *Vibrio* species are included in the Harveyi clade and are well-known as pathogenic for marine-farmed organisms (Austin and Zhang 2006; Cano-Gomez et al. 2011; Gomez-Gil et al. 2004), including *S. senegalensis* in the case of *V. harveyi* (Arijo et al. 2005; Zorrilla et al. 2003). However, it is frequent in the presence of virulent and non-virulent strains of *V. harveyi* (Rico et al. 2008; Zorrilla et al. 2003), and for this reason, more studies are necessary to evaluate the potential virulence of the strains of these species and how diet formulation may modulate bacterial presence in the intestine. On the other hand, the dietary probiotic supplementation in PP35 diet (PP35_PROB) induced the presence of *S. saprophyticus*. *S. saprophyticus* is associated to microorganisms that play a critical role in fish fermentation and is known to be resistant to different antibiotics (Sergelidis et al. 2014; Zhang et al. 2015). In the current study, *S. saprophyticus* were detected solely in fish fed probiotic diets, especially in low PP groups. Although there are no reports that *S. saprophyticus* caused diseases in fish, Sun et al. (2011) speculate that it may be a potential harmful bacterium as it is often implicated in human urinary tract infections (Kuroda et al. 2005). Its presence in the intestinal microbiota of fish must be considered as a route for the transmission of antibiotic resistance (Chajecka-Wierzchowska et al. 2015).

In conclusion, Senegalese sole was able to cope with high plant ingredient content in replacement of marine-derived ingredients. After 2 and 17 days, diets with high PP content seem to acutely stimulate the innate immune defence of the fish, as suggested by lysozyme and ACH50 activities. However, after 73 days of feeding, fish fed PP72 showed reduced the number of the goblet cells and low hepatic glycogen content, indicating a lower capacity to overcome stress situations.

Our data demonstrates that it is possible to modulate the intestinal microbiota of Senegalese sole by dietary supplementation with probiotic or autolysed *S. cerevisiae*, even if *Vibrio* sp. are the predominant bacteria present in sole intestine. The diets with selected probiotics or autolysed yeast were able to reduce *Vibrio* load, but the appearance of *V. harveyi* and *S. saprophyticus* still has associated a potential health risk.

Dietary inclusion of the multispecies probiotic bacteria or autolysed yeast used in this work should be regarded with caution for Senegalese sole, since there was a negative impact on fish growth performance and no clear health benefits were observed at the end of the trial. More studies should be performed to evaluate if the selected probiotics' capacity to modulate gut microbiota results in clear health benefits for the fish.

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