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Histological and micro-/macro-morphological evaluation of intestine in sharpsnout seabream (*Diplodus puntazzo*) fed soybean meal-based diets added with MOS and inulin as prebiotics

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Abstract The effects of mannanoligosaccharides (MOS) and inulin (INU) inclusion (8 g kg^{-1}) in a soybean meal (SBM)-containing diet on histology, macro- and micro-morphology of proximal and distal intestine of sharpsnout seabream (*Diplodus puntazzo*) were evaluated. 144 sharpsnout seabream (100 g average initial body weight) were fed with 4 isolipidic and isoproteic diets. At the end of the experimental period (114 days), four fish per diet were randomly sampled. The partial substitution of fish meal (FM) with SBM affected the intestine histology and macromorphology of sharpsnout seabream and modified the enterocyte parameters. The addition of prebiotics has not produced positive effects, leading in some cases to a worsening, especially for INU diet that markedly altered the intestine histology. The micromorphological values were in both proximal and distal intestines significantly affected by the diet: in proximal tract, the nucleus was closer to the apex of the enterocyte in soy-fed fish (SBM, IMU, MOS diets), while fish fed with MOS diet showed the lowest value of enterocyte height, significantly different from the FM diet. In distal tract of intestine, fish fed with FM diet showed the highest value for all considered parameters, while MOS diet resulted in the lowest enterocyte height and supranucleus height among the four diets. SBM-containing diets (SBM, IMU, MOS) resulted in lower values than FM diet for the features of enterocyte nucleus. Microvilli height was not affected by the diet in both proximal and distal tracts. In conclusion in this study, the partial substitution of FM with SBM (40 % on protein basis) induced inflammatory reaction of the gut in sharpsnout seabream, evidenced by histological analysis and changes in morphometric characters of villi and enterocytes, not amended by the addition of MOS and even worsened in the case of inulin.

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Abbreviations

FM	Fish meal
SBM	Soybean meal
MOS	Mannanoligosaccharides
INU	Inulin
HE	Enterocyte height
hMV	Microvilli height
hSN	Supranucleus height
hN	Nucleus height
wN	Nucleus width
HF	Fold height
SPC	Soy protein concentrate

Introduction

Due to its protein/amino acid composition, competitive price and security of supply, soybean meal (SBM) is one of the most used protein source alternative to fish meal in aquafeed formulations (Lim et al. 1998; Hardy 1999; Storebakken et al. 2000; Swick 2002; Gatlin et al. 2007). Nevertheless, its utilization presents some problem of palatability (Papatryphon and Soares 2001) and has been demonstrated to contain anti-nutritional factors for fish (Gomes et al. 1995; Kaushik et al. 1995; Hardy 1996; Gomes da Silva and Oliva-Teles 1998; Francis et al. 2001; Gomez-Requeni et al. 2004; Ogunji 2004; Tibbetts et al. 2006; Tibaldi et al. 2006; Gatlin et al. 2007; Collins 2014), inducing histological changes of the fish gastrointestinal tract such as enteritis, increased susceptibility to bacterial infection, increased presence of inflammatory cells, villi shortening and reduced microvilli density and length (Baeverfjord and Krogdahl 1996; Krogdahl et al. 2000, 2003, 2009, 2011; Heikkinen et al. 2006; Bakke-McKellep et al. 2007).

Among the further possible candidates for mariculture diversification, sharpsnout seabream (*Diplodus puntazzo*) represents one of the most promising species to be managed in hatchery conditions (Micale et al. 1996; Garcia and Garcia 2010; Parisi et al. 2014) Sharpsnout seabream is an omnivorous species, and in the wild, it feeds on seaweeds, worms, molluscs and shrimps (Sala and Ballesteros 1997). The enzymatic pattern of *D. puntazzo* justifies its omnivorous habit and suggests a high enzymatic potential for both protein and vegetable polysaccharide digestion (Tramati et al. 2005).

The possible use of soybean meal as a substitute for fish meal in sharpsnout seabream diets was investigated by Hernandez et al. (2007), which tested different SBM inclusion levels in the diet (0, 20, 40, 60 %). These authors found a decrease in final weights as the soybean meal inclusion increased starting from 40 up to 60 %. Similarly, as the soybean meal content increased, feeding efficiency and protein utilization of the diets decreased, as an effect of the smaller digestibility coefficient observed for the diets containing soybean meal compared to the FM-based diet.

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon (Ringø et al. 2010). In the last years, several studies have indicated prebiotics as interesting feed supplements able to improve performances of farmed fish, enhancing their innate immune parameters, such as alternative complement activity (ACH50), lysozyme activity, natural haemagglutination activity, respiratory burst, superoxide dismutase activity and phagocytic activity, bacterial infections' resistance and health by the improvement of the ultrastructure of the intestine mucosa as well as the activation of health-promoting bacteria in the intestine, such as *Lactobacillus* and *Bifidobacterium*, and concentration of whole body protein (Genc et al. 2007a, b; Torrecillas et al. 2007; Yilmaz et al. 2007; Zhou et al. 2010; Ringø et al. 2010).

Aim of the research was to verify through histological analysis and morphometric measurements of distal and proximal intestine tracts, whether the supplementation with MOS or FOS in form of inulin could improve the health status of intestine in sharpsnout seabream amending the negative effects deriving by the administration of a soybean meal-based diet.

Materials and methods

The trial was carried out in the indoor partially recirculating water system (total volume 8 m^3) of the Department of Veterinary Medicine and Animal Production (University of Napoli Federico II, Italy), using 144 sharpsnout seabream of about 100 g (98.8 ± 2.5 g) initial body weight obtained from the Maricoltura Mattinatese s.r.l. company (Mattinata, Italy). After a short period of adaptation (15 days) in the quarantine tanks, during which fish were fed a commercial diet (FM), fish were randomly distributed in 12 fibreglass tanks (180 L each). The system was provided with thermostatic control and regulation of water temperature, mechanical sand filter, biological filter and UV lamp apparatus, and a constant and optimal environment quality was ensured to sharpsnout seabream (daily water renewal, 5 %; artificial day length, 12 h; temperature, 21.9 ± 1.6 °C; salinity, 30.0 ± 2 g L^{-1}; dissolved oxygen, 6.4 ± 1.5 mg L^{-1}; pH 7.5 ± 0.5; total ammonia nitrogen, <0.15 mg L^{-1}; nitrite nitrogen, <0.05 mg; nitrate nitrogen, <40 mg L^{-1}). Testing conditions included 12 fish per tank, with each diet being experimentally tested in triplicate. The experimental period lasted 114 days. Water temperature, pH and dissolved oxygen were measured daily using a mercury thermometer, Orian digital pH metre and oxygen metre (WTW, OXI330, Weilheim, Germany). Total ammonia nitrogen ($N\text{-}NH_3$), nitrite nitrogen ($NO_2\text{-}N$) and nitrate nitrogen ($NO_3\text{-}N$) were determined biweekly by colorimetric methods using commercial kits and a spectrophotometer (Hanna Instruments, C-203, Leighton Buzzard, UK). The tanks were inspected once daily for mortalities, and dead fish were removed immediately from the tanks after detection.

Diets and feeding

Four isolipidic (crude lipid about 14 % as fed) and isoproteic diets (crude protein about 49 % as fed) were formulated based on sharpsnout seabream nutritional requirements using commercial ingredients (Table 1). In the control diet (FM), "999" fish meal was the sole protein source. In the second diet (SBM), 38 % of fish meal protein was replaced by soybean meal. MOS and INU diets were prepared by adding 8 g kg^{-1} of mannanoligosaccharides (ECHOMOS; Mazzoleni Prodotti Zootecnici, Cologno al Serio, BG, Italy) and inulin [INULINA (F.O.S), Methodo Chemicals, Novellara, RE, Italy] to the SBM diet, respectively. The diets were produced in the laboratories of the Department of Veterinary

Table 1 Ingredient and proximate composition of experimental diets

Diets	FM	SBM	MOS	FOS
Ingredient (g kg^{-1})				
Fish meal, herring	695.0	415.0	415.0	415.0
Soybean meal	–	408.0	408.0	408.0
Fish oil	85.0	98.0	98.0	98.0
Starch	180.0	35.0	27.0	27.0
Vitamin mix	17.5	17.5	17.5	17.5
Mineral mix	2.5	2.5	2.5	2.5
Methionine	–	4.0	4.0	4.0
Binder	10	10	10	10
Mannanoligosaccharide	–	–	8.0	–
Fructooligosaccharide	–	–	–	8.0
Proximate composition (g kg^{-1})				
Dry matter	926.0	919.2	916.0	923.1
Crude protein	485.8	488.7	488.1	486.8
Crude lipid	135.5	148.5	145.0	145.9
Gross energy (MJ kg^{-1})	20.10	20.47	20.30	20.43

Medicine and Animal Production, University of Napoli Federico II, Italy. All ingredients were ground through a 0.5-mm sieve before final mixing and dry pelleting through a 3-mm dye.

Fish were daily hand-fed with two meals (9:00 and 16:00) ad libitum (i.e. until the first feed item was refused). The feed was administered over the whole water surface in the tanks in order to be accessible simultaneously for all the fish.

Histological analysis and morphometric evaluation of the gut

Sampling protocol

At the end of the experimental period, four fish per diet were killed by overdose of methanesulphonate tricaine (MS222), dissolved in water at the dose of 250 mg L^{-1} and weighted, and from each fish, the whole intestine was isolated and fixed in 10 % buffered formalin for histology. Proximal and distal segments (0.5–1 cm length) of the intestinal tube were collected, dehydrated by serial ethanol baths at increasing concentration (70°, 80°, 90°, 100°), further dehydrated in xylene and embedded in paraffin wax. The intestinal segments were then cut in two parts to have one longitudinal and one cross section. Slides were 4 μm thick and stained by Haematoxylin–Eosin stain.

Study of morphometric characters of villi (macromorphology)

To assess the modifications of the mucosal folds, sections were observed by a light microscope (Zeiss Axioskop 2 MOT) and for each section 10 pictures were taken by a digital camera (AxioCam MRc5). For both the distal and proximal parts, the longitudinal axis of 5 intestinal villi was measured considering the intestinal epithelium (HF = fold height), from the lamina propria to the luminal extremity, according to protocols described in the literature (Van den Ingh et al. 1991; Baeverfjord and Krogdahl 1996; Refstie et al. 2000, 2001; Escaffre et al. 2007).

Study of morphometric characters of enterocytes (micromorphology) (Fig. 1)

The height of enterocytes (HE), 20 measures from the proximal part and 10 from the distal part of intestine in each fish, was also taken. Further measures according to Escaffre et al. (2007) have been considered:

- height of microvilli (hMV) (ten measures from the proximal part and ten from the distal part);
- supranucleus height (hSN) (ten measures from the proximal part and ten from the distal part),
- nucleus height (hN) (eight measures from the proximal part and eight from the distal part).
- nucleus width (wN) (eight measures from the proximal part and eight from the distal part).

Statistical analysis

For each parameter, the mean value for each fish was processed using the following model (PROC GLM, SAS 2000): $y_{ij} = \mu + D_i + \varepsilon_{ij}$ where y represents each parameter, μ the

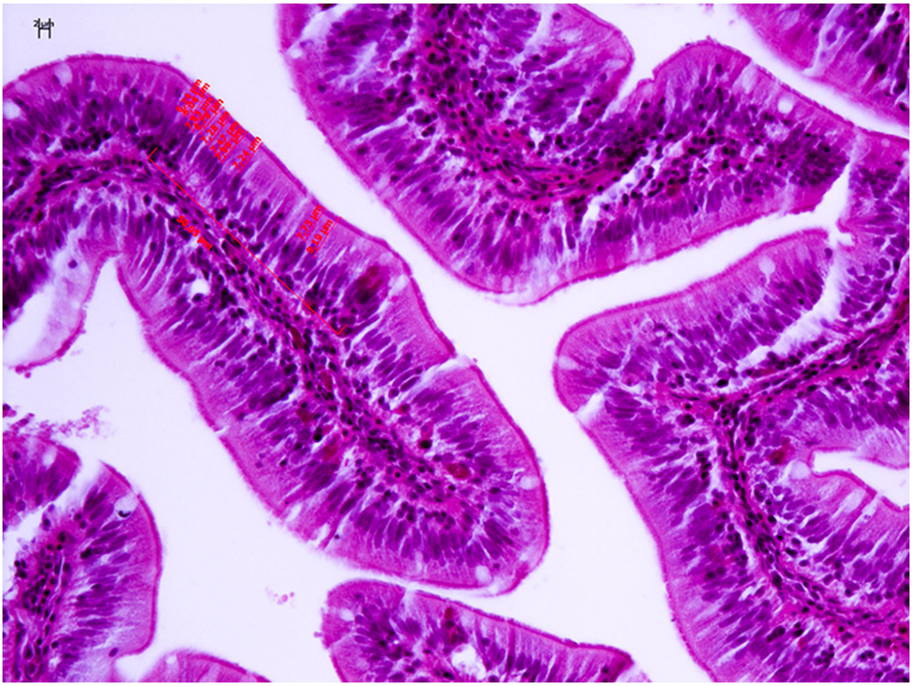

Fig. 1 Morphometric characters of enterocytes (micromorphology): supranucleus height (hSN) measures from the proximal part of intestine in *D. puntazzo* fed INU diet

mean, D the diet effect ($i = 1$–4) and ε the error terms. The differences among means were tested for significance using T test and were considered significant at $P < 0.05$.

Results

During the experimental period, mortality was 4.16 % and there was no statistical difference among groups.

Histological analyses are as follows.

Fish meal all the structure of villi was normal with a monolayer of enterocytes and a physiological presence of mucosal cells. Few grouped lymphocytes, and eosinophilic granular cells were observed within the lamina propria (Fig. 2a).

Soybean meal in all the examined fish both in proximal and distal segments, the top of the villi showed mild epithelial sloughing off. Few eosinophilic granular cells and several lymphocytes infiltrated the *lamina propria* and the villi (Fig. 2b).

Inulin in all the fish, distal and proximal segments showed marked changes in the villi, sometime scalloped and in some case fused together. Enterocytes were normal, mucosal

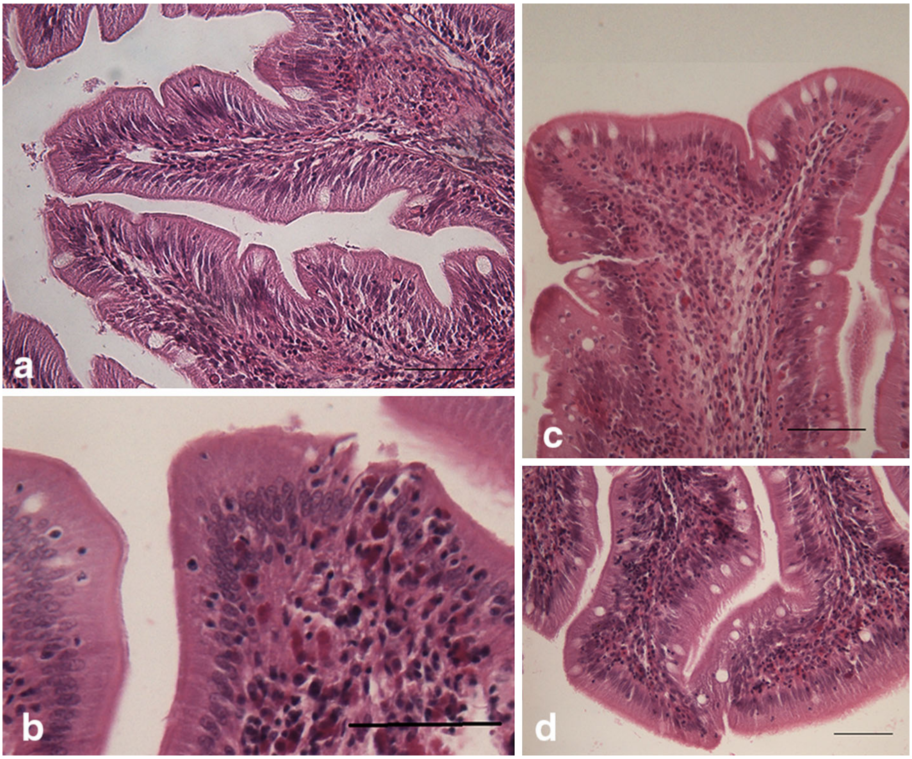

Fig. 2 Histological cross sections of the gut from *D. puntazzo* (EE, *scale bar* 50 µm): **a** fish meal (FM): intestinal villum with normal structure and few lymphocytes and eosinophilic granular cells; **b** soybean meal (SBM): epithelial sloughing off on the topo of the villum and lymphocyte infiltration of the *lamina propria*; **c** inulin (INU): thickening of the *lamina propria* with diffuse lymphocyte infiltration; **d** mannanoligosaccharide (MOS): lymphocytes and eosinophilic granular cell infiltration in the *lamina propria* and villum axis

cells were slightly hypertrophic and the *lamina propria* was thicker with diffuse lymphocyte infiltration (Fig. 2c).

MOS in all the fish both in proximal and distal segments, the villi showed modifications only in the hypertrophy of mucosal cells and lymphocytes and eosinophilic granular cell infiltrations in the *lamina propria* and villus axis (Fig. 2d).

Macromorphology of the intestine

In the intestine proximal tract, fish fed on SBM diet showed the highest HF value (1186.7 μm) and it was significantly different from fish fed on INU diet that showed the lowest value. In the distal intestine, diet did not affect fold height (Table 2).

Micromorphology of intestines

The micromorphological values were in the same range in proximal and distal intestines.

The structure of both proximal and distal epithelia was significantly affected by the diet.

In proximal tract, the nucleus was closer to the apex of the enterocyte in soy-fed fish (6.67, 6.50 and 6.49 μm for SBM, INU and MOS, respectively) compared to the control diet (8.81 μm; $P < 0.05$). MOS diet showed the lowest value for HE parameter (10.26 μm), significantly different from FM diet (12.81 μm). The features of enterocyte nucleus (HN an WN) were unaffected by the diets (Table 3).

In distal tract of intestine, the three SBM-containing diets showed lower values compared to control diet that showed as expected the highest value for all considered parameters. In particular, for HE and HSN, MOS diet showed significantly lower values (10.06 and 5.12 μm, respectively) compared to both FM (16.57 and 8.91 μm) and SBM diets (13.47 and 7.51 μm). INU diet, instead, resulted significantly lower just than FM diet showing intermediate values between MOS and SBM diets (12.07 and 6.27 μm). For the features of enterocyte nucleus (HN and WN), the general trend was the same with SBM-containing diets showing lower values than FM diet (Table 4).

Microvilli height was not affected by the diet in both proximal and distal tracts.

Table 2 Height of the villi (macromorphology) in intestine distal and proximal tracts (μm)

	FM	MOS	INU	SBM	MSE
Fish average body weight (g)	133.6	134.4	129.8	140.5	295.46
HFprox	1042.6^{ab}	1028.6^{ab}	936.0^{b}	1186.7^{a}	25,571.82
HFdist	763.6	922.7	753.5	773.2	23,920.48

Means followed by different superscript letters are significantly different ($P < 0.05$)

Table 3 Micromophology of proximal intestine (μm)

Dieta	HSN	HN	WN	HE	HMV
FM	8.61^{a}	3.82	1.59	12.81^{a}	1.50
INU	6.50^{b}	4.36	1.51	11.34^{ab}	1.21
MOS	6.49^{b}	4.12	1.66	10.26^{b}	1.43
SBM	6.67^{b}	4.17	1.67	11.74^{ab}	1.67
MSE	1.1674	0.1765	0.0342	1.4814	0.1124

Means in a column followed by different superscript letters are significantly different ($P < 0.05$)

Table 4 Micromophology of distal intestine (μm)

Dieta	HSN	HN	WN	HE	HMV
FM	8.91^{a}	5.95^{A}	1.74^{a}	16.57^{Aa}	1.33
INU	6.27^{bc}	4.1^{B}	1.65^{ab}	12.07^{bc}	1.74
MOS	5.12^{c}	3.99^{B}	1.52^{ab}	10.06^{Bc}	1.53
SBM	7.51^{ab}	4.62^{B}	1.50^{b}	13.47^{b}	1.44
MSE	1.1483	0.1637	0.0124	1.2661	0.1262

Means in a column followed by different superscript letters are significantly different (^{a,b} $P < 0.05$; ^{A,B} $P < 0.01$)

Discussion

In the present study, the partial substitution of FM with SBM altered the histology and macromorphology of sharpsnout seabream intestine and modified the enterocyte morphology.

In fish species, a different sensibility to the anti-nutritional factors, such as soybean trypsin inhibitor, saponins and lectins, is reported to be higher in the carnivorous fish such as Atlantic salmon (*Salmo salar*) (Krogdahl et al. 2003) and rainbow trout (*Oncorhynchus mykiss*) (Heikkinen et al. 2006), lower or negligible in other species such as cod (*Gadus morhua*) (Refstie et al. 2006), halibut (*Hippoglossus hippoglossus*) (Grisdale-Helland et al. 2002), gilthead seabream (*Sparus aurata*) (Bonaldo et al. 2008, Dimitroglou et al. 2010), European sea bass (*Dicentrarchus labrax*) (Bonaldo et al. 2008), cobia (*Rachycentron canadum*) (Romarheim et al. 2008) and sole (*Solea aegyptiaca*) (Bonaldo et al. 2006).

Changes typically associated with soy-induced enteritis observed in the present study were in accordance with previous descriptions in Atlantic salmon in which shortening of heights of the intestinal mucosal foldings, loss of the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium, widening of the central stroma within the mucosal folding with increased amounts of connective tissue, profound infiltration of inflammatory cells constituted by lymphocytes, macrophages and polymorphonuclear leucocytes in the lamina propria and an increased number of eosinophilic granular cells were reported (Van den Ingh et al. 1991, 1996a, b; Baeverfjord and Krogdahl 1996; Refstie et al. 2001) and in contrast with the results reported by Escaffre et al. (2007) in a trial carried out on rainbow trout (*O. mykiss*).

However, in our study, the addition of SBM to the diet, despite having led to changes in the histology of intestine, has not had a negative effect on the final weights of the fish. This could suggest that the magnitude of these changes was not able to have an impact on growth performance. In several cases, SBM caused flogistic reactions in the gut directly proportional to the amount of SBM used in the feed. Otherwise, it has also been observed that inflammatory reactions start with the administration of a SBM-based diet and progressively decrease for an adaptation of the fish to the vegetal component of the feed. This adaptation is manifested histologically through the reduction of lymphocyte infiltration and a histological recovery of the physiological aspect of villi and epithelial cells of intestinal mucosa (Urán et al. 2008a, b). The adaptation to the SBM seems to be more efficient in the omnivorous fish species as sharpsnout seabream, if compared to the carnivorous.

In fact in the literature, similar studies were carried out on *Cyprinus carpio* (Urán et al. 2008a, b) and *S. salar* (Baeverfjord and Krogdahl 1996; Urán et al. 2008a, b), the former showing a milder enteritis recovering almost completely the epithelial integrity after an adaptation period of 5 weeks, the latter a non-infectious sub-acute enteritis of the distal epithelial mucosa without recovering until the interruption of the SBM administration.

In our study, lasted 16 weeks, we noticed pathological signs such as inflammatory reaction and infiltrated lymphocytes in the lamina propria in all the diets containing SBM (SBM, MOS, INU).

Especially in fish fed diets containing inulin, we had a thickening of *lamina propria* with several infiltration of lymphocytes and increasing of goblets cells. Further study aimed to monitor the evolution of the observed modifications over time and to link these latter to the growth performance of fish is required.

However, these modifications were partially confirmed in the micrometric evaluation of the villi and enterocytes.

Olsen et al. (2001) reported changes in the organization of microvilli and the presence of intracellular lamellar bodies in distal intestine enterocytes in Arctic charr fed on inulin-added diets (15 %). The same authors also reported increased vacuolization of distal intestine enterocytes (measured as percentage of cell volume).

On the contrary, recent studies carried out on Atlantic salmon fed with 7.5 % of inulin supplementation did not show significant differences on the histological features of intestine (Refstie et al. 2006; Bakke-McKellep et al. 2007).

Even if in our trial the percentage of inulin inclusion (almost 1 %) was significantly lower than that used in the mentioned trials, it is possible to assume that the inclusion of inulin did not lead to improvements compared to SBM diet or even led to worse results.

On the contrary, in fish fed MOS diet the proximal and distal tracts of intestine did not show modification of the macromorphology of villi.

MOS utilization is reported to produce in some cases improvements on enterocytes and in particular on the brush border. In European sea bass fed a diet containing 0.4 % of MOS, no variation was observed (Torrecillas et al. 2007), instead of Senegalese sole (Sweetman and Davies 2005) and rainbow trout (Yilmaz et al. 2007) in which an improved microvilli alignment and an increase of villi length of the proximal intestine were noticed, respectively.

Other authors assessed that any level of supplementation of MOS in the feed did not show any alteration of intestinal morphology in rainbow trout and gilthead seabream (Torrecillas et al. 2007; Yilmaz et al. 2007; Bonaldo et al. 2008) and moreover increased density and length of enterocyte brush border in rainbow trout (Dimitroglou et al. 2008). The differences among the various authors could be due to the different doses used in the various studies, the species involved in the trials, the bacterial flora present in different fish species, different rearing conditions or methodological approaches used. In our study in MOS-fed fish, we did not observe any modification of the structure of the villi compared to SBM diet. The presence of lymphocyte infiltration in the lamina propria and the villi could be consequent to the SBM anti-nutritional effect not modified by MOS supplementation. Further studies could consider higher inclusion levels of MOS supplementation to better understand the real effectiveness of this prebiotic in this fish species.

For what concerns micromorphology parameters, Escaffre et al. (2007), in a trial on rainbow trout fed soy protein concentrate (SPC), found that the diet did not affect the height of villi and the height of enterocytes in the proximal part of intestine. Otherwise, in the distal intestine, the diet influenced the height and the width of enterocytes; this measure was significantly lower in SPC diet than in control diet. The enterocyte nucleus was closer to the apex in the proximal and distal intestines in SPC-fed fish, but the features of the nucleus were not affected by the diet. The same effect has been observed by other authors when soybean meal is substituted to fish meal, but only at the distal and not the proximal intestine level (Van den Ingh et al. 1991, 1996a, b; Baeverfjord and Krogdahl 1996).

Our study highlighted that all the micromorphology parameters were affected by the presence of SBM in the diet. The addition of prebiotics did not modify this condition in some cases leading to a worsening. In particular, the height of villi (HF) of the proximal gut was influenced negatively by the presence of inulin in the diet, while in distal tract MOS diet resulted in significantly lower values of HE and HSN even if compared to SBM. On the other hand, the addition of MOS and inulin to the diets did not have positive effects on fish growth performance. These results are in agreement with those previously obtained on other fish species such as Gulf sturgeon (Pryor et al. 2003), hybrid tilapia (Genc et al. 2007a), Atlantic salmon (Grisdale-Helland et al. 2008) and gilthead seabream (Dimitroglou et al. 2010), and in contrast to the results on rainbow trout (Staykov et al. 2007), green tiger prawn (Genc et al. 2007b) and European sea bass (Torrecillas et al. 2007), which showed MOS improving growth performance in these species. The lack of growth response inulin is in agreement with the results on turbot larvae (Mahious et al. 2006), Atlantic salmon (Refstie et al. 2006; Grisdale-Helland et al. 2008) and juvenile red drum (Buentello et al. 2010). Our results could be also ascribed to the dose/concentration at which prebiotics were used in our trial. In particular, MOS concentration merits further studies, while the negative effects registered for inulin at histological and morphological level seem to suggest that an increased concentration of this prebiotic in the diet is not recommended for this fish species at least.

In conclusion, this study confirms that soybean meal contains factors inducing inflammatory reaction of the gut also in an omnivorous species such as sharpsnout seabream. The impact of the use of soybean meal is marked at both histological and cellular levels, even if such intestinal alterations were not such as to generate negative effects on the growth performance of fish in this trial. The addition of prebiotics such as inulin and MOS did not lead to the expected results. On the contrary, inulin resulted in marked changes of the villi in both distal and proximal segments even at a low rate of inclusion ($\sim$1 %). MOS, instead, at the tested level did not modify histological parameters but induced significant modifications at enterocyte level.

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