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Increased parasite resistance of greater amberjack (*Seriola dumerili* Risso 1810) juveniles fed a cMOS supplemented diet is associated with upregulation of a discrete set of immune genes in mucosal tissues

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ABSTRACT

The main objective of this study was to determine the effect of two forms of mannan oligosaccharides (MOS: Bio-Mos® and cMOS: Actigen®, Alltech Inc, USA) and their combination on greater amberjack (*Seriola dumerili*) growth performance and feed efficiency, immune parameters and resistance against ectoparasite (*Neobenedenia girellae*) infection. Fish were fed for 90 days with 5 g kg^{-1} MOS, 2 g kg^{-1} cMOS or a combination of both prebiotics, in a *Seriola* commercial base diet (Skretting, Norway). At the end of the feeding period, no differences were found in growth performance or feed efficiency. Inclusion of MOS also had no effect on lysozyme activity in skin mucus and serum, but the supplementation of diets with cMOS induced a significant increase of serum bactericidal activity. Dietary cMOS also reduced significantly greater amberjack skin parasite levels, parasite total length and the number of parasites detected per unit of fish surface following a cohabitation challenge with *N. girellae*, whereas no effect of MOS was detected on these parameters. Of 17 immune genes studied cMOS dietary inclusion up-regulated hepcidin, defensin, Mx protein, interferon-γ (IFNγ), mucin-2 (MUC-2), interleukin-1β (IL-1B), IL-10 and immunoglobulin-T (IgT) gene expression in gills and/or skin. MOS supplementation had a larger impact on spleen and head kidney gene expression, where piscidin, defensin, iNOS, Mx protein, interferons, IL-1β, IL-10, IL-17 and IL-22 were all upregulated. In posterior gut dietary MOS and cMOS both induced IL-10, IgM and IgT, but with MOS also increasing piscidin, MUC-2, and IL-1β whilst cMOS induced hepcidin, defensin and IFNγ. In general, the combination of MOS and cMOS resulted in fewer or lower increases in all tissues, possibly due to an overstimulation effect. The utilization of cMOS at the dose used here has clear benefits on parasite resistance in greater amberjack, linked to upregulation of a discrete set of immune genes in mucosal tissues.

1. Introduction

Seriola aquaculture has traditionally been focused on yellowtail kingfish (*S. lalandi*) and Japanese amberjack (*S. quinqueradiata*) [1]. In Europe, greater amberjack (*Seriola dumerili*, Risso 1810) is considered an emerging aquaculture species due to its high commercial value and fast-growth [2], where under appropriate culture conditions they can reach 6 kg in 2.5 years [3]. Nevertheless, greater amberjack production in sea cages is limited by several bottlenecks, with monogenean ectoparasite outbreaks a key concern [4–6].

Neobenedenia girellae is a monogenean ectoparasite that has become one of the main causes of greater amberjack parasitic infections. It is characterised by a broad host range and wide distribution in warm waters, with an important prevalence in aquaculture farms [4,7]. Its lifecycle is highly dependent of seasonal temperature [8–11] which promotes the parasite attachment to the host. Furthermore, parasite attachment to fish skin produces important alterations [5,12] such as wounds and ulcers, promoting secondary infections [13], thereby

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increasing mortality. To fight secondary infections, especially those caused by fungi and bacteria, several different strategies have been adopted, mainly based on the use of antibiotics and topical treatments that have some risks [14]. Nowadays, one of the most common strategies to avoid the use of antibiotics is to boost the immune system to enable fish to overcome pathogen infections [15,16]. These strategies include dietary inclusion of prebiotics and use of functional feeds, some of which have been shown to affect ectoparasite prevalence [17,18].

Prebiotics are commonly used in the animal production industry due to their effects on the immune system leading to pathogen protection [19]. It has been well established that the by-products produced when beneficial commensal bacteria ferment prebiotics play a key role in improving host health [20]. New prebiotics have been showing successful results [21], including mannan oligosaccharide (MOS) by-products [22–25]. Studies of MOS beneficial effects have focused on growth performance and health, especially the modulation of intestinal microbiota and promotion of gut integrity in adult and juvenile fish [23,26]. However, MOS effects are known to be highly dependent upon the biotic parameters of the cultured fish, including the species, culture conditions, duration of the supplementation, age and size [21,27].

Previous studies have shown that an inclusion level of 4g MOS kg^{-1} in diets increases growth performance, feed efficiency and feed intake in salmonids and seabass after 67 days of supplementation [22,28]. In contrast, in gilthead seabream and channel catfish no effect was observed on these parameters using this inclusion level during 63 and 42 days respectively [29,30], but changes of the immune system were found. Similarly, in rainbow trout [31] fed a functional diet with 2 g MOS kg^{-1} during 42 and 90 days improved antibody production and lysozyme activity were found, and in Japanese flounder, after 56 days of dietary inclusion of 5 g MOS kg^{-1} gave higher lysozyme activity, although no differences were observed in the numbers of cells undergoing phagocytosis or the phagocytic index [32]. However, in Atlantic salmon (200g) fed a diet supplemented with 10 g MOS kg^{-1} for 4 months no effects on the innate immune system were seen [33]. Such studies suggest that the effects are not consistent between species or that there is a limited duration of the MOS effect on the host immune response. Recently the study of key regulatory cytokines as markers has also become a useful indicator of the immune system status in fish. For instance, previous studies with Atlantic cod showed that MOS dietary inclusion produces changes in gut cytokine expression levels after 35 days of supplementation [34]. Clearly future studies on cytokines are warranted to shed light on MOS effects.

Little information is available about the immune system of greater amberjack [10,35] and few studies have investigated the use of immunostimulants with this species [36–38], with none using MOS or concentrated MOS (cMOS) inclusion. For this reason, the objective of the present work was to determine the effect of MOS and cMOS (Bio-Mos® and Actigen®) and their combination on greater amberjack juveniles, focusing on immune parameters, protective effects against a *N. girellae* and any impact on growth/feed efficiency.

2. Materials and methods

The present study was conducted at the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals and were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria. For the whole trial, a tank is considered as an experimental unit.

2.1. Experimental fish and conditions

Two hundred and sixteen fish (mean weight 331.4 ± 30 g) were distributed in twelve cylindroconical 1000 L tanks with an open circulation (18 fish/tank). Water conditions were monitored daily, maintaining salinity at 37 mg L^{-1}, oxygen values at 6.0 ± 1 ppm O_2 and temperature at 23 °C ± 0.3 during July, August and September. Fish were fed by hand 3 times per day to apparent satiety. Uneaten pellets were recovered, dried and weighed.

2.2. Diets

The diets used combined a *Seriola* base diet designed by Skretting (Stavanger, Norway) and containing 55% protein, 55% fish meal and 10% fish oil, with two different prebiotics, namely MOS and cMOS (Bio-Mos® and Actigen® developed by Alltech, Inc.). Diet C (control) was composed exclusively of the *Seriola* base diet, the MOS diet included 5 g Bio-Mos® kg^{-1}, the cMOS diet 2 g Actigen® kg^{-1}, and the MOS + cMOS diet had 5 g Bio-Mos® kg^{-1} and 2 g Actigen® kg^{-1}. Each diet was randomly assigned to triplicate groups of fish (n = 3 × 3).

2.3. Sampling procedures

Sampling was conducted after 0, 30 days, 60 days and 90 days of feeding, where growth and feed utilization parameters were evaluated. Additionally, at the end of the feeding trial head kidney, spleen, gills, posterior gut and skin of 3 fish per tank were sampled for immune gene expression analysis. Skin mucus and blood (serum) were also collected from 3 fish per tank. Finally, a parasite challenge against *N. girellae* was performed (as outlined below).

2.4. Fish performance parameters

Specific growth rate (SGR) and feed efficiency were calculated as follows:

SGR = (Ln (final weight) – Ln (initial weight))*100/feeding time (days)

Feed efficiency = (feed intake/weight gain)

2.5. Gene expression analyses

Samples for gene expression analyses were collected in RNAlater and stored for 48 h at 6 °C. Total RNA was subsequently extracted using the Trizol reagent method (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry measuring the absorbance at 260 and 280 nm (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain). Electrophoresis in agarose gels was conducted to check extracted RNA quality by visualization of RNA bands. DNase treatment was applied to the extracted RNA, according to the manufacturer's instructions, to remove possible contaminating genomic DNA (AMPD1–1 KT, Sigma–Aldrich, Broendby, Denmark). Total RNA was reverse transcribed in a 20 μL reaction volume containing 2 μg total RNA, using a ThermoScript ™ Reverse Transcriptase (Invitrogen) kit, until cDNA was obtained in a thermocycler (Mastercycle® nexus GSX1, Eppendorf AG, Hamburg, Germany) run according to the manufacturer's instructions. The samples were then diluted 1:20 in miliQ water and stored at −20 °C.

Specific primers were designed to target genes found in genbank from species phylogenetically related with *S. dumerili* (Table 1), following the methodology described in Ref. [39]. The primers were used to amplify products using amberjack cDNA obtained from a pool of gill, posterior-gut, head kidney and spleen tissue, and the products cloned and sequenced. At least a partial sequence was obtained for all the target genes and these partials were sufficient in length to determine gene identity and develop qPCR primers. qPCR was conducted with SYBRgreen and truestar taq following a programme of: 1 cycle of 6 min denaturalization at 95 °C, 45 cycles of amplification (25 s at 95 °C, 30 s at the annealing temperature, 25 s at 70 °C for the extension, and 5 s at 82 °C), 1 cycle for the melting curve of 5 s at 95 °C and 1 min at

Table 1
Primers used for gene expression analysis by RT-qPCR in skin, gill, posterior gut, head kidney and spleen of greater amberjack juveniles (*Seriola dumerili*) fed MOS and cMOS (t = 90 days).

Gene	Name	Ann. temp. (°C)*	Product size (bp)	Forward Sequence	Reverse Sequence
Hep	Hepcidin	61	99	GATGATGCCGAATCCCGTCAGG	CAGAAACCGCAGCCCTTGTTGGC
Pis	Piscidin	58	112	ATC GTC CTG TTT CTT GTG TTG TCA C	CGC TGT GGA TCA TTT TTC CAA TGT GAA A
Def	Defensin	60	133	ATGAGGCTGCATCCTTTCCATG	AGAAAATGAGATACGCAACACAAGAAGCC
iNOS	Inducible Nitric oxide synthase	60	151	TGTTTGGCCTTGGCTCCAGGG	GCCCAAGTTCTGAATGACTCCTCCTG
TNFα	Tumor necrosis factor α	62	212	GAAAACGCTTCATGCCTCTC	GTTGGTTTCCGTCCACAGTT
MX Prot	Interferon-inducible Mx protein	61	211	GGCTACATGATTGTGAAGTGCAGGG	CTTCCAGTCGAGGCAGAGATTTCTCAATGT
IFN γ	Interferon γ	59	163	AACTTGGTTTCACGGTGCAG	TCACAACACCGAGAAAGTCCT
IFN d	Interferon type I	59	111	GTCAGGGTGCAGCTGAGTTA	ACAGAAACGGCAGCTCAAAC
MUC-2	Mucin-2	62	342	ATT GAG TTT GGC AAC AAA CAG AAA GCC C	TAC AGC ACA GAA CTG AGG TGT CCT C
IL-1β	Interleukin 1β	62	205	TGATGGAGAACATGGTGGAA	GTCGACATGGTCAGATGCAC
IL-8	Interleukin 8	58	164	GAAGCCTGGGAGTAGAGCTG	GGGGTCTAGGCAGACCTCTT
IL-10	Interleukin 10	58	134	CTC AAG AGT GAT GTC ACC AAA TGT AGA AAC T	AGC AAA TCC AGC TCG CCC ATT
IL-17F	Interleukin 17F	62	120	GGTGGCCCCAGAGGATCTCC	GGAGGACCAAAACCTGGTAGTAGATGG
IL-17D	Interleukin 17D	62	111	CGGTCTACGCTCCCTCCGTG	GCGGCACACAGGTGCATCCC
IL-22	Interleukin 22	61	146	GCC AAC ATC CTC GAC TTC TAC CTG AAC	TGG TCG TGG TAG TGA GTC ACA TTG C
IgM	Immunoglobulin M	58	148	CTCTTTGATAGGAATACCGGAGGAGAG	CAACTAGCCAAGACACGAAAACCC
IgT	Immunoglobulin T	59	196	TGGACCAGTCGCCATCTGAG	GGGAAACGGCTTTGAAAGGA
β-Actin	β-Actin	61	212	TCT GGT GGG GCA ATG ATC TTG ATC TT	CCT TCC TTC CTC GGT ATG GAG TCC
EF1 α	Elongation factor 1α	60	194	TGC CAT ACT GCT CAC ATC GCC TG	ATT ACA GCG AAA CGA CCA AGA GGA G

*Ann. temp: annealing temperature.

75 °C, ending with 1 cycle of cooling for 1 min at 40 °C. MUC-2 was only analysed in the mucosal tissues and not head kidney and spleen.

2.6. Blood and mucus immunological parameters

Serum was obtained by centrifuging the collected blood after clotting overnight at 4 °C. Skin mucus was obtained following the methodology described by Guardiola et al. [40] with some modifications. Skin mucus was collected by gently scrapping the surface of the fish skin with autoclaved microscopy slides and diluted 1:1 with filtered and autoclaved salt water. Lysozyme activity was determined as described by Ellis [41]. Lysozyme activity was expressed in units ml^{-1}, were one unit of lysozyme was considered as the quantity of enzyme needed for reducing absorbance by 0.001 per millilitre of serum and mucus per minute. Bactericidal activity was measured with a modification of the method described by Sunyer and Tort [42], using *Photobacterium damselae*.

2.7. Parasite infection

The parasite source was a tank (10,000 L) of *S. dumerili* naturally infested with *Neobenedenia girellae* at high parasite density. Nets (0.14 mm pore diameter) were placed into the tank to entangle the eggs and collect them. After 24 h eggs were introduced into a 1000 L tank with 200 uninfected *S. dumerili* juveniles. After 10 days, all the fish were infected to the same degree. Then, 96 infected animals from the source tank were placed into twelve 0.03 m^3 cages (8 infected fish per cage and one cage per experimental tank) for 15 days, to enable a cohabitation challenge after 100 days of prebiotic inclusion. After 15 days of cohabitation, the remaining one hundred eighty experimental fish were sampled, and a visual evaluation of infection level for each fish was carried out by 3 different trained researchers. The levels were scored between 0 (no parasites observed), 1 (between 1 and 5 parasites), 2 (between 6 and 15) and 3 (more than 15). After that, the fish were introduced into freshwater to release all of the attached parasites, and the parasites counted and measured. The number of parasites per fish was converted into the number of parasites per square centimetre of fish surface area, calculated following the method described in Ohno et al. [43]. Total length of 50 adult parasites per tank was recorded using a profile projector (Mitutoyo, PJ-A3000).

2.8. Statistical analyses

The statistical analyses followed the methods outlined by Sokal and Rolf [44], with means and standard deviations (SD) calculated for each parameter measured. All data were tested for normality and homogeneity of variance. Data were subjected to one-way ANOVA and differences were considered significant when $P < 0.05$. Two-way ANOVA was conducted for MOS, cMOS and the interaction among treatments. If the variances were not normally distributed, data were transformed (log_{10}) and the Kruskall-Wallis non-parametric test applied. Kruskall-Wallis analysis was also used for range-comparison statistical analyses. Analyses were performed using SPSS software (SPSS for windows 10).

Multivariant analyses and their plots were performed using PRIMER 7 and PERMANOVA. The number of permutations was established at 999. PERMANOVA analysis considered differences significant when the permutation p-value (p perm.) was below 0.05 (See Figs. 1-5).

3. Results

3.1. Growth performance

No effect of MOS, cMOS or their combination was observed in final weight, SGR or feed efficiency among fish fed the different dietary treatments ($p > 0.05$), although fish fed the cMOS diet tended to perform better (+4% SGR) (Table 2).

3.2. Serum and skin mucus immunological parameters

After 90 days of feeding, two way-ANOVA analysis revealed a significant increase in serum bactericidal activity in fish fed MOS ($F = 6.68$, $P = 0.04$) and cMOS ($F = 17.56$, $P = 0.02$), whereas no effect was detected when measured in mucus (Table 2). Lysozyme activity in mucus and serum was not affected by MOS or cMOS dietary supplementation. No interaction between MOS and cMOS was detected for the mucus and serum immune parameters evaluated (Table 2).

3.3. Parasite challenge

Greater amberjack given dietary supplementation of cMOS for 90 days had significantly reduced skin parasite levels ($F = 6.17$, $P = 0.01$),

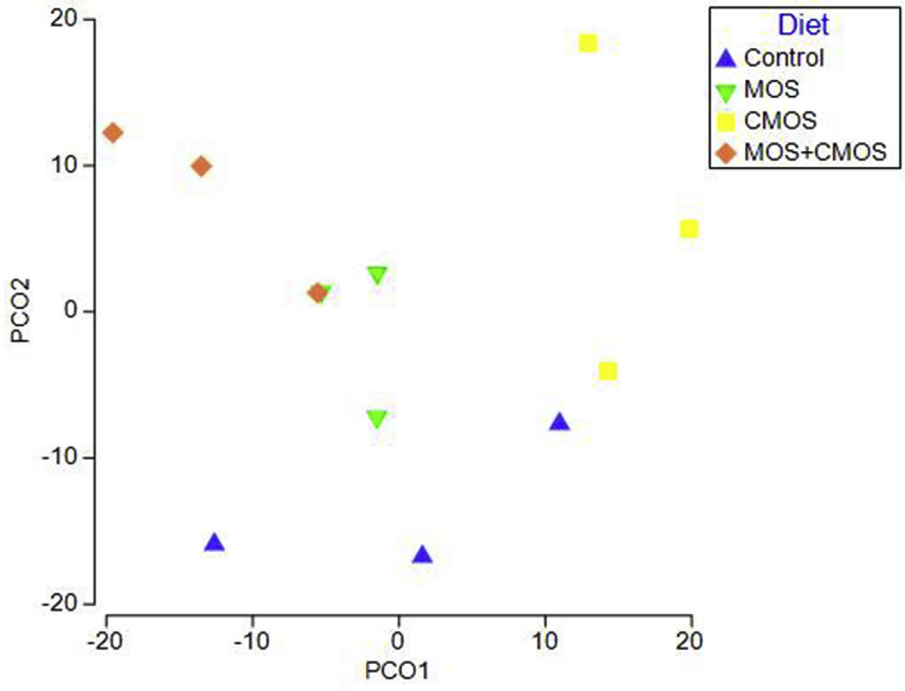

Fig. 1. Skin principal coordinates analyse (PCO).

parasite total length (F = 15.47, P = 0.01) and the number of parasites by unit of fish surface (F = 52.36, P = 0.01) following challenge with *N. girellae*. No specific effect of MOS was found on these parameters (Table 2) and no interaction between MOS and cMOS was detected.

3.4. Gene expression

At the end of the feeding trial (90 days), two way-ANOVA analyses showed that dietary cMOS up-regulated skin hepcidin, MUC-2, IL-1β, IL-10 and IgT (Table 3). On the other hand, a down-regulation of skin iNOS gene expression was detected after dietary MOS supplementation, and supplementation with both products resulted in a down-regulation of skin IL-10, IL-17D and IgT and a reduced impact on IFN expression vs the single supplements (Table 3).

In gills, dietary cMOS up-regulated hepcidin, defensin, Mx protein and IFNγ transcript levels (Table 4). No effects of dietary MOS were found. However, supplementation with both products resulted in down-regulation of gill IgT and reduced the cMOS effect on defensin and Mx protein gene expression in gills (Table 4).

Regarding fish posterior gut, two way-ANOVA analysis showed that

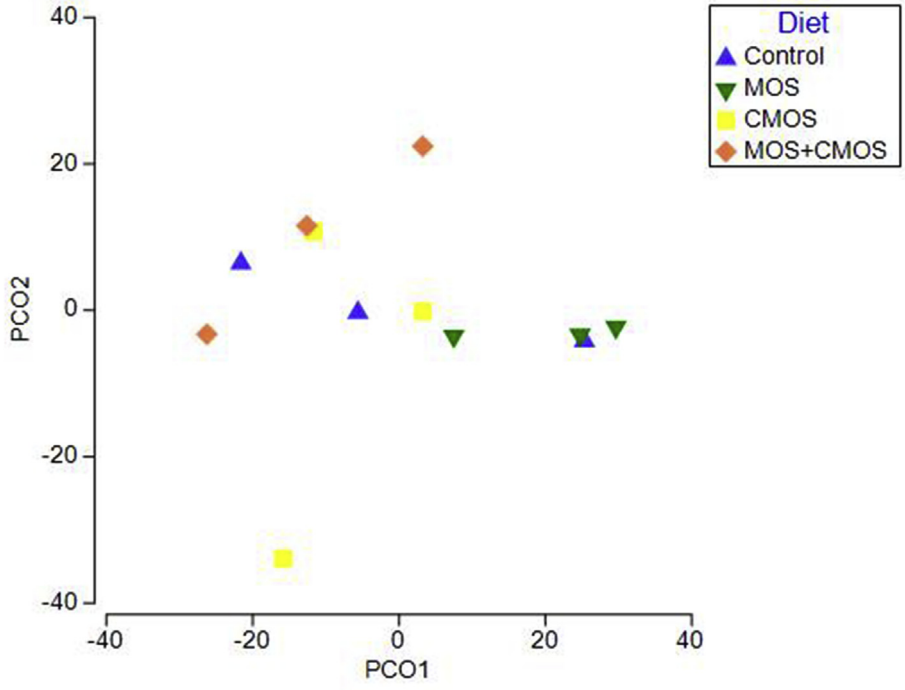

Fig. 2. Gills principal coordinates analyse (PCO).

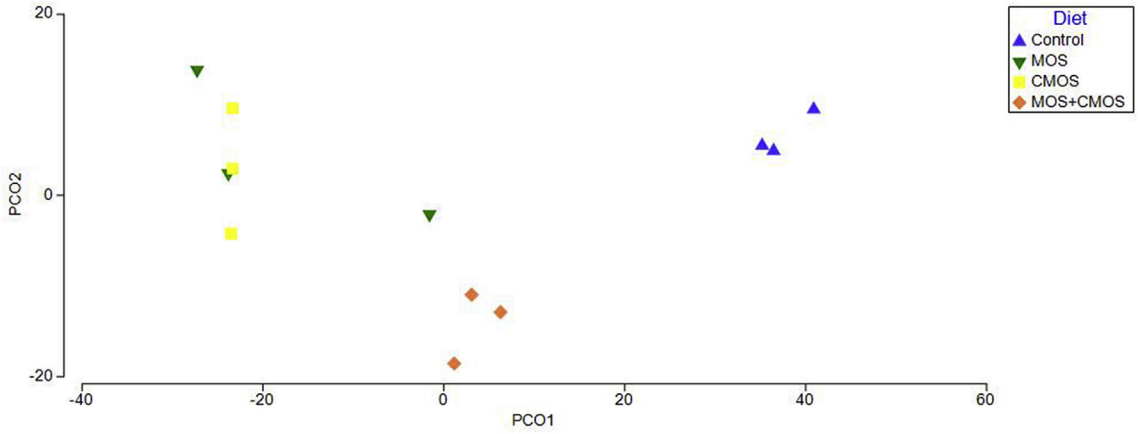

Fig. 3. Posterior gut principal coordinates analyse (PCO).

dietary cMOS up-regulated expression of hepcidin, defensin, IFNγ, IL-10, IgM and IgT. Additionally, dietary MOS up-regulated piscidin, MUC-2, IL-1β, IL-10, IgM and IgT gene expression. However, supplementation with both products down-regulated IFNγ ($F = 1.09$, $P = 0.02$) and IgM ($F = 2.41$, $P = 0.02$) gene expression and lost the effects on IL-10 and IgT (Table 5).

Head kidney gene expression analyses showed that dietary cMOS up-regulated hepcidin, IFNd, IL-10 and IL-22, while MOS up-regulated iNOS, Mx protein, IFNd, IL-10, IL-17D and IL-22. Supplementation with both products resulted in up-regulation of defensin and Mx protein but down-regulated IL-10 transcript levels relative to single supplementation (Table 6). In addition, the effects on IFNd and IL-22 were lost.

Lastly, cMOS down-regulated spleen hepcidin gene expression whilst dietary MOS induced expression of piscidin, defensin, IFNγ, IL-1β and IL-17D in this tissue. Supplementation with both products further increased defensin expression (Table 7).

Multivariant analyses comparing gene expression data presented different responses for each tissue and are presented in Annex 1 (supplementary files). Principal coordinates analysis (PCO) of skin clearly separated responses in fish fed the cMOS diet from fish fed the other dietary treatments, with the main sources of variation due to anti-microbial peptides (AMPs) (piscidin and defensin), MUC-2, iNOS, TNFα, Mx Protein, IL-8, IL-10, IL-17 and IFN genes. PERMANOVA analysis indicated differences in gene expression between MOS and cMOS, with an interaction effect more related to PC1 (p-perm. < 0.05).

PCO analysis in gill partially separated the MOS and cMOS effects due to AMPs and IFNs. Nonetheless, PERMANOVA analysis showed no difference between MOS and cMOS in this tissue (p-perm. > 0.05).

PCO analysis of posterior gut clearly separated dietary treatments into three different groups: control, MOS and cMOS, and MOS + cMOS. This variation was due to the effect on AMPs, IL-10, IFNs and iNOS gene expression. Hence, the posterior gut PCO PERMANOVA analysis found differences between MOS, cMOS and an interaction effect more related to PC2 (p-perm. < 0.05).

PCO analysis of head kidney discriminated cMOS from the other treatments due to the effect of this prebiotic on Igs and AMP gene expression. MOS treatment was also differentiated from the other treatments in the spatial distribution by PCO analysis due to effects on IFNs, ILs, defensin and TNFα gene expression. PERMANOVA comparisons showed differences in the MOS and cMOS dietary effects and also on interaction (p-perm. < 0.05).

In spleen PCO analysis discriminated MOS from the other treatments mainly due to its effect on piscidin and IgM gene expression. PERMANOVA analysis only showed a difference for the MOS treatment (p-perm. < 0.05).

Fish fed cMOS were differentiated from other groups in skin and posterior gut, together with MOS in this last tissue, with differences found using PERMANOVA (p-perm. < 0.05) in terms of increasing immune parameters compared with control fish. Fish fed dietary MOS showed an up-regulation in immune parameters in spleen and head

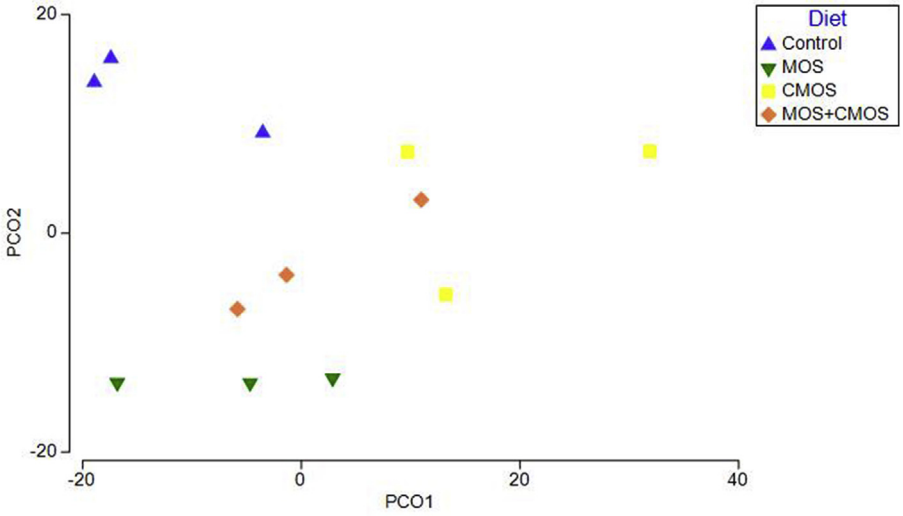

Fig. 4. Head kidney principal coordinates analyse (PCO).

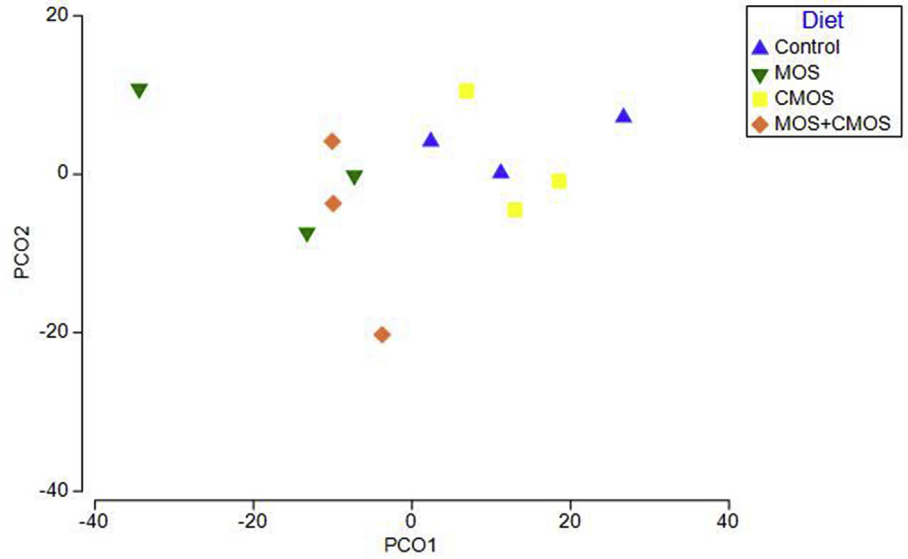

Fig. 5. Spleen principal coordinate analyse (PCO).

kidney (p-perm. < 0.05), with cMOS responsible for increased Ig levels.

4. Discussion

The present study examined the effects of dietary supplementation with MOS and cMOS on greater amberjack growth, immunity and disease resistance. No effects on growth performance were found, in agreement with previous studies on hybrid tilapia (*Oreochromis niloticus x O. aureus*) or channel catfish (*Ictalurus punctatus*) [30,45]. In contrast, in studies conducted with European sea bass (*Dicentrarchus labrax*), MOS and cMOS enhanced fish growth performance and improved FCR [22,23]. Similarly, in fresh water species such as rainbow trout (*Oncorhynchus mykiss*), MOS dietary inclusion increases growth performance and reduces FCR [31]. These effects are likely related with the enhanced nutrient availability due to changes in digestive enzyme activity or in gut morphology, that subsequently increase absorption efficiency [46]. However, such differences in the impact of MOS on growth parameters among species suggest that these effects are highly dependent on the supplementation level, fish species and age, rearing conditions and diet composition [27].

An increase in mucus production has been shown to be a key factor for reducing ectoparasite adhesion in fish species such as Atlantic salmon (*Salmo salar*) [47]. MOS promotes both the enhancement of the innate immune system and mucus production (for reviews see Refs. [16,27,46]), reducing bacterial and parasite adherence to the host. In the present study, cMOS induced an up-regulation of skin MUC-2 compared with fish fed the other dietary treatments, suggesting it promotes mucus production. Dietary MOS showed a similar effect on the gut, in agreement with previous results in European sea bass [23]. Whilst the impact of prebiotics on ectoparasite resistance is poorly studied [18], cMOS showed a clear effect on parasite adhesion in the present work. cMOS not only prevented parasite attachment but also reduced the growth and development of the parasites concomitant with increased immune responses (see below). A mobilization of fish

Table 2
Growth performance, serum and skin mucus immunological parameters (lysozyme activity and bactericidal activity) and parasite data of greater amberjack juveniles (*Seriola dumerili*) after 90 days on the feeding trial.

	Dietary treatments				Two way ANOVA		
	C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*CMOS
Growth performance							
Final Weight (g)	1046.75 ± 129.61	1024 ± 161.17	1090.37 ± 135.49	1036.55 ± 126.88	NS	NS	NS
SGR (%)	1.09 ± 0.04	1.09 ± 0.06	1.13 ± 0.09	1.08 ± 0.07	NS	NS	NS
Feed efficiency	0.654 ± 0.06	0.656 ± 0.01	0.698 ± 0.04	0.704 ± 0.08	NS	NS	NS
Skin mucus							
Lysozyme activity (U/ml)	103.92 ± 17.64	114.25 ± 28.1	124.55 ± 31.64	121.9 ± 11.97	NS	NS	NS
Bactericidal activity (%)	3.72 ± 1.86	5.03 ± 1.21	6.54 ± 0.89	5.22 ± 2.61	NS	NS	NS
Serum							
Lysozyme activity (U/ml)	301.61 ± 42	348.76 ± 52.1	253.88 ± 25.86	287.69 ± 39.04	NS	NS	NS
Bactericidal activity (%)	4.89 ± 1.06	5.91 ± 1.70	8.27 ± 1.05	9.51 ± 1.27	P = 0.04 F = 6.68	P = 0.02 F = 17.56	NS
Parasite challenge							
Parasitisation level (range)	2–3	2	1–2	1–2	NS	P = 0.01 F = 6.17	NS
Parasite total length (mm)	4.44 ± 0.31	3.9 ± 0.43	3.32 ± 0.40	3.56 ± 0.43	NS	P = 0.01 F = 15.47	NS
No parasites/fish surface (cm^2)	0.101 ± 0.01	0.087 ± 0.02	0.015 ± 0.01	0.042 ± 0.01	NS	P = 0.01 F = 52.36	NS

Diet C (control diet, non-supplemented), MOS (MOS supplemented diet), cMOS (cMOS supplemented diet), MOS + cMOS (combined MOS and cMOS supplemented diet). Values expressed in mean ± SD (n = 3 tanks/diet). Two-way ANOVA comparation (P < 0.05). SGR: Specific growth rate; parasitation level: ranged among 1 (lower) to 3 (higher).

Table 3
RT-qPCR gene expression in skin of *Seriola dumerili* juveniles after 90 days on the feeding trial.

Gene	Dietary treatments				Two-way ANOVA		
	Diet C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*cMOS
Hep	3.05 ± 1.16	3.67 ± 1.26	6.11 ± 2.25	2.01 ± 0.34	NS	P = 0.04, F = 2.13	NS
Pis	507.47 ± 184.49	1825.94 ± 992.81	2961.56 ± 969.37	3448.44 ± 657.29	NS	NS	NS
Def	181.69 ± 85.59	422.65 ± 179.34	472.89 ± 215.85	285.93 ± 79.93	NS	NS	NS
iNOS	354.02 ± 132.51	56.34 ± 15.48	514.35 ± 208.57	83.7 ± 31.8	P = 0.01, F = 9.34	NS	NS
TNFα	10.78 ± 2.50	10.88 ± 0.97	18.65 ± 4.72	8.21 ± 2.67	NS	NS	NS
MX prot	571.15 ± 279.59	362 ± 272.29	805.57 ± 460.93	112.64 ± 38.44	NS	NS	NS
IFN γ	31.25 ± 5.57	100.13 ± 46.91	130.18 ± 66.74	41.97 ± 16.39	NS	NS	P = 0.01, F = 3.89
IFN d	9.83 ± 2.01	29.20 ± 10.77	44.04 ± 21.61	12.71 ± 3.11	NS	NS	P = 0.01, F = 2.35
MUC-2	9.96 ± 4.18	8.48 ± 2.85	24.74 ± 6.08	8.94 ± 5.51	NS	P = 0.04, F = 3.27	NS
IL-1β	4.32 ± 0.87	4.48 ± 1.34	9.52 ± 5.36	2.08 ± 0.39	NS	P = 0.02, F = 5.52	NS
IL-8	10.50 ± 2.73	9.85 ± 3.16	21.04 ± 2.50	11.99 ± 4.48	NS	NS	NS
IL-10	1468.17 ± 398.19	1521.55 ± 364.18	2724.73 ± 812.56	231.37 ± 167.44	NS	P = 0.01. F = 9.52	P = 0.01, F = 4.81
IL-17F	25.37 ± 7.1	13.36 ± 2.58	29.66 ± 9.83	10.69 ± 2.78	NS	NS	NS
IL-17D	6.19 ± 1.52	10.65 ± 3.77	65.30 ± 36.34	4.63 ± 1.50	NS	NS	P = 0.01, F = 5.23
IL-22	2.26 ± 0.9	4.72 ± 3.98	3.29 ± 2.57	1.56 ± 0.75	NS	NS	NS
IgM	1008.43 ± 246.86	1074.15 ± 502.02	921.66 ± 545.95	2155.97 ± 835.60	NS	NS	NS
IgT	5.25 ± 0.97	6.32 ± 1.40	12.14 ± 3.51	3.05 ± 0.87	NS	P = 0.04, F = 3.27	P = 0.01, F = 2.23

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS + cMOS (5 g kg^{-1} of MOS and 2 g $kg^{-1}$of cMOS). Data are presented as means ± SD. N = 3 tanks/diet. Two-way ANOVA analyses are presented when P < 0.05. NS= Not significant.

defences to the skin mucus has been described as an effect of prebiotics [48], and could prevent the correct development of parasites as they attempt to overcome the first physical and chemical barriers of the host. In line with this, red drum (*Sciaenops ocellatus*) show a reduced mortality and parasite level after challenge with *Amyloodinium ocellatum*, when receiving a diet supplemented with MOS at 10 g kg^{-1} for 30 days [17]. Similarly, Atlantic salmon fed for 98 days with 4 g MOS kg^{-1} had a significantly reduced parasite load [18].

MOS has shown a more consistent effect on the immune system, improving parameters such as lysozyme activity in fish species including channel catfish, Japanese flounder (P*aralichthys olivaceus*), rainbow trout or European sea bass when supplemented at similar doses [46]. Whilst skin mucus and serum lysozyme activity were unaffected by dietary MOS in the present study, serum bactericidal activity was increased in fish fed the supplemented diets. This indicates that other molecules within the innate immune system that effect antimicrobial responses are affected by these prebiotics [49]. Indeed, the results of the present study show there is upregulation of antimicrobial peptide (AMP) gene expression in all of the tissue studied, and these molecules are an important part of the innate immune system in fish. AMPs are stored in cells so that they are readily available after an infection [50,51]. That MOS mainly increased piscidin whilst cMOS mainly increased hepcidin and defensin is curious. It is known that different cytokines can have unique specificity regarding AMP gene induction [53–55] and may be a factor here. The kinetics of AMP induction can also vary, as seen in rainbow trout after dietary inclusion of peptidoglycans [56].

Adaptive immunity also plays a key role in the host response against ectoparasites [47,52]. IgT is considered a mucosal associated immunoglobulin in fish [57–59]. The increase of IgT transcript levels in skin after feeding cMOS in the present study supports the key role of this immunoglobulin at mucosal surfaces, and could be related with the reduction of the parasite load induced by cMOS. The mode of action of this immunoglobulin is not completely understood, although an up-regulation in IgT expression in skin has been observed as a response to sea lice infection in Atlantic salmon [60], as well as to parasites in the gills and gut [57,61].

Key genes of the immune system have traditionally been selected as

Table 4
RT-qPCR gene expression in gills of *Seriola dumerili* juveniles after 90 days on the feeding trial.

Gene	Dietary treatments				Two-way ANOVA		
	Diet C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*cMOS
Hep	3.83 ± 1.09	8.07 ± 3.58	20.74 ± 6.40	3.05 ± 0.63	NS	P = 0.01, F = 3.22	NS
Pis	123559.34 ± 57885.09	49101.56 ± 20481.84	145655.85 ± 39802.15	220796.87 ± 115335.48	NS	NS	NS
Def	154.83 ± 57.91	269.99 ± 117.38	1538.51 ± 560.83	352.82 ± 181.47	NS	P = 0.01, F = 7.48	P = 0.03, F = 2.59
iNOS	22.57 ± 13.59	287.96 ± 200.29	591.18 ± 261.70	41.52 ± 26.58	NS	NS	NS
TNFα	30.65 ± 12.60	68.12 ± 20.59	91.23 ± 32.13	32.95 ± 4.90	NS	NS	NS
MX prot	24.50 ± 5.55	366.77 ± 244.42	1125.22 ± 336.94	49.10 ± 33.21	NS	P = 0.02, F = 2.27	P = 0.03, F = 4.37
IFN γ	57.11 ± 9.1	118.43 ± 54.38	220.41 ± 52.02	69.04 ± 18.96	NS	P = 0.03, F = 3.86	NS
IFN d	18.63 ± 8.95	43.70 ± 19.93	102.95 ± 21.84	20.83 ± 3.73	NS	NS	NS
MUC-2	1138.31 ± 250.05	1149.28 ± 633.21	522.40 ± 212.30	592.53 ± 226.38	NS	NS	NS
IL-1β	12.90 ± 6.21	16.20 ± 6.23	15.92 ± 4.82	5.92 ± 1.79	NS	NS	NS
IL-8	17 ± 2.92	27.21 ± 5.20	74.62 ± 28.04	31.26 ± 12.15	NS	NS	NS
IL-10	2347.06 ± 824.85	2373.74 ± 203.84	5078.92 ± 2726	4073.95 ± 2180.10	NS	NS	NS
IL-17F	7.47 ± 3.96	11.69 ± 4.62	21.20 ± 8.31	4.78 ± 1.64	NS	NS	NS
IL-17D	18.19 ± 4.07	69.54 ± 28.57	63.85 ± 15.36	16.28 ± 5.56	NS	NS	NS
IL-22	15.51 ± 5.79	19.75 ± 6.01	21.95 ± 4.96	10.18 ± 2.65	NS	NS	NS
IgM	65941.75 ± 43329.10	16665.56 ± 5287.38	1575.48 ± 1071.64	144185.46 ± 56001.67	NS	NS	NS
IgT	8.72 ± 2.17	14.04 ± 4.26	20.80 ± 5.31	7.98 ± 2.12	NS	NS	P = 0.01, F = 9.88

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS + cMOS (5 g kg^{-1} of MOS and 2 g $kg^{-1}$of cMOS). Data are presented as means ± SD. N = 3tanks/diet. Two-way ANOVA analyses are presented when P < 0.05. NS= Not significant.

Table 5
RT-qPCR gene expression in posterior gut of *Seriola dumerili* juveniles after 90 days on the feeding trial.

Gene	Dietary treatments				Two-way ANOVA		
	Diet C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*cMOS
Hep	3.77 ± 1.05	9.43 ± 0.86	103.78 ± 44.61	12.18 ± 2.88	NS	P = 0.02, F = 6.23	NS
Pis	18159.99 ± 6184.25	70552.71 ± 20631.15	16733.78 ± 2690.02	100257.10 ± 47228.58	P = 0.03, F = 7.35	NS	NS
Def	103.35 ± 60.43	526.85 ± 272.52	5909.07 ± 2592.71	1721.73 ± 483.36	NS	P = 0.03, F = 2.98	NS
iNOS	352.72 ± 56.94	699.99 ± 361.29	615.43 ± 278.11	1183.26 ± 418.29	NS	NS	NS
TNFα	32.27 ± 12.53	64.93 ± 19.44	108.79 ± 43.23	71.82 ± 17.04	NS	NS	NS
MX prot	410.19 ± 47.28	831.96 ± 175.50	955.28 ± 867.09	1962.94 ± 909.99	NS	NS	NS
IFN γ	34.21 ± 16.26	427.99 ± 193.55	1241.66 ± 542.39	284.67 ± 41.87	NS	P = 0.04, F = 3.32	P = 0.02, F = 1.09
IFN d	7.49 ± 3.48	57.75 ± 19.91	81.64 ± 30.78	38.93 ± 9.07	NS	NS	NS
MUC-2	2800.02 ± 511.50	6819 ± 1350.56	3375.84 ± 993.78	2701.72 ± 810.98	P = 0.04, F = 17.72	NS	NS
IL-1β	6.73 ± 1.08	66.97 ± 28.67	14.06 ± 8.09	18.65 ± 6.65	P = 0.02, F = 3.52	NS	NS
IL-8	20.75 ± 6.67	53.22 ± 12.95	191.49 ± 99.69	75.67 ± 14.98	NS	NS	NS
IL-10	1578.98 ± 194.29	9495.37 ± 4244.02	107128.07 ± 45885.12	17241.35 ± 6641.88	P = 0.03, F = 9.48	P = 0.02, F = 2.79	NS
IL-17F	8.53 ± 5.53	21.20 ± 8.05	11.73 ± 4.99	7.20 ± 1.49	NS	NS	NS
IL-17D	31.43 ± 10.75	71.53 ± 15.36	74.54 ± 28.76	64.69 ± 23.07	NS	NS	P = 0.01, F = 5.23
IL-22	19.61 ± 7.73	22.75 ± 9.25	13.19 ± 3.38	13.42 ± 3.26	NS	NS	NS
IgM	73788.21 ± 41586.91	$29.2 \times 10^5 \pm 14.2 \times 10^5$	$22.3 \times 10^5 \pm 80.3 \times 10^4$	573173.42 ± 319410.84	P = 0.01, F = 8.24	P = 0.02, F = 6.14	P = 0.02, F = 2.41
IgT	18.47 ± 13.64	70.28 ± 16.82	56.15 ± 19.18	34.52 ± 13.48	P = 0.01, F = 2.78	P = 0.02, F = 3.11	NS

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS + cMOS (5 g kg^{-1} of MOS and 2 g $kg^{-1}$of cMOS). Data are presented as means ± SD. N = 3tanks/diet. Two-way ANOVA analyses are presented when $P < 0.05$. NS= Not significant.

Table 6
RT-qPCR gene expression in head kidney of *Seriola dumerili* juveniles after 90 days on the feeding trial.

Gene	Dietary treatments				Two-way ANOVA		
	Diet C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*cMOS
Hep	8.38 ± 3.65	11.90 ± 3.86	23.23 ± 5.85	31.40 ± 6.99	NS	P = 0.01, F = 10.96	NS
Pis	113233.81 ± 40305.31	107678.83 ± 50906.83	224992.61 ± 92470.72	102237.60 ± 19950.65	NS	NS	NS
Def	187.37 ± 71.37	3198.20 ± 1666.94	2407.93 ± 1279.35	1518.66 ± 793.53	NS	NS	P = 0.03, F = 2.19
iNOS	1809.69 ± 689.22	12437.49 ± 2634.66	1164.43 ± 150.86	4101.22 ± 791.51	P = 0.02, F = 8.32	NS	NS
TNFα	57.46 ± 30.26	84.34 ± 22.70	115.74 ± 60	45.05 ± 8.84	NS	NS	NS
MX prot	727.39 ± 183.65	10088.19 ± 1439.72	1252.17 ± 62.92	4399.24 ± 1623.50	P = 0.01, F = 8.48	NS	P = 0.03, F = 3.65
IFN γ	216.77 ± 108.53	355.11 ± 101.34	294.81 ± 213.67	352.18 ± 102.91	NS	NS	NS
IFN d	29.62 ± 6.46	71.28 ± 10.82	62.30 ± 15.11	55.16 ± 18.84	P = 0.02, F = 4.23	P = 0.02, F = 7.15	NS
IL-1β	52.81 ± 29.24	262.49 ± 117.76	121.57 ± 63.93	62.41 ± 9.84	NS	NS	NS
IL-8	8.05 ± 1.99	32.80 ± 9.16	22.41 ± 9.72	105.56 ± 48.61	NS	NS	NS
IL-10	852.98 ± 203.37	5077.33 ± 2249.74	3090.39 ± 1025.95	577.80 ± 117.01	P = 0.02, F = 5.28	P = 0.02, F = 7.68	P = 0.01, F = 9.51
IL-17F	7.22 ± 2.41	21.74 ± 2.96	25.56 ± 8.95	14.47 ± 3.03	NS	NS	NS
IL-17D	36 ± 18.68	139.82 ± 32.31	15.47 ± 2.31	58.98 ± 16.09	P = 0.04, F = 1.67	NS	NS
IL-22	4.79 ± 0.79	43.93 ± 9.44	36.33 ± 10.45	24.68 ± 8.25	P = 0.03, F = 4.89	P = 0.02, F = 9.93	NS
IgM	18166.59 ± 386.67	44482.19 ± 18652.10	298249.40 ± 112084.40	99464.83 ± 24438.90	NS	NS	NS
IgT	48.93 ± 27.35	60.15 ± 32.54	80.64 ± 38.20	62.13 ± 15.32	NS	NS	NS

Diets: C (control diet), MOS (5 g kg-1), cMOS (2 g kg-1), MOS + cMOS (5 g kg-1 of MOS and 2 g kg-1of cMOS). Data are presented as means ± SD. N = 3 tanks/diet. Two-way ANOVA analyses are presented when $P < 0.05$. NS= Not significant.

Table 7
RT-qPCR gene expression in spleen of *Seriola dumerili* juveniles after 90 days on the feeding trial.

Gene	Dietary treatments				Two-way ANOVA		
	Diet C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*cMOS
Hep	61.72 ± 19.67	64.87 ± 13.07	33.35 ± 6.53	147.64 ± 48.89	NS	P = 0.01, F = 7.39	NS
Pis	165899.47 ± 86928.35	326203.01 ± 92385.94	68108.33 ± 27208.51	313455 ± 159603.02	P = 0.01, F = 9.95	NS	NS
Def	276.29 ± 82.03	1416.65 ± 289.94	450.51 ± 207.30	2815.82 ± 1277.64	P = 0.01, F = 8.61	NS	P = 0.01, F = 7.35
iNOS	1830.64 ± 504.84	2494.56 ± 945.27	1089.47 ± 407.30	778.71 ± 279.99	NS	NS	NS
TNFα	411.98 ± 82.99	925.46 ± 257.80	203.52 ± 63.54	429.05 ± 146.70	NS	NS	NS
MX prot	1021.71 ± 307.35	2182.75 ± 821.69	860.82 ± 350.61	439.08 ± 155.54	NS	NS	NS
IFN γ	405.34 ± 107.19	870 ± 130.56	300.82 ± 62.79	434.63 ± 140.21	P = 0.02, F = 3.29	NS	NS
IFN d	30.54 ± 3.73	75.70 ± 21.16	27.95 ± 6.56	84.80 ± 27.22	NS	NS	NS
IL-1β	18.91 ± 5.84	43.14 ± 7.19	12 ± 1.45	19.54 ± 2.35	P = 0.01, F = 14.36	NS	NS
IL-8	23.68 ± 5.68	61.37 ± 23.18	19.51 ± 7.61	44.89 ± 16.51	NS	NS	NS
IL-10	2268.78 ± 944.39	5478.43 ± 2040.92	2305.29 ± 1080.20	6791.58 ± 2267.62	NS	NS	NS
IL-17F	9.75 ± 2.30	15.45 ± 4.67	3.76 ± 0.88	11.38 ± 6.08	NS	NS	NS
IL-17D	14.42 ± 4.08	40.39 ± 12.23	14.12 ± 3.22	43.94 ± 22.28	P = 0.04, F = 1.36	NS	NS
IL-22	6.15 ± 1.53	26.11 ± 10.74	6.46 ± 1.91	19.35 ± 7.50	NS	NS	NS
IgM	152198.50 ± 42526.77	28665.69 ± 6833.63	104560.74 ± 35002.44	51173.12 ± 15474.28	NS	NS	NS
IgT	26.17 ± 10.84	63.59 ± 14.76	16.13 ± 3.46	38.16 ± 10.21	NS	NS	NS

Diets: C (control diet), MOS (5 g kg-1), cMOS (2 g kg-1), MOS + cMOS (5 g kg-1 of MOS and 2 g kg-1of cMOS). Data are presented as means ± SD. N = 3tanks/diet. Two-way ANOVA analyses are presented when P < 0.05. NS= Not significant.

markers of immune system activation by prebiotics, including TNFα, IL-1β, IL-8, IL-10, iNOS, IFNs, IgM, TLRs and MHC [62]. As discussed above, there is a direct linkage between MOS administration and innate immune system modulation [15,26], with the skin a key point of entry of potential pathogens in fish [63]. In humans an increase of TNFα expression with no IL-10 response is associated with an increase of mucosal IL-17 [46,64,65], similar to the results obtained in the present study. A balanced pro and anti-inflammatory response in the skin is linked to an increased inflammatory response at the moment of parasite attachment, and gives lower parasite levels in Atlantic salmon infected with sea lice [66]. Indeed, our PCO analysis showed a higher effect of cMOS in skin, relative to MOS, mainly due to upregulation of AMPs (hepcidin, defensin, piscidin), MUC-2, TNFα, Mx Protein, IL-8, IL-10, IL-17 and IFNs as revealed by PERMANOVA.

In studies of prebiotics, especially MOS, the gut is the main tissue where the effects of the prebiotic take place. Although cMOS induced higher hepcidin, defensin, IFNγ, IL-10, IgM and IgT, the stimulatory effect of MOS was equal to or even higher for IL-10, IgM and IgT and also impacted piscidin, MUC-2 and IL-1β unlike cMOS. This modulation of the expression of these selected genes reveals an increased cytokine response and enhanced mucus production [26,46,67]. Hence both MOS and cMOS could potentially have positive effects on resistance to gut parasites and this should be explored in future studies.

The impact of dietary MOS was also assessed in head kidney and spleen, two important systemic immune tissues in fish that play a key role in the maturation of B-cells and phagocytic cells [68]. The importance of the head kidney and spleen response during parasite infections has been described in many studies where systemic responses help coordinate the fight against secondary infections and participate in the wound healing process [47]. Furthermore, upregulation of proinflammatory cytokines such as IL-1β, IL-17 and TNFα in head kidney and spleen has been associated with reductions in sea lice load in pink salmon [69,70], akin to the results found in spleen in the present study where IL-1β, IFNγ and IL-17D were increased. cMOS is a more purified product than MOS, and some components of the outer cell wall of *Saccharomyces cerevisiae* strains (probably β-glucans) could have been removed during the production process, as suggested by Torrecillas et al. [71]. Since β-glucans are potent PAMPs able to trigger innate immunity [72], this would explain the higher stimulation of innate immune parameters with MOS but not cMOS. On the other hand, B-cell stimulation will lead to increased adaptive immunity, with Ig transcripts notably increased by dietary cMOS in the present study. Indeed, the dispersion patterns seen in the head kidney PCO analysis in the cMOS dietary group were explained by the increased number of Ig transcripts, which separated cMOS from the other dietary groups. Tadiso et al. [60] found that immunological changes in spleen affected the skin response, strengthening the relationship between systemic and mucosal immune responses.

The combination of MOS and cMOS showed similar results to the control diet group for most of the genes analysed. PCO and PERMANOVA analyses typically showed an interaction between MOS and cMOS, probably related to a loss of effect by overstimulation. It has been reported previously that the combination of two different prebiotics, like MOS and peptidoglycans, can have positive synergic effects in the immune system when suitable doses are used [73]. In the case of cMOS, it is a second generation MOS, therefore the pathways of action of these two prebiotics should be similar. Thus, the combination of both prebiotics likely induces effects similar to using a high dietary inclusion of these prebiotics alone, and may result in receptor overload or immune fatigue related to a high energy cost of continued immunostimulation [74–76].

In conclusion, the utilization of dietary cMOS at 2 g kg^{-1} increased protection against *N. girellae* after 90 days of feeding, by reducing the parasite level and parasite total length. This protection was associated with up-regulation of several proinflammatory cytokines, AMPs, MUC-2 and IgT genes in skin and enhanced serum bactericidal activity. In contrast, dietary MOS at 5 g kg^{-1} stimulated AMPs, IFNs and proinflammatory cytokines in head kidney and spleen, but had little effect in skin and these fish had a higher parasite level compared with fish fed the cMOS diet. The posterior gut also showed immune stimulation with dietary MOS and cMOS, in terms of effects on expression of AMPs, proinflammatory cytokines, IgM and IgT. However, the combination of MOS and cMOS appears to have delivered an over stimulation of the immune system, resulting in a lack of effect.

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Appendix A. Supplementary data

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