New species for EU aquaculture

Deliverable Report

Deliverable No:	D25.5	**Delivery Month:**	57
Deliverable Title	Impact of Oral Administration of Greater Amberjack with mucus stimulation products on immune resistance to parasitic infections and development of molecular markers for its evaluation.		
WP No:	25	**WP Lead beneficiary:**	P5.UNIABDN
WP Title:	Fish Health – Greater Amberjack		
Task No:	25.2	**Task Lead beneficiary:**	P2. FCPCT
Task Title:	Promoting resistance to parasitic incidence on Greater Amberjack.		
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Status:	Delivered	**Expected month:**	58
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Objective: The objective of this Deliverable is to determine the impact of oral administration of Greater Amberjack with mucus stimulation products on immune resistance to parasitic infections and development of molecular markers for its evaluation, showing evidence whether the inclusion of Mucus Stimulation Ingredients modulates the gene expression of selected immune markers.

Description: Description of the work done and results

Introduction

Neobenedenia girellae is a monogenean ectoparasite that has become one of the main causes of greater amberjack parasitic infections. The parasite attachment to fish skin produces important alterations (Hirayama *et al*., 2009, Hirazawa *et al*., 2016) such as wounds and ulcers, promoting the appearance of secondary infections (Hagiwara *et al*., 2011), thereby increasing mortality. The use of dietary prebiotics and functional feeds has been shown to affect ectoparasite prevalence (Buentello *et al*., 2010; Dimitroglou *et al*., 2011).

Prebiotics, including mannan oligosaccharides (MOS) by-products, are commonly used in the animal production industry due to their effects on the immune system leading to pathogen protection (Torrecillas *et al*., 2014; Guerreiro *et al*., 2017). 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 Torrecillas *et al*., 2011; 2013).

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 and feed efficiency.

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.

Experimental fish and conditions

Two hundred and sixteen fish (mean weight 331.4 ± 30 g) were distributed in twelve cylindroconical 1,000 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.

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=3x3).

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).

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)

Gene expression analyses

Samples for gene expression analyses were collected in RNAlater and stored for 48 h at 6ºC. 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 (NanoDrop2000, 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 (AMPD1–1KT, 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) running according to manufacturer's instructions. The samples were then diluted 1:20 in miliQ water and stored at -20ºC.

Specific primers were designed according to target genes found in genbank from species phylogenetically related with *S. dumerili* (**Table 1**), following the methodology described in deliverable 25.1 "*Marker genes of mucosal immunity in greater amberjack cloned and ways to increase their expression level determined*". The primers were used to amplify products using amberjack cDNA obtained from a pool of gill, mid-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 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.

Table 1. Primers used for gene expression analysis by real-time PCR 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)*	Forward Sequence	Reverse Sequence
Hep	Hepcidin	61	GATGATGCCGAATCCCGTCAGG	CAGAAACCGCAGCCCTTGTTGGC
Pis	Piscidin	58	ATC GTC CTG TTT CTT GTG TTG TCA C	CGC TGT GGA TCA TTT TTC CAA TGT GAA A
Def	Defensin	60	ATGAGGCTGCATCCTTTCCATG	AGAAAATGAGATACGCAACACAAGAAGCC
iNOS	Inducible Nitric oxyde synthase	60	TGTTTGGCCTTGGCTCCAGGG	GCCCAAGTTCTGAATGACTCCTCCTG
TNFα	Tumor necrosis factor α	62	GAAAACGCTTCATGCCTCTC	GTTGGTTTCCGTCCACAGTT
MX Prot	Interferon-inducible Mx protein	61	GGCTACATGATTGTGAAGTGCAGGG	CTTCCAGTCGAGGCAGAGATTTCTCAATGT
IFN γ	Interferon γ	59	AACTTGGTTTCACGGTGCAG	TCACAACACCGAGAAAGTCCT
IFN δ	Interferon δ	59	GTCAGGGTGCAGCTGAGTTA	ACAGAAACGGCAGCTCAAAC
MUC-2	Mucin-2	62	ATT GAG TTT GGC AAC AAA CAG AAA GCC C	TAC AGC ACA GAA CTG AGG TGT CCT C
IL-1β	Interleukin 1β	62	TGATGGAGAACATGGTGGAA	GTCGACATGGTCAGATGCAC
IL-8	Interleukin 8	58	GAAGCCTGGGAGTAGAGCTG	GGGGTCTAGGCAGACCTCTT
IL-10	Interleukin 10	58	CTC AAG AGT GAT GTC ACC AAA TGT AGA AAC T	AGC AAA TCC AGC TCG CCC ATT
IL-17F	Interleukin 17F	62	GGTGGCCCCAGAGGATCTCC	GGAGGACCAAAACCTGGTAGTAGATGG
IL-17D	Interleukin 17D	62	CGGTCTACGCTCCCTCCGTG	GCGGCACACAGGTGCATCCC
IL-22	Interleukin 22	61	GCC AAC ATC CTC GAC TTC TAC CTG AAC	TGG TCG TGG TAG TGA GTC ACA TTG C
IgM	Immunoglobulin M	58	CTCTTTGATAGGAATACCGGAGGAGAG	CAACTAGCCAAGACACGAAAACCC
IgT	Immunoglobulin T	59	TGGACCAGTCGCCATCTGAG	GGGAAACGGCTTTGAAAGGA
β-Actin	β-Actin	61	TCT GGT GGG GCA ATG ATC TTG ATC TT	CCT TCC TTC CTC GGT ATG GAG TCC
EF1 α	Elongation factor 1α	60	TGC CAT ACT GCT CAC ATC GCC TG	ATT ACA GCG AAA CGA CCA AGA GGA G

*Ann. temp: anneling temperature

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.* (2014) 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 (1990). Bactericidal activity was measured with a modification of the method described by Sunyer and Tort (1995), using *Photobacterium damselae*.

Parasite infection

The parasite source was a tank (10,000 L) of previously *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 recollect them. After 24 h eggs were introduced into a 1,000 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 0.03m^3 cages (8 infected fish per cage and one cage per experimental tank) for 15 days, to enable a cohabitation challenge. After 15 days of cohabitation, the experimental fish were sampled, and an evaluation of infection level for each fish was carried out by a judge panel (composed of 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*., (2008). Total length of 50 adult parasites per tank was recorded using a profile projector (Mitutoyo, PJ-A3000).

Statistical analyses

The statistical analyses followed the methods outlined by Sokal and Rolf (1995), 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.

Results

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**).

Table 2. Growth performance, serum and skin mucus immunological parameters (lysozyme activity and bactericidal activity) and parasitation data of greater amberjack juveniles after 90 days of 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 (µg/ml)	15.45 ±1.71	14.45 ±4.74	13.45 ±0.94	13.7 ±5.98	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 (µg/ml)	37.81 ±4.08	42.4 ±2.5	34 ±3.50	36.45 ±3.79	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
Parasitation challenge							
Parasitation 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).

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 it was 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**).

Parasite challenge

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

Gene expression

At the end of the feeding trial (90 days), two way-ANOVA analyses showed that dietary cMOS up-regulated skin hepcidin (F=2.13, P=0.04), MUC-2, IL-1ß, 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**).

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

	Dietary treatments				Two-way ANOVA		
Gene	**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 δ	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 presented as mean ± SD. N=3 tanks/diet. Two-way ANOVA analyses (P<0.05). NS= No significant.

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**).

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

	Dietary treatments				Two-way ANOVA		
Gene	**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 δ	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 presented as mean ± SD. N=3tanks/diet. Two-way ANOVA analyses (P<0.05). NS= No significant.

Regarding fish posterior gut, two way-ANOVA analysis showed that 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 reduced their effects on 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**).

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

	Dietary treatments				Two-way ANOVA		
Gene	**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 δ	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	$29.2x10^{3}$ ±$14.2x10^{3}$	$22.3x10^{3}$ ±$80.3x10^{4}$	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 presented as mean ± SD. N=3tanks/diet. Two-way ANOVA analyses (P<0.05). NS= No significant.

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 decreased IL-10 transcript levels relative to single supplementation. In addition, the effects on IFNd and IL-22 were lost (**Table 6**).

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

	Dietary treatments				Two-way ANOVA		
Gene	**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 δ	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 presented as mean ± SD. N=3 tanks/diet. Two-way ANOVA analyses (P<0.05). NS= No significant.

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).

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

	Dietary treatments				Two-way ANOVA		
Gene	**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 δ	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 presented as mean ± SD. N=3tanks/diet. Two-way ANOVA analyses (P<0.05). NS= No significant.

Multivariant analyses comparing gene expression data presented different responses for each tissue. 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 (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). (**Fig.1**).

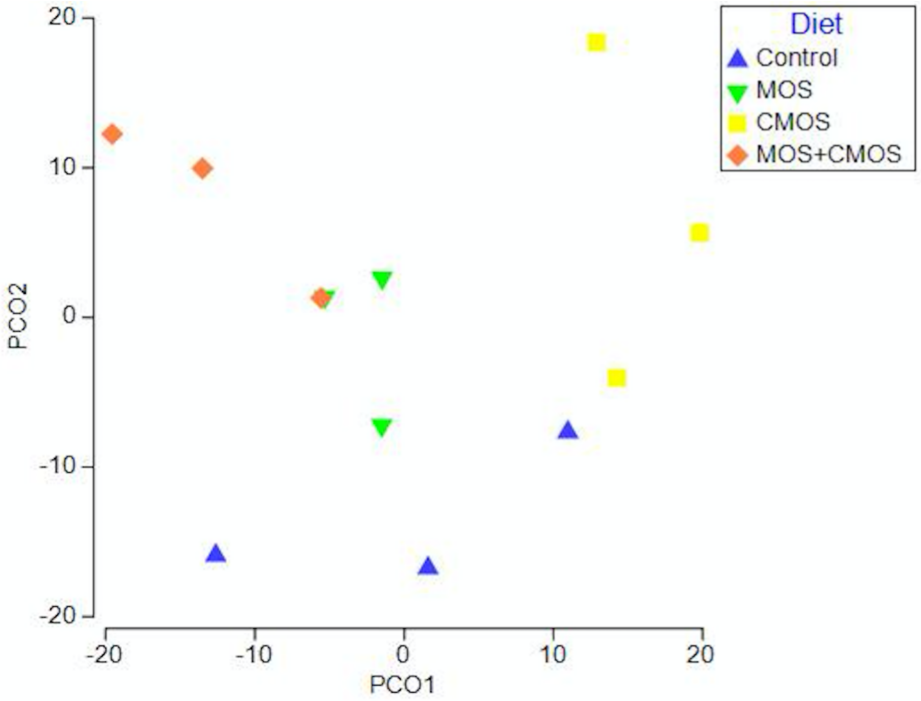

Figure 1. Principal coordinates analyse of skin (PCO).

PCO analysis in gills 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) (**Fig. 2**)

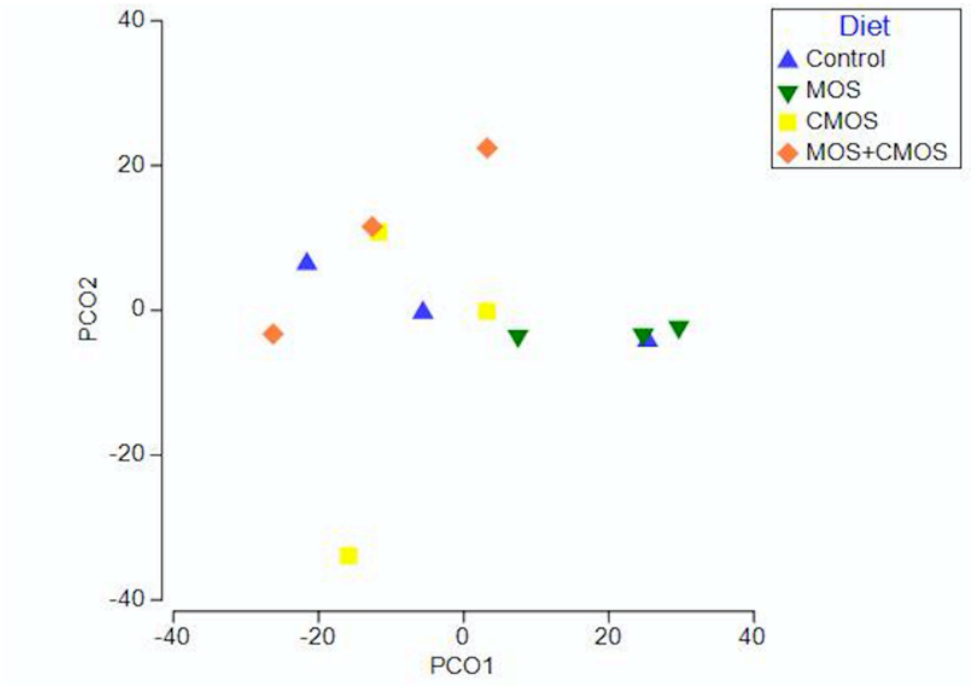

Figure 2. Principal coordinates analyse of gills (PCO)

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) (**Fig. 3**).

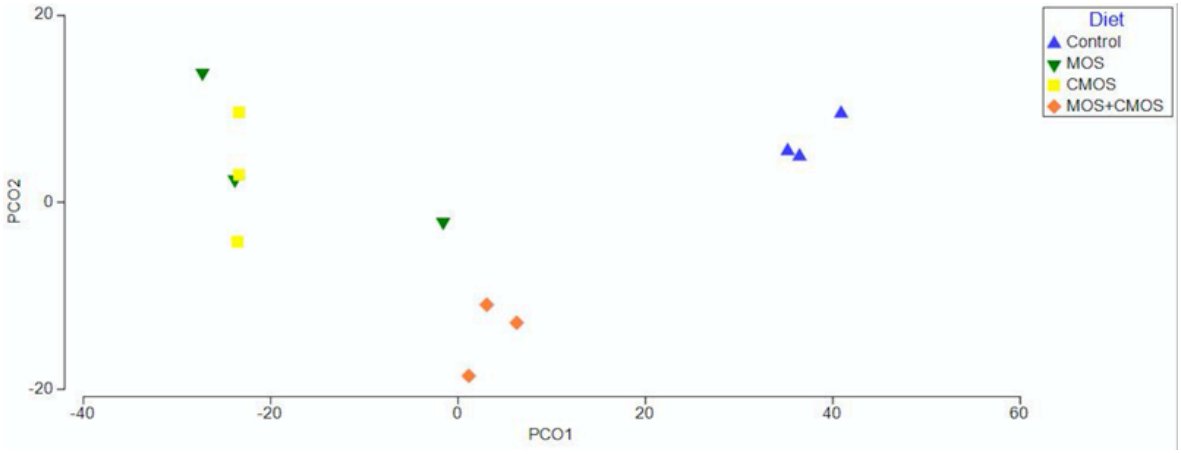

Figure 3. Principal coordinates analyse of posterior gut (PCO)

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) (**Fig. 4**).

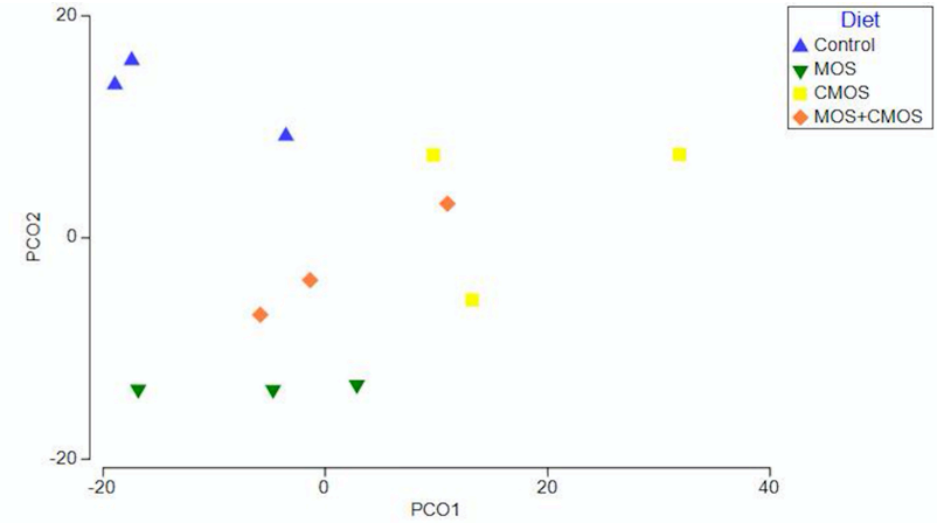

Figure 4. Principal coordinates analyse of anterior kidney (PCO)

Lastly, 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) (**Fig. 5**).

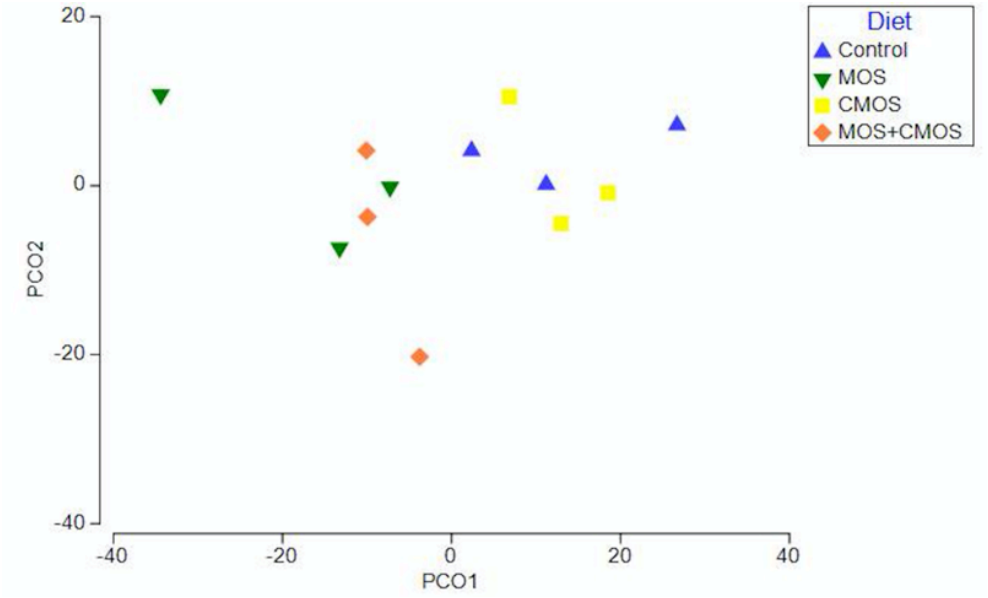

Figure 5. Principal coordinates analyse of spleen (PCO)

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

Discussion & Conclusions

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*) (Peterson *et al*., 2010; Genc *et al*., 2007).

An increase in mucus production has been shown to be a key factor for reducing ectoparasite adhesion in fish species such as Atlantic salmon (Fast, 2014). MOS promotes both the enhancement of innate immunity and mucus production (for review see Torrecillas *et al*., 2014), 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 rest of the dietary treatments, suggesting it promotes mucus production. Dietary MOS showed a similar effect on gut (Torrecillas *et al*., 2011). Whilst the impact of prebiotics on ectoparasite resistance is poorly studied (Dimitroglou *et al*., 2011), 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 defences to the skin mucus has been described as an effect of prebiotics (Sheikhzadeh *et al*., 2012), and could affect the correct development of parasites as they attempt to overcome the first physical and chemical barriers of the host.

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 (*Paralichthys olivaceus*), rainbow trout

or European sea bass when supplemented at similar doses (Torrecillas *et al*., 2014). 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 (Ellis, 1990). 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 (Terova et al., 2009). 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 (Costa et al., 2011; Hong et al., 2013; Wang et al., 2018) and may be a factor here.

Adaptive immunity also plays a key role in the host response against ectoparasites in fish (Fast 2014). IgT is considered a mucosal associated immunoglobulin in fish (Zhang *et al*., 2010). 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.

As discussed above, there is a direct linkage between MOS administration and innate immune system modulation, with the skin a key point as one of the main portals of entry of potential pathogens in fish (Torrecillas *et al*., 2013). In humans an increase of TNFα expression with no IL-10 response is associated with an increase of mucosal IL-17 (Torrecillas *et al*., 2014), 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 the parasite attachment, and gives lower parasite levels in Atlantic salmon infected with sea lice (Poley *et al*., 2013). Indeed, our PCO analysis showed a higher effect of cMOS in skin, relative to MOS, mainly due to AMPs (hepcidin, defensin, piscidin), MUC-2, iNOS, 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 studied tissue where the many of the effects of the prebiotic take place. Although cMOS induced higher stimulation of hepcidin, defensin, IFNγ, IL-10, IgM and IgT, the stimulatory effect of MOS response was equal to or even higher for IL-10, IgM and IgT and also impacted piscidin, MUC-2 and IL-1β unlike cMOS, in agreement with previous studies with other marine species (Torrecillas *et al*., 2011). This modulation of the expression of these selected genes reveals an increased cytokine response and enhanced mucus production (Torrecillas et al., 2014). 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 (Secombes et al., 2001). 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 processes (Fast 2014). 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 (*Oncorhynchus gorbuscha*) (Jones et al., 2007), akin to the results found in spleen in the present study where IL-1β, IFNγ and IL-17D were increased.

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. However, 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 (Yousefian *et al*., 2009).

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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