Animal Microbiome

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Longitudinal study on the effects of a synbiotic supplement to Atlantic salmon diets on performance, gut microbiota and immune responses during antibiotic treatment and subsequent recovery

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Abstract

Background Antibiotic use has undesirable side-effects on the host, including perturbations of gut microbiota, immunity, and health. Mammalian studies have demonstrated that concomitant/post antibiotic use of pro-, pre-, and synbiotics could re-establish gut microbiota and prevent detrimental host effects. However, studies evaluating similar effects in fish are scanty. This study evaluated the effects of dietary supplementation with a synbiotic mixture on the post-smolt Atlantic salmon gut microbiota, growth performance, and health during antibiotic treatment and subsequent recovery. Fish in five tanks each were fed either a commercial control diet or a synbiotic diet containing *Pediococcus acidilactici* and fructo-oligosaccharides, for 6 weeks (S1). Then, fish in three tanks per treatment were fed with medicated diets, containing 3500 ppm florfenicol coated onto the control or synbiotic diets, for 2 weeks (S2) and refed with the respective nonmedicated diets for another 3 (S3) and 5 (S4) weeks of recovery period. The fish not subjected to medication were fed the control or synbiotic diets throughout the experimental period. Samples were collected at S1-S4 from both the nonmedicated and medicated fish.

Results Florfenicol decreased the feed intake in control group. It reduced the growth rate in both control and synbiotic groups with lesser reduction in synbiotic group. Florfenicol did not significantly affect observed taxa and Shannon indexes. Bacterial composition before and after medication clustered distinctly in control and clustered together in synbiotic groups. *Lactobacillus* dominated in control while *Lactobacillus* and *Pediococcus* dominated in synbiotic group during medication and recovery. Florfenicol did not significantly influence the immune or stress response marker gene expressions, though the expression patterns differed between diet groups. Florfenicol did not cause inflammation in the distal intestine or change hepatosomatic index.

Conclusions This study highlighted the negative impact of a two-week florfenicol treatment on feed intake and growth performance in Atlantic salmon, with moderate effects on gut microbiota and gene expression. Concomitant use of a synbiotic diet helped to maintain the gut microbial composition and influenced the performance positively and immune gene expressions differently during medication. This study indicates the importance of nutritional

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interventions through synbiotic supplementation as a possible strategy for managing Atlantic salmon during antibiotic treatment.

Keywords Antibiotics, Florfenicol, Synbiotics, Gut microbiota, Atlantic salmon, *Pediococcus acidilactici*, FOS

Introduction

Salmon aquaculture industry has rapidly expanded over the last decades with Norway and Chile being the main contributors [18]. The rapid expansion of the industry has triggered concerns regarding environmental impacts and sustainability [41]. Among the sustainability issues are factors affecting fish welfare and health including sea lice and disease outbreaks leading to chemical and antibiotic usage [7, 26, 41]. With effective disease control measures including vaccination and improved fish welfare status, the main producer of Atlantic salmon, *Salmo salar*, Norway was able to reduce antibiotic usage to a minimum by 2019 [50]. However, some salmon farming areas still depend on antibiotics to treat infectious diseases. One important disease is the salmonid rickettsial septicemia for which efforts to develop an efficient vaccine with long term protection have so far not succeeded [19].

Antibiotic usage has raised concerns regarding the development of antibiotic resistance within the host and environmental microbiota, as well as posing other health challenges to the host [9, 26, 45]. Antibiotics can trigger perturbations in the host gut microbiome resulting in dysbiosis and consequential changes in host immune homeostasis, leading to detrimental health effects [30, 48, 72]. Antibiotics cause many changes in host-immunity including antimicrobial defenses, immune cell activities, mucosal immunity and associate with several intra and extra intestinal diseases [20, 42, 72]. A healthy gut microbiome is important for a wide spectrum of host physiological processes including energy metabolism, growth, health, and performance. Based on the few studies carried out in fish it seems that perturbations in gut microbiota triggered by antibiotics could impair gut health, as shown in zebrafish [76], may increase susceptibility to secondary infection and/or reduce growth, as observed in western mosquitofish, *Gambusia affinis*, zebrafish and southern catfish, *Silurus meridionalis* [12, 75, 76], and increase mortality, as documented in zebrafish larvae [56]. Perturbations in gut microbiota has been reported for Atlantic salmon treated with the widely used antibiotics, florfenicol and oxytetracycline [25, 47]. However, in depth understanding of antibiotic-induced dysbiosis and related health and physiological effects in Atlantic salmon is lacking, in particular since we still lack a comprehensive understanding of the taxonomical and functional elements that define a healthy microbiome in salmon [4].

In recent years, the salmon aquaculture industry has introduced a holistic approach which takes into consideration managemental, nutritional, as well as environmental conditions to promote the robustness of the farmed fish. Robust fish can be a key for disease management and continuing the work towards further reducing the antibiotic usage. In this regard, development of feed ingredients that maintain robustness of fish during the medication regime is important. Synbiotics are mixtures of probiotic and prebiotic agents, made to have beneficial affects to the host by increasing the survival and activity of probiotics and indigenous health promoting bacteria in the gut [22]. Several previous studies have described beneficial effects of synbiotics in fish including salmonids [1, 29, 59, 60]. We have recently reported that dietary application of functional ingredients with prebiotic fructo-oligosaccharides (FOS) in combination with a probiotic, *P. acidilactici* (FOS-BC diet) had a moderate effect and replacing FOS with galacto-oligosaccharides in FOS-BC diet had a marked effect on distal intestine gut microbiota, transcriptome and metabolite profiles of post-smolt Atlantic salmon [15]. Mammalian studies have shown that concomitant or post antibiotic use of probiotics, prebiotics and synbiotics could re-establish gut microbiota and prevent antibiotic resistance as well as antibiotic associated gastrointestinal disorders and intestinal disruptions [27, 36, 40, 44, 52, 53, 64]. However, studies on this topic conducted in fish are scarce. A study reported that application of probiotics after antibiotic treatment increased the survival of black molly, *Poecilia sphenops* after challenge with *Vibrio anguillarum* [62].

This study evaluated the effects of nutritional management through a synbiotic strategy on growth performance, gut health, gut microbiota, and immune and stress gene expression of post-smolt Atlantic salmon during antibiotic treatment and subsequent recovery. In the present work, florfenicol, a broad-spectrum bacteriostatic antibiotic widely used in veterinary medicine, was employed. The study analyzed longitudinal microbiota changes by using the digesta samples from pit-tagged fish. A summary of the experimental design can be found in Fig. 1.

Results

This study used digesta collected by faecal stripping to perform a longitudinal analysis of the gut microbiota in a consistent group of fish over the course of the feeding

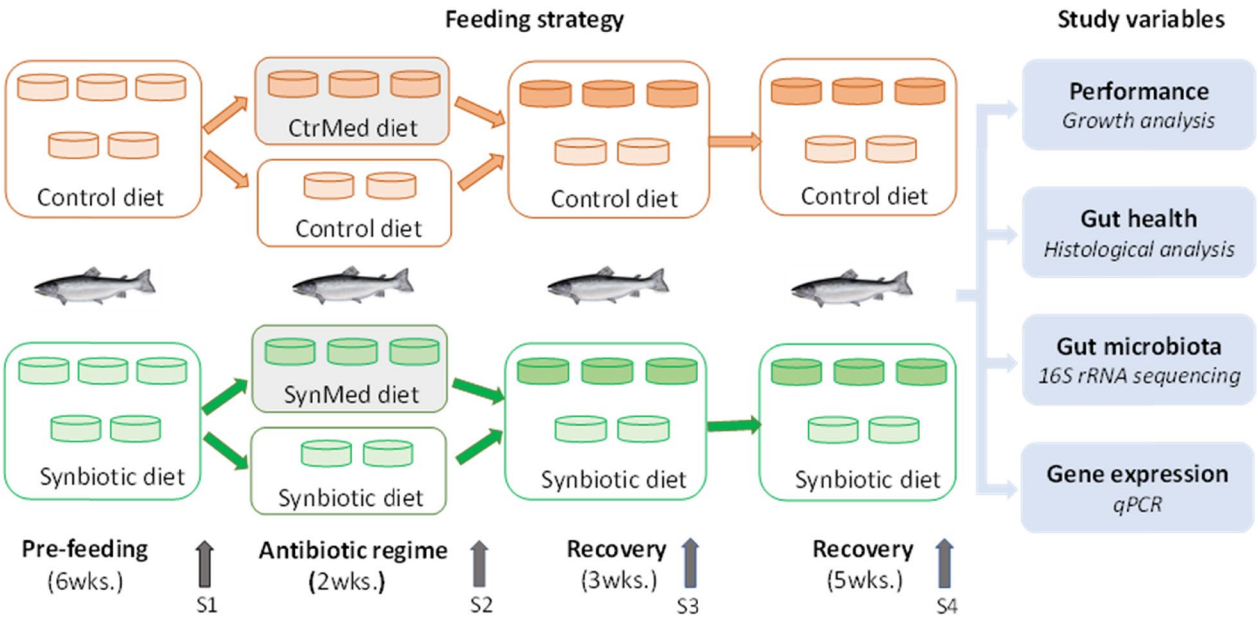

Fig. 1 Experimental design. This study evaluated the effects of nutritional management through a synbiotic strategy on growth performance, gut health, gut microbiota, and immune and stress gene expression of post-smolt Atlantic salmon during antibiotic treatment and subsequent recovery. Fish in five tanks each were fed either a commercial control diet (Ctr) or a synbiotic diet (Syn) containing FOS and *P. acidilactici* for a period of 6 weeks. After pre-feeding period (S1), fish in three tanks from each treatment were fed a medicated diet prepared by coating respective control and synbiotic diets with 3500 ppm florfenicol in oil (medicated group, Med, CtrMed and SynMed diets) for 2 weeks. Thereafter, those fish were refed the respective control and synbiotic diets for a recovery period of 3 or 5 weeks. The fish not subjected to antibiotic treatment (nonmedicated group, NonMed) were fed with control or synbiotic diets throughout the experimental period. Four different variables were analyzed before the start of antibiotic treatment (S1), after antibiotic treatment (S2), and after 3 weeks (S3, except gut microbiota) and 5 weeks (S4) of recovery period both from the medicated and nonmedicated groups as detailed in the materials and methods section. Longitudinal microbiota changes (S1-S2-S4) were analyzed using the digesta samples collected from pit-tagged fish

trial. Therefore, all results are presented separately for the fish which were not subjected to antibiotic treatment (nonmedicated group) and the fish which were subjected to antibiotic treatment (medicated group). Findings from comparisons between the nonmedicated and medicated groups are presented when appropriate. Results regarding effects on growth performance, histology, microbiota, and gene expression changes in fish which were not subjected to medication provide information on how the synbiotic diet affected those parameters. Comparison of those parameters from S1 (after 6 weeks of pre-feeding or before the start of the antibiotic treatment) to S2 (termination of the antibiotic treatment) time points allowed us to understand the possible effect of florfenicol treatment on control and synbiotic group as well as how the synbiotic strategy could influence changes triggered by antibiotic treatment. The changes from the S2 to S3 and S4 sampling points provided information about possible recovery of intestinal microbiota and host parameters which may have been disturbed by the antibiotic treatment.

Zootechnical performance

Nonmedicated group

For specific growth rate (SGR), effects of diet ($p=0.016$) and time ($p<0.0001$) were significant, but the interaction effect was not significant. SGR was significantly higher in fish fed both diets in the first 6 weeks and gradually decreased during the trial period (Fig. 2a). SGR of the fish fed synbiotic diet tended ($p=0.08$) to have higher values in the period from S1-S2 and S2-S3 compared to the fish fed control diet (Fig. 2a).

Feed intake (percentage of specific feeding rate, SFR) was significantly affected by the diet ($p=0.004$) and time ($p=0.0009$). In the control group, there was a general decrease in SFR along the trial period with a significant decrease from the period S1-S2/S2-S3 to S3-S4 (Fig. 2c).

For the feed conversation ratio (FCR), effect of time was significant ($p=0.0003$) and increased throughout the trial period (Fig. 2e). No significant effect of diet was observed.

Relative weight of the liver (hepatosomatic index) was not affected by diet or time (Supplementary Fig. 1a).

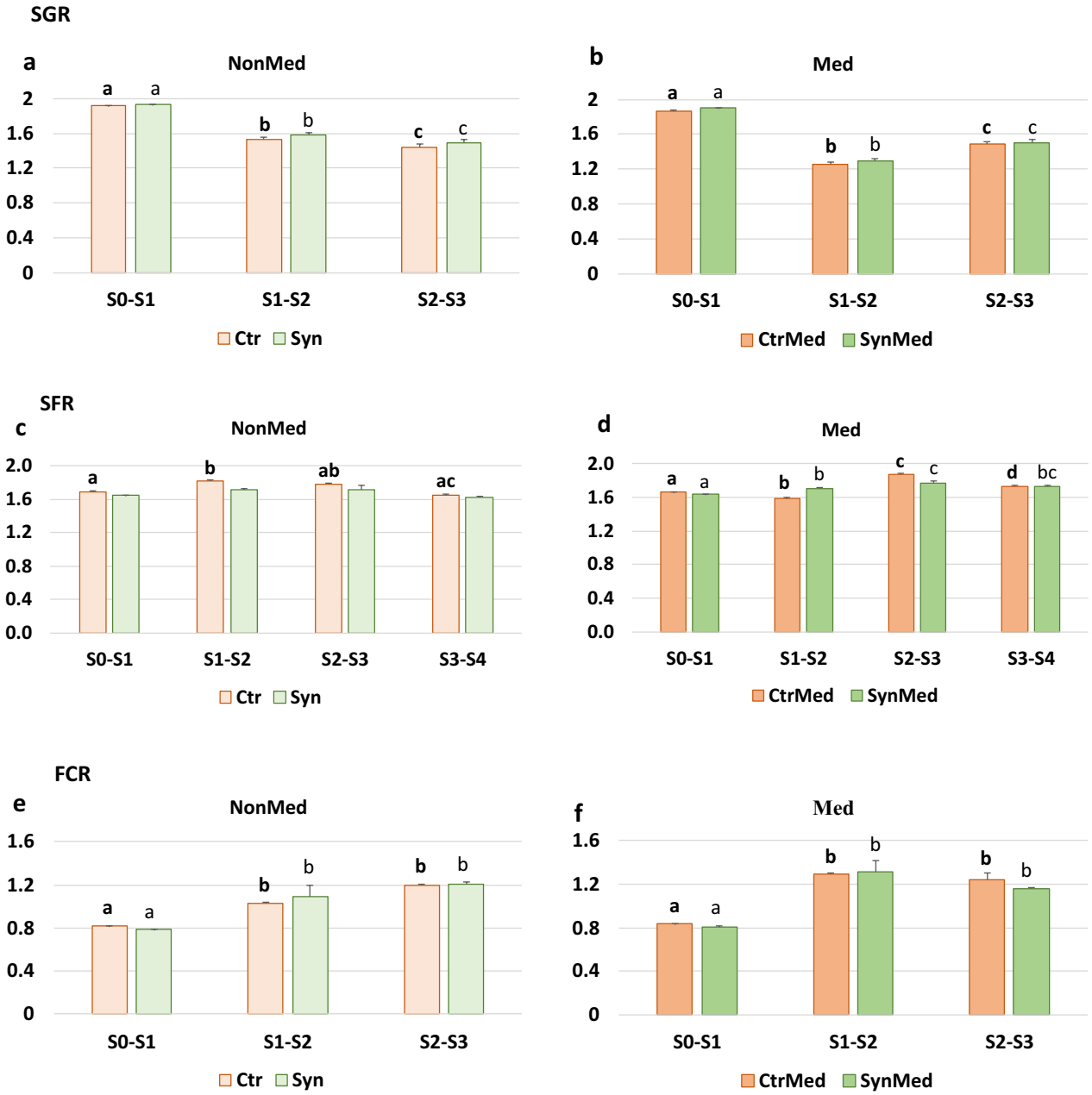

Fig. 2 Specific growth rate (SGR), percentage specific feeding rate (SFR) and feed conversion ratio (FCR). Performance data presented as an average per group/tank. Error bars represent standard error of mean (S.E.M.). See the materials and methods section for number of fish and tanks used per diet group at each time points and Fig. 1 legend for description of the diet groups and sampling time points. Different letters among values indicate statistically significant differences ($p < 0.05$) in each diet group among the sampling points. Values sharing the same letters are not statistically significant. Any significant differences between the diet groups at a sampling point are reported in the text

Medicated group

For SGR, effects of diet ($p = 0.03$) as well as time ($p < 0.0001$) were significant as in the nonmedicated group. Antibiotic treatment significantly reduced the growth in fish fed both the control and synbiotic diets (Fig. 2b), and this reduction tended to be less for the fish fed synbiotic diet ($p = 0.089$). Generally, fish subjected to antibiotic treatment showed lower SGR compared to the fish not subjected to antibiotic treatment during the period S1-S2 (see the Fig. 2a and b). After three weeks of feeding the respective control or synbiotic diets (S2-S3), SGR increased in the medicated

group and those values were similar to the SGRs of the nonmedicated groups (see the Fig. 2a and b).

SFR was affected by interaction of the diet and time ($p=0.0004$). Antibiotic treatment significantly reduced SFR in the fish fed control diet whereas it was significantly increased in the fish fed synbiotic diet (Fig. 2d). Further, fish fed the synbiotic diet showed significantly higher SFR ($p=0.0001$) for the period fish underwent antibiotic treatment (S1-S2). Fish subjected to antibiotic treatment showed generally lower values in the control group compared to their counterparts in nonmedicated group (1.8 ± 0.02 S.E.M in nonmedicated control group and 1.6 ± 0.01 S.E.M in medicated control group) during the S1-S2 period. On the other hand, SFR remained at 1.7 ± 0.01 S.E.M for the nonmedicated and medicated synbiotic groups during the S1-S2 period. During the recovery period, SFR increased in both groups (Fig. 2d).

Considering the antibiotic consumption, fish fed control and synbiotic diets consumed respectively 43 and 46 mg of florfenicol per kg of body weight (BW) per day, during the medicated period (S1-S2).

For FCR, effect of time was significant ($p<0.0001$) similar to that observed for the nonmedicated group. Antibiotic treatment increased FCR in fish fed both control and synbiotic diets (Fig. 2f). Those values were relatively higher compared to the respective nonmedicated groups (S1-S2, see the Fig. 2e and f). During the recovery period FCR remained the same as the period of antibiotic treatment. FCR did not show any significant difference between fish fed the control and synbiotic diets at any of the periods.

Relative weight of the liver was significantly affected by interaction of the diet and time ($p=0.02$, Supplementary Fig. 1b). Antibiotic treatment did not significantly change the hepatosomatic index in both groups. During the recovery period it remained the same as the period of antibiotic treatment, but the values at S3 were significantly higher compared to the values at S1 in fish fed synbiotic diet (Supplementary Fig. 1b). At S1, hepatosomatic index of fish fed control diet was higher compared to the fish fed synbiotic diet ($p=0.02$).

Histomorphological analysis of the distal intestine

Most of the fish evaluated was observed with normal and healthy distal intestine morphology. However, histopathological changes associated with inflammatory reactions, i.e. villi atrophy, loss of enterocyte supranuclear vacuolization, hyperemia and lamina propria hemorrhage were observed in a proportion of the evaluated fish, with uneven distribution among sampling points (Additional File 1. Fig. S2).

Nonmedicated group

Fish fed both the control and synbiotic diet showed moderate to marked inflammatory changes after the pre-feeding period (S1) in more than 30% of the fish observed (Additional File 1. Fig. S2). For both diet groups, the prevalence and the severity of the inflammatory changes were reduced at the S2 sampling compared to S1 (significant for the control group), whereas the symptoms tended to increase again in both groups at the later S3 and S4 time points.

Medicated group

Similar to the nonmedicated group, moderate to marked inflammation were observed in more than 25% fish fed both the control and synbiotic diets after the pre-feeding period (S1, Additional File 1. Fig. S2). Similar as observed for the nonmedicated group, the prevalence and the severity of the inflammatory changes were reduced at the S2 sampling compared to S1 (significant for the synbiotic group), whereas the symptoms tended to increase again in both groups at the later S3 and S4 time points.

Microbiota profiling

Alpha diversity

Alpha diversity measured using observed taxa (i.e., number of taxa), and Shannon index (i.e., number of taxa and their relative abundance) at amplicon sequence variant (ASV) levels are shown in Fig. 3. The relationship between alpha diversities and predictor variables, diet group and sampling time and their interaction analyzed form linear mixed effect model (LME), are presented in Table 1.

Nonmedicated group

The statistical analyses of observed taxa showed significant interaction between diet and time (Table 1 and Fig. 3a). At S2, the result was significantly higher ($p=0.005$) for the control fed fish than the synbiotic fed fish, whereas at S1 and S4 the results did not differ clearly. Regarding the Shannon index (Table 1 and Fig. 3b), effects of diet group was significant, with higher values for fish fed control than those fed synbiotic diet, and a strong trend ($p=0.053$) towards a decreasing effect of time. Shannon index was significantly higher ($p=0.012$) for fish fed control diet compared to the fish fed synbiotic diet at S2 sampling time point.

Medicated group

Observed taxa was significantly affected by interaction between diet and time (sampling point from S1 to S2

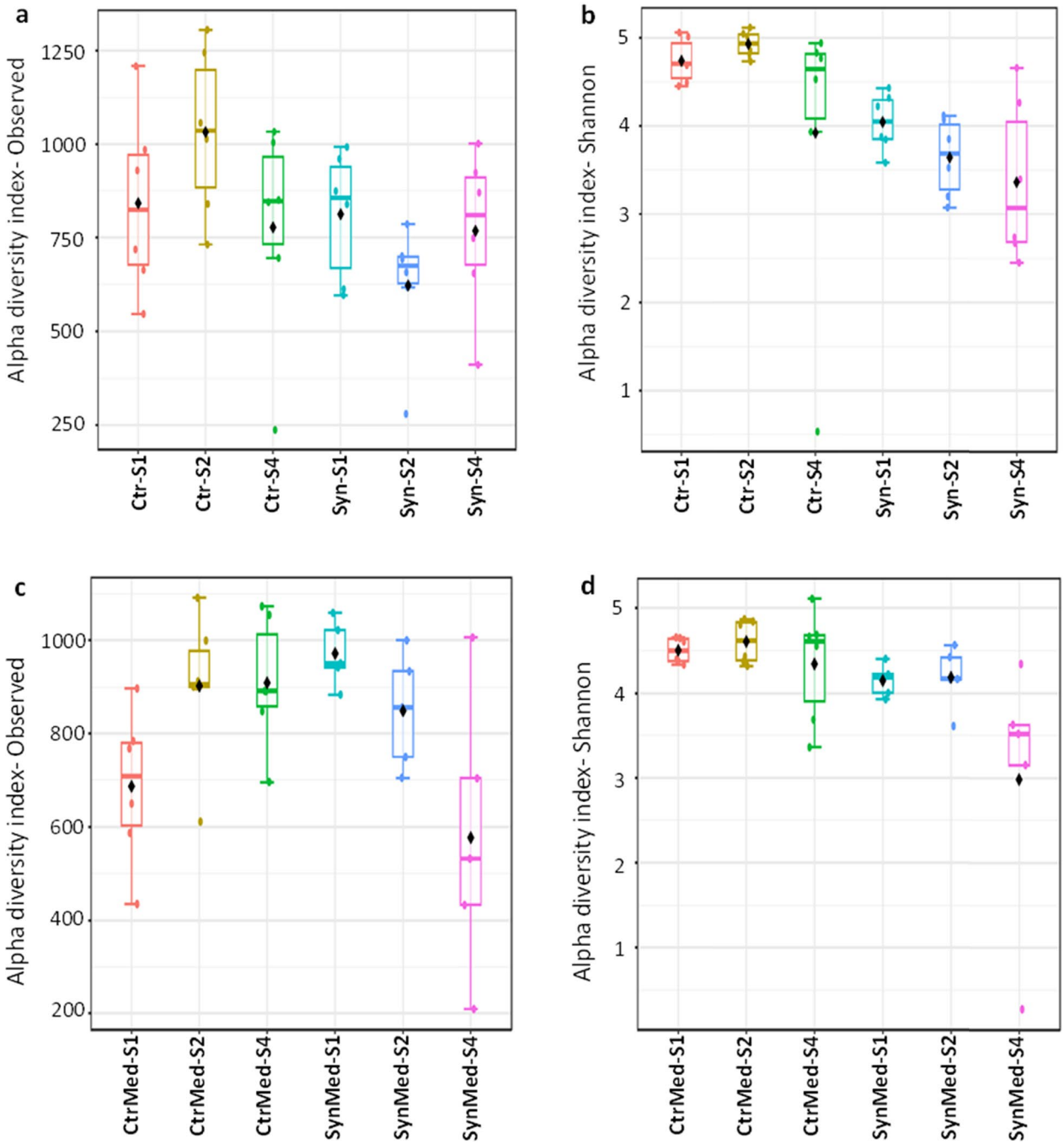

Fig. 3 The alpha diversity indices for digesta at ASV level. Alpha diversity of digesta microbiota of Atlantic salmon that were not subjected to antibiotic treatment (nonmedicated group, **a** and **b**) and that were subjected to antibiotic treatment (medicated group, **c** and **d**). Digesta samples were striped before the start of antibiotic treatment (S1), after antibiotic treatment (S2), and after 5 weeks (S4) of recovery period from pit-tagged fish in both the nonmedicated and medicated groups. See Fig. 1 legend for description of the diet groups and sampling time points

included the effect from antibiotic treatment and S2 to S4 included the effect from subsequent recovery, Table 1, Fig. 3c). Observed taxa tended to increase in control ($p = 0.087$) and it was unchanged in fish fed synbiotic diet ($p = 0.243$) during the antibiotic treatment (Fig. 3c). Observed taxa did not significantly change during the recovery period (S2 to S4) in fish fed the control diet but it was significantly changed in the

Table 1 LME analysis of the relationship between alpha diversity indices and diet and sampling time

Alpha diversity indices	Predictors	DF1	DF2	F	*P*
Nonmedicated					
Observed	Diet	1	9.97	2.35	0.157
	Time	2	20.50	0.31	0.735
	Diet:time	2	20.50	4.25	0.029
Shannon	Diet	1	9.93	7.36	0.022
	Time	2	20.76	3.39	0.053
	Diet:time	2	20.76	0.74	0.490
Medicated					
Observed	Diet	1	3.85	0.13	0.742
	Time	2	23.08	1.84	0.181
	Diet:time	2	23.08	9.43	0.001
Shannon	Diet	1	3.76	8.38	0.048
	Time	2	23.14	3.63	0.043
	Diet:time	2	23.14	1.70	0.205

DF1 and DF2, degree of freedom 1 and 2; F, F ratio; P, *p* value

fish fed the synbiotic diet ($p=0.036$). Further, observed taxa tended to be higher ($p=0.087$) in fish fed control diet and were significantly lower in the fish fed synbiotic diet ($p=0.003$) at S4 sampling point compared to the start of the antibiotic treatment S1. Observed taxa significantly differed between the fish fed control and synbiotic diets at S1 ($p=0.014$) and S4 time points ($p=0.009$).

For Shannon index, effects of diet group as well as time were significant (Table 1, Fig. 3d). In control group, Shannon index was not significantly influenced by antibiotic treatment or subsequent recovery for 5 weeks. In fish fed synbiotic diet too, it was not significantly affected by antibiotic treatment. However, in fish fed synbiotic diet, Shannon index decreased significantly (Fig. 3d) at S4 compared to the S2 ($p=0.038$) and S1 ($p=0.038$). Fish fed control and synbiotic diets showed significantly different Shannon index after recovery period (S4, $p=0.004$).

Beta diversity

Differences in bacterial composition, assessed using Bray–Curtis index (abundance of taxa) and weighted UniFrac distance (UniFrac) distance (abundance with considering phylogenetic relationship) are presented in principal coordinates analysis (PCoA) ordination plots in Fig. 4 and Additional File 1. Figure S3 respectively. The relationship between beta diversities and predictor variables, diet group and sampling time, and their interaction analyzed by permutational multivariate analysis of variance (PERMANOVA) are presented in the Table 2.

Nonmedicated group

Both Bray–Curtis index and weighted UniFrac distance were significantly affected by diet and time, and the results also showed close to significant interaction (Table 2). PCoA ordination plots indicated that microbial taxa at the S4 time point clustered separately from other sampling points (Fig. 4a and Additional File 1. Fig.

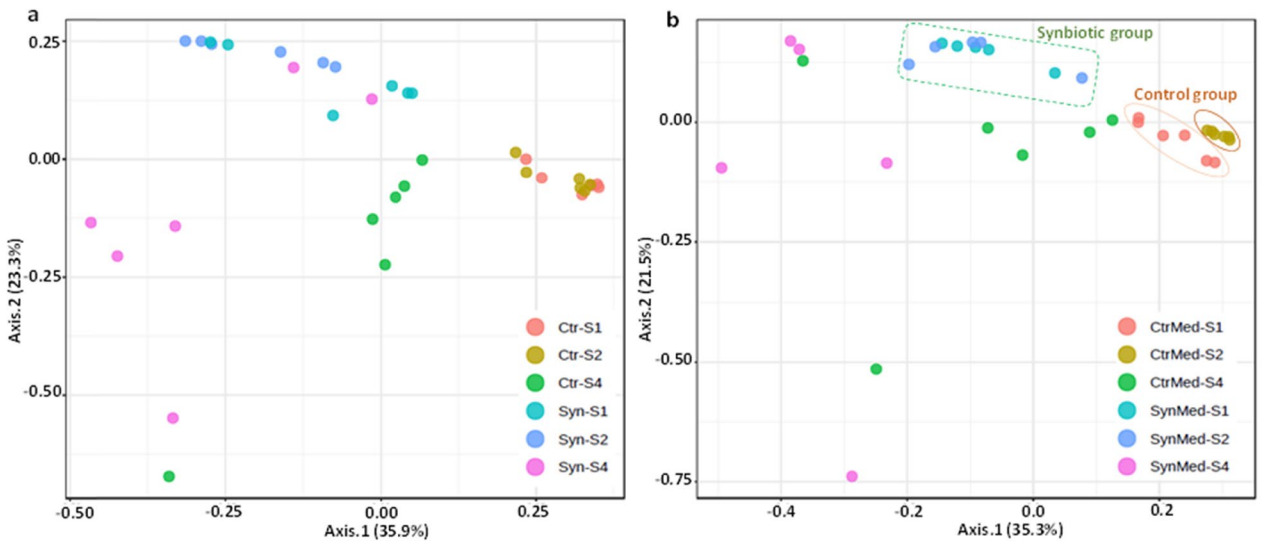

Fig. 4 PCoA plots based on Bray–Curtis dissimilarity matrix showing beta diversity at ASV level. Bacterial community structures (beta diversity) in the digesta of the fish, **a** Not subjected to antibiotic treatment (nonmedicated group) and **b** Subjected to antibiotic treatment (medicated group). The whole bacterial community of each sample is represented by a dot in the figure. Samples with similar bacterial compositions are close to each other. See Fig. 1 legend for description of the diet groups and sampling time points

Table 2 The PERMANOVA analysis of the relationships between beta diversity indices and diet and sampling time

Beta diversity indices	Predictors	R^2 (%)	F	*P*
Nonmedicated				
Bray–Curtis	Diet	11.54	4.85	0.003
	Time	9.79	2.06	0.003
	Diet:time	7.26	1.52	0.055
Weighted Unifrac				
	Diet	14.29	7.20	0.028
	Time	14.87	3.75	0.022
	Diet:time	11.33	2.85	0.053
Medicated				
Bray–Curtis	Diet	6.75	2.33	0.186
	Time	11.49	1.98	0.022
	Diet:time	3.40	0.59	0.922
Weighted Unifrac	Diet	2.85	0.99	0.091
	Time	17.43	3.02	0.019
	Diet:time	1.90	0.33	0.939

R^2, Eta-squared; F, F ratio; *P*, *p* value

S3a). However, pairwise analysis, controlling for random effect from fish id, did not show significant difference in Bray-Curtis dissimilarity index among the sampling time points per diet group or between the diet groups per sampling time point. On the other hand, weighted UniFrac distance indicated significant difference in microbial composition at S4 compared to the S1 ($p=0.03$) and S2 ($p=0.03$) time points in fish fed the control diet.

Medicated group

Bacterial composition assessed by both the Bray–Curtis index and weighted UniFrac distance matrices were significantly affected by time (sampling point from S1 to S2 also included the effect from antibiotic treatment and S2 to S4 included the effect from subsequent recovery, Table 2). In the fish fed control diet, microbiota after antibiotic treatment (S2) clustered separately from the microbiota before the antibiotic treatment (S1) as indicated by PCoA plot based on Bray–Curtis index (Fig. 4b). Similar separation could not be observed in fish fed synbiotic group in which microbiota from S1 and S2 time points clustered together. However, PCoA plots based on weighted UniFrac distance did not show such distinct clusters (Additional File 1. Fig. S3b). In fish fed both the control and synbiotic diets, microbiota from S4 sampling point scattered distinctly from the S1 and S2 time points (Fig. 4b and Additional File 1. Fig. S3b). Pairwise analysis, controlling the random effect from fish id, did not show significant difference in both beta diversity indices among the sampling time points in a diet group or between the diet groups in a sampling time point.

Taxonomic composition

Nonmedicated group

At the phylum level, *Firmicutes* dominated in fish fed both the diets irrespective of the time point (Additional File 1. Fig. S4a). In fish fed both diets, it showed highest abundance at S2 comprising 86% and 94% of the total abundance in control and synbiotic fed fish, respectively. *Firmicutes* decreased in relative abundance at S4 (51% and 58% respectively in fish fed control and synbiotic diets). On the other hand, *Photobacteria* increased to 33% in fish fed control diet and 38% in fish fed synbiotic diet at S4. *Actinobacteriota* showed higher abundance in fish fed control diet at S1 and S4 comprising around 13% (S1)-14% (S4). It ranged from 6% (S1) to 4% (S4) in fish fed synbiotic diet.

At genus level, *Lactobacillus* was present in high abundance in fish fed the control diet at S1 (49%) and S2 (48%) sampling points while fish fed the synbiotic diet had 32% and 24% at the respective sampling points (Fig. 5a). It decreased in abundance at S4 sampling point (control, 19% and synbiotic 18%). As expected, *Pediococcus* predominated in fish fed the synbiotic diet comprising 32%, 51% and 26% at S1, S2 and S4 sampling points. *Pediococcus* were present in low abundance in fish fed the control diet comprising 1%, 3% and 7% at respective time points. *Staphylococcus* comprised 6%, 7% and 14% in fish fed the control diet and 3%, 3% and 2% in fish fed synbiotic diet respectively at S1, S2 and S4 sampling points. *Aliivibrio* dominated at the S4 sampling point consisting of 19% and 29% respectively in fish fed control and synbiotic diets. However, this dominancy was observed mostly in fish fed synbiotic diet and in only one individual in fish fed control diet.

Medicated group

Firmicutes dominated also in the medicated group, comprising 90% (S1) and 85% (S2) in fish fed control diet and 92% (S1) and 82% (S2) in fish fed synbiotic diet (Additional File 1. Fig. S4b). In fish fed both diets, it decreased at S4 time point to 72% in fish fed control diet and 59% in fish fed synbiotic diet. In the medicated group too, *Photobacteria* increased to 19% in fish fed control diet and 35% in fish fed synbiotic diet at S4. *Actinobacteriota* comprised 8–10% in fish fed control diet and 6–8% in fish fed synbiotic diet.

At genus level, *Lactobacillus* were also abundant in medicated group at S1 and S2 sampling point consisting of 53% (S1), 55% (S2) in fish fed control diet and 36% (S1), 31% (S2) in fish fed synbiotic diet (Fig. 5b). Its abundance was only slightly influenced by antibiotic treatment (S1 to S2) in fish fed both diets. Similar to nonmedicated group, *Lactobacillus* abundance was also reduced at S4

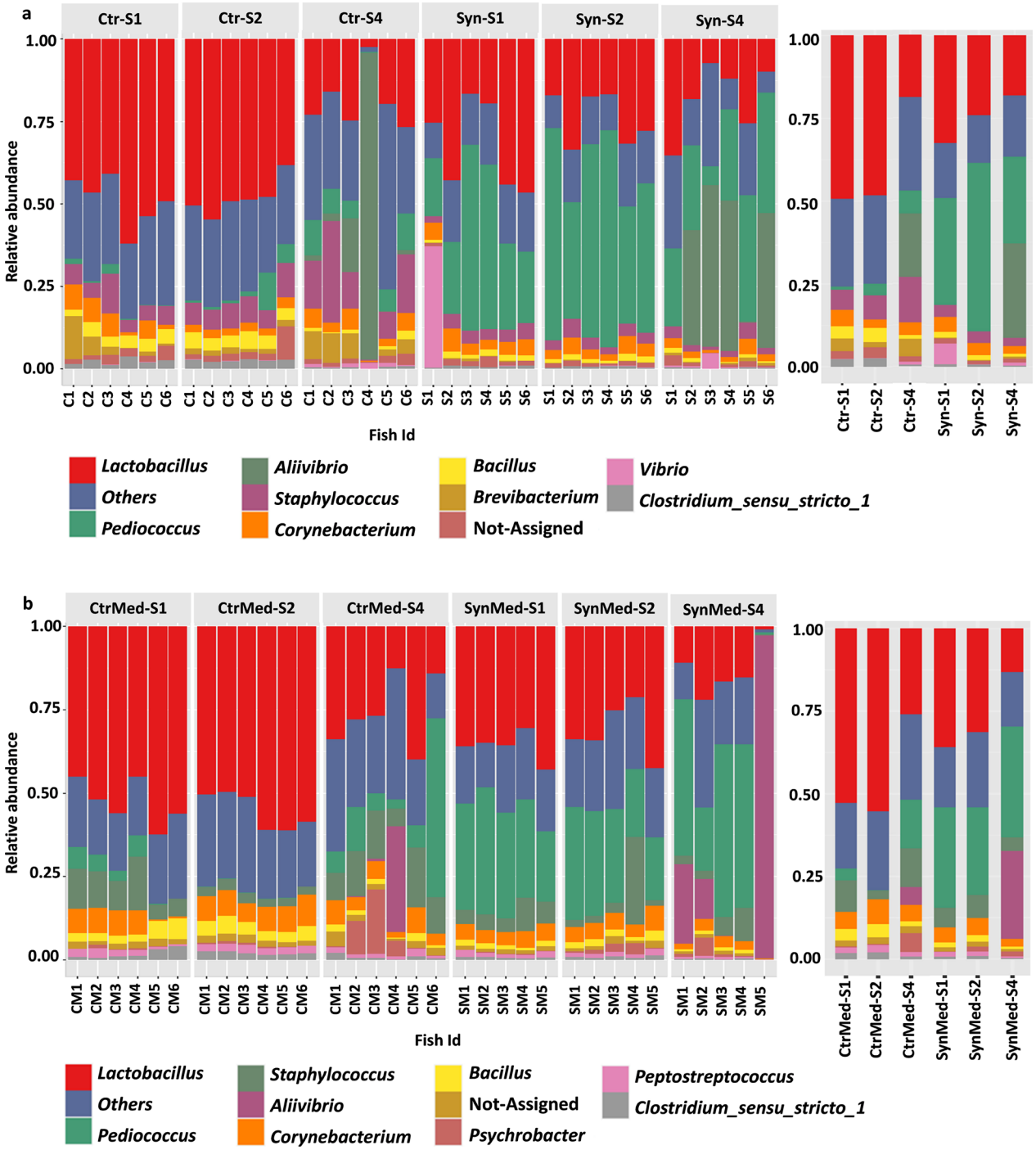

Fig. 5 Top 10 most abundant genera in the digesta. **a** Fish not subjected to antibiotic treatment (nonmedicated group). **b** Fish subjected to antibiotic treatment (medicated group). The relative abundance of genera per individual fish from respective diet group at a sampling point is presented on the left side and the mean relative abundances of genera per diet group at a sampling point is presented on the right side, respectively. See Fig. 1 legend for description of the diet groups and sampling time points

sampling point (26% and 13%, respectively, in fish fed control and synbiotic diets). The control fed fish showed low abundance of *Pediococcus* at start of the antibiotic treatment (S1, 3%). It diminished upon antibiotic treatment (S2, 0.1%), but increased considerably after the recovery period (S4, 15%). *Pediococcus* predominated in

the synbiotic fed fish at all the time points (Fig. 5b). It was slightly reduced upon antibiotic treatment (S1, 30% to S2, 27%) which was not the case in nonmedicated group in which it increased considerably from S1 to S2. However, it increased after the recovery period (S4, 33%). Relative abundance of *Staphylococcus* was also slightly affected by antibiotic treatment, decreasing from 9% (S1) to 3% (S2) in the fish fed control diet, whereas it was stable in the corresponding group not subjected to antibiotic treatment. On the other hand, its abundance was fairly stable in the synbiotic fed fish upon antibiotic treatment (S1, 6%, and S2, 7%). Similar to the nonmedicated group, *Alivibrio* increased at S4 sampling point in fish fed both the control (6%) and synbiotic (27%) diets.

As anticipated. *P. acidilactici* showed higher abundance in fish fed the synbiotic diet in both the nonmedicated and medicated groups (Fig. 6). *P. acidilactici* levels increased from S1 to S2 in nonmedicated group, while it was reduced with antibiotic treatment, the reduction was high in fish fed the control diet. For both diet groups, *P. acidilactici* increased after the recovery period after antibiotic treatment.

Gene expression analysis

Results from gene expression analysis performed for the samples collected from sacrificed fish at S1, S2, S3, and S4 time points (Fig. 1), are presented in Figs. 7 and 8. The relationship between gene expression and predictor variables, diet and sampling time points, and their interaction, are presented in the Additional File 2. Table S1.

Nonmedicated group

In the nonmedicated fish, the expressions of the pro-inflammatory cytokines, interleukin 1β, *il1β*, interleukin 17a, *il17a,* interleukin 8, *il8* and interferon γ, *ifnγ* showed significant interaction between diet and time (Additional File 2. Table S1). Fish fed the control diet showed no significant difference in expression levels between the sampling time points, whereas fish fed the synbiotic diet displayed elevated expression at S2 and S4 (Fig. 7a–d). The expression levels were also significantly higher compared to the fish fed the control diet ($p < 0.05$) except for expression of *ifnγ* at S2 which showed increased trend ($p = 0.07$).

The anti-inflammatory cytokines, interleukin 10, *il10* and transforming growth factor β, *tgfβ* also showed a significant interaction between diet and time (Additional File 2. Table S1). In fish fed the control diet, they were not significantly changed between the sampling time points whereas in fish fed the synbiotic diet, they were significantly elevated at S2 and/or S4 (Fig. 7e and f) and showed significantly higher values ($p < 0.05$) compared to the fish fed the control diet.

Expression of the T-cell markers, cluster of differentiation 3γδ, *cd3γδ* and cluster of differentiation 8β, *cd8β* were significantly influenced and showed an interaction

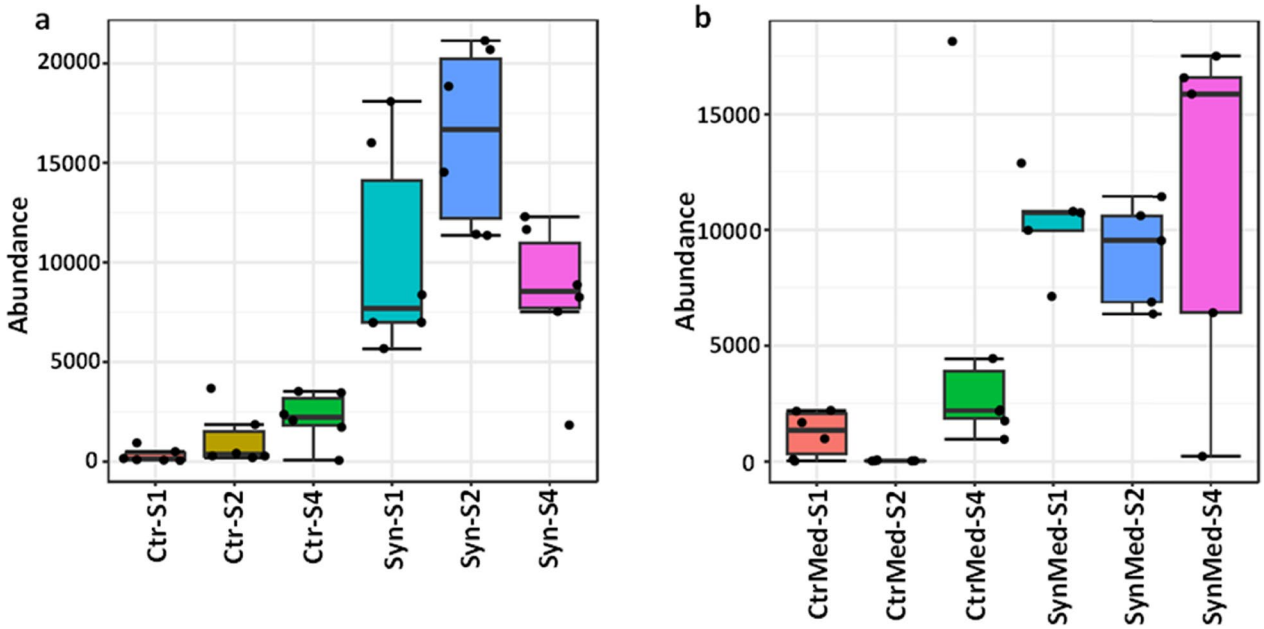

Fig. 6 Box plots showing filtered absolute counts of *P. acidilactici* in the digesta. Abundance of *P. acidilactici*, **a** Of fish not subjected to antibiotic treatment (nonmedicated group) and **b** Of fish subjected to antibiotic treatment (medicated group). See Fig. 1 legend for description of the diet groups and sampling time points

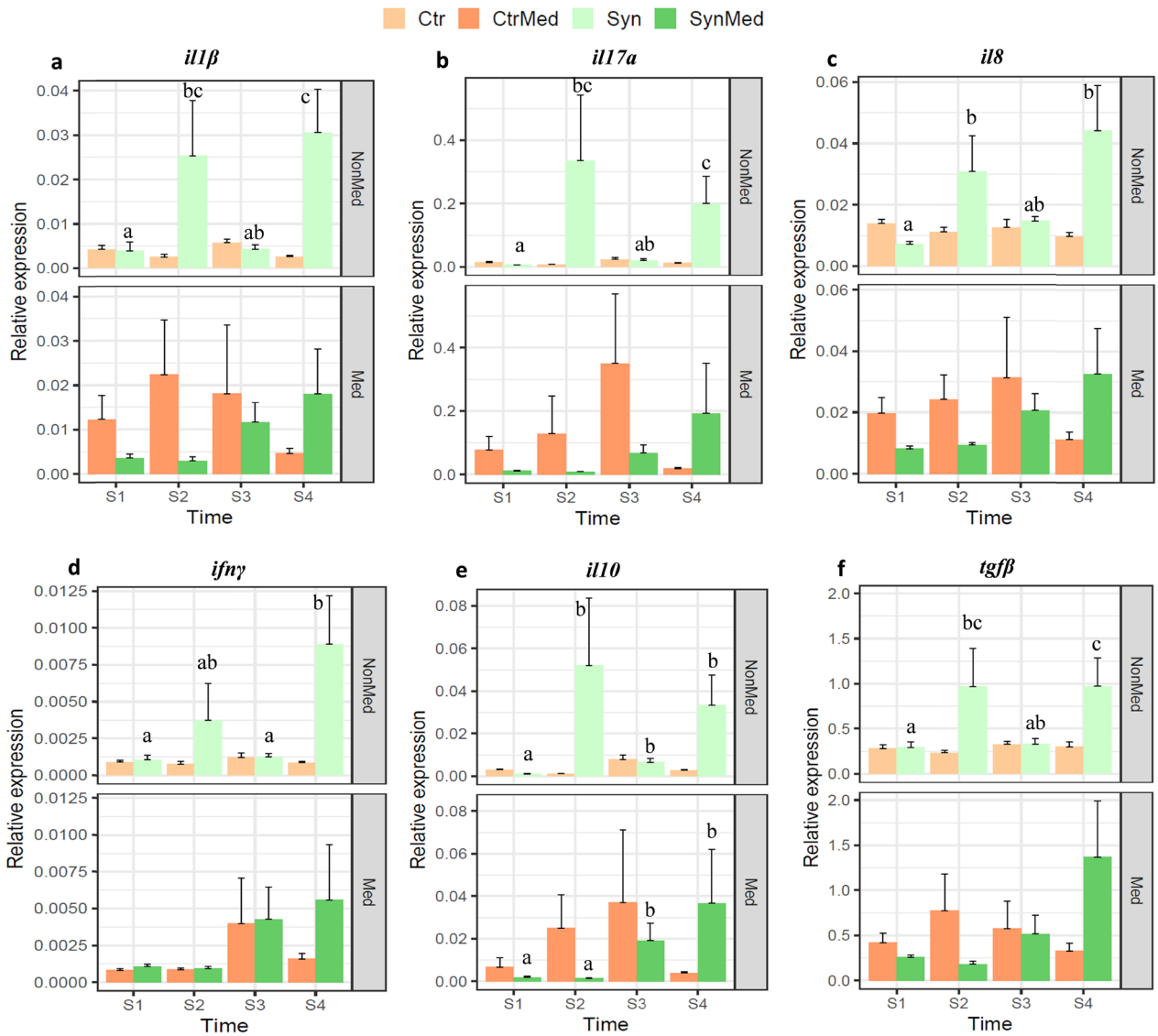

Fig. 7 Relative expression of cytokines. Relative expression of pro-inflammatory cytokines, **a** interleukin 1β, *il1β*, **b** interleukin 17a, *il17a,* **c** interleukin 8, *il8* and **d** interferon γ, *ifnγ*, and anti-inflammatory cytokines, **e** interleukin 10, *il10* and **f** transforming growth factor β, *tgfβ*. Transcripts were quantified by qPCR and normalized using the geometric average of the selected reference genes. Values are presented as means ± S.E.M. (n = 6). Different letters among values indicate statistically significant differences ($p < 0.05$) in each group among the sampling points. Values sharing the same letters are not statistically significant. Any significant differences between the diet groups at a sampling point are reported in the text. See Fig. 1 legend for description of the diet groups and sampling time points

(See figure on next page.)

Fig. 8 Relative expression of immune, antioxidant defense and stress response genes. T-cell markers, **a** cluster of differentiation 3γδ, *cd3γδ*, **b** cluster of differentiation 8β, *cd8β,* other immune genes, **c** myeloid differentiation primary response gene 88, *myd88*, **d** major histocompatibility complex, class 1, *mhc1*, and **e** lysozyme C II, *lysozyme C II*, and goblet cell marker, **f** mucin 2, *muc2,* and antioxidant defense and stress response, **g** superoxide dismutase 1, *sod1*, **h** catalase, *cat*, and **i** heat shock protein 70, *hsp70*. Transcripts were quantified by qPCR and normalized using the geometric average of the selected reference genes. Values are presented as means ± S.E.M. (n = 6). Different letters among values indicate statistically significant differences ($p < 0.05$) in each group among the sampling points. Values sharing the same letters are not statistically significant. Any significant differences between the diet groups at a sampling point are reported in the text. Please refer to Fig. 1 legend for the description of the diet groups and sampling time points

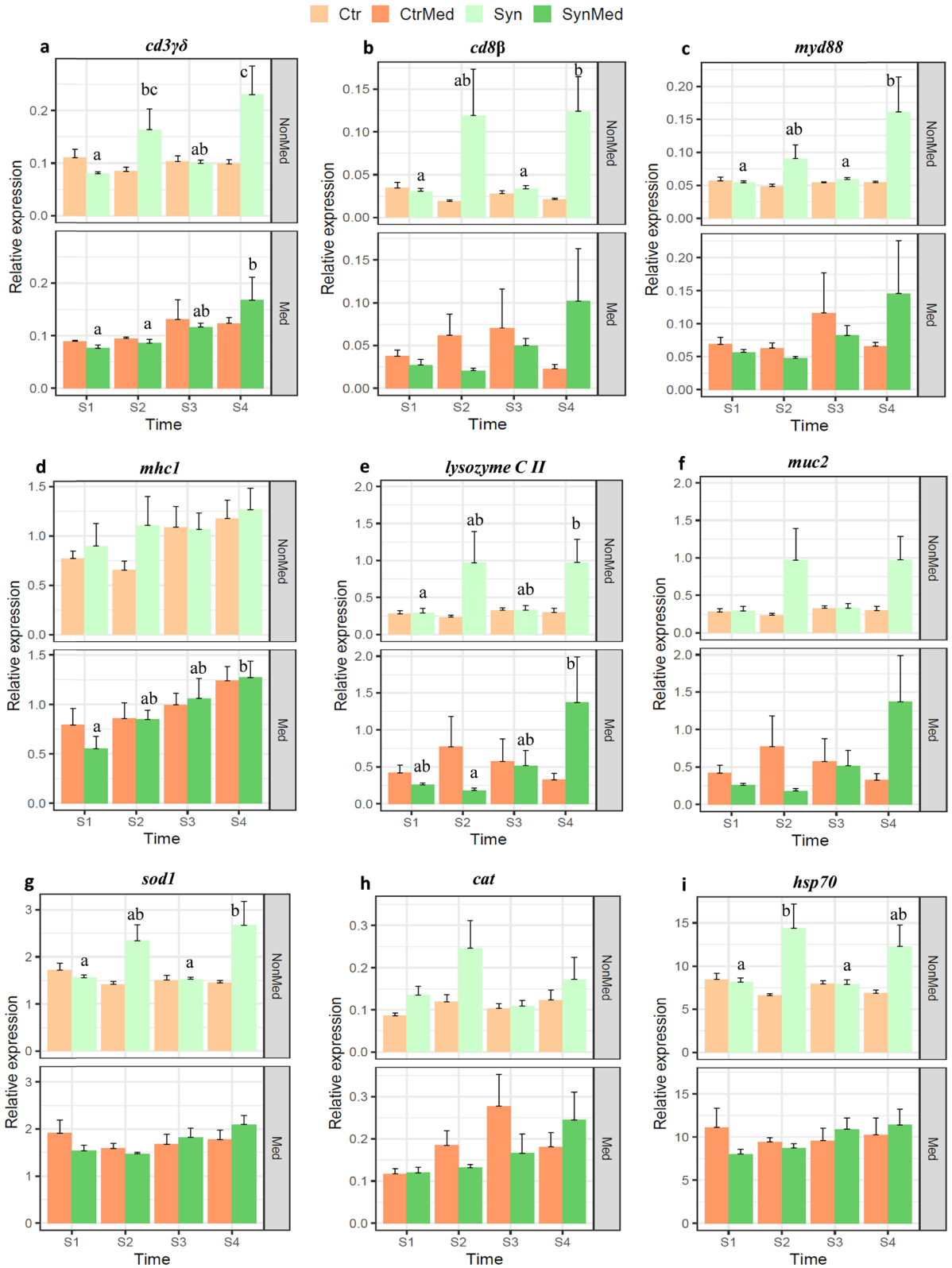

Fig. 8 (See legend on previous page.)

between diet and time (Additional File 2. Table S1). Fish fed the control diet showed no significant difference in expression levels between the sampling time points, whereas fish fed the synbiotic diet showed elevated values at S2 and S4 (Fig. 8a and b). The expression levels were also higher for fish fed control diet compared to those fed synbiotic diet at S2 and S4 except the higher trend for *cd3γδ* at S2 ($p=0.059$).

Expression of immune genes, myeloid differentiation primary response gene 88, *myd88* and *lysozyme C II* showed close ($p=0.07$) to significant interaction between diet and time (Additional File 2. Table S1). Expression of the above immune genes as well as major histocompatibility complex, class 1, *mhc1* (Fig. 8c–e) did not significantly change between the sampling time points in fish fed the control diet whereas in synbiotic fed fish, *myd88* and *lysozyme C II* showed elevated values at S4 (Fig. 8c and e). Expression levels of *myd88* and *lysozyme C II* were also significantly higher at S2 and S4 in synbiotic fed fish compared to the control fed fish, except *lysozyme C II* which showed a higher trend at S2 ($p=0.056$).

Expression of the goblet cell marker, mucin 2, *muc2* was not significantly changed over the time either in fish fed control or synbiotic diets (Fig. 8f) but showed relatively elevated values at S2 and S4 in synbiotic fed fish. However, the values were significantly higher in the synbiotic fed fish compared to the control fed fish at S4.

Among the genes responsible for antioxidant defense and stress response, superoxide dismutase 1, *sod1* and heat shock protein 70, *hsp70* showed significant interaction between diet and time (Additional File 2. Table S1). Expressions of *sod1*, catalase, *cat* and *hsp70* did not significantly change between sampling the time points in fish fed control diet (Figs. 8g–i). In fish fed synbiotic diet, those genes were elevated at S2 and S4 time points and some of them showed significant elevation. Expression levels of *sod1* and *hsp70* were significantly different between control and synbiotic fed fish at S2 and S4 except *hsp70* showed a differing trend at S4 ($p=0.07$).

Medicated group

Among the pro-inflammatory cytokines, *il8* showed significant interaction between diet and time and *ifnγ* was significantly affected by time (Additional File 2. Table S1). None of the pro-inflammatory cytokines tested were differentially expressed between the sampling time points either in fish fed control or synbiotic diets e.g., either by antibiotic treatment or during the recovery period (Fig. 7a–d). However, there were relative increase in expressions of those genes at S2 and S3 in fish fed the control diet and at S3 and S4 in the fish fed the synbiotic diet.

Among anti-inflammatory cytokines, *il10* showed a trend ($p=0.084$) towards interaction between diet and time (Additional File 2. Table S1). In control fed fish it was not significantly changed between the time points while in synbiotic fed fish, it significantly increased during recovery period at S3 and S4 compared to the just before (S1) and after (S2) the antibiotic treatment (Fig. 7e). Albeit not significant, a similar pattern of expression was observed for *tgfβ* (Fig. 7f).

Expression of *cd3γδ* was significantly affected by time while *cd8β* was significantly affected by interaction between diet and time (Additional File 2. Table S1). In the fish fed control diet, there were no significant difference in expression levels of those genes between the sampling time points, whereas in the fish fed synbiotic diet, *cd3γδ* was significantly increased at S4 compared to just before (S1) and after (S2) antibiotic treatment (Fig. 8a). Further, fish fed the synbiotic diet tended to have higher values for *cd8β* at S4 ($p=0.055$) compared to the fish fed the control diet.

When considering the expression of immune genes *myd88*, *mhc1* and *lysozyme C II*, only *mhc1* was significantly affected by time (Additional File 2. Table S1). In control fed fish, those immune genes were not significantly changed after antibiotic treatment or during the recovery period (Fig. 8). On the other hand, in synbiotic fed fish, *mhc1* was increased over the time and values were significantly higher at S4 compared to S1 (Fig. 8d). Expression levels of *lysozyme C II* increased during the recovery period and showed a significant increase at S4 (Fig. 8e), and the value also tended to be higher compared to the control fed fish ($p=0.058$).

Expression levels of *muc2* were not significantly changed after antibiotic treatment or during the recovery period either in fish fed control or synbiotic diets (Fig. 8f).

None of the antioxidant defense and stress response genes analyzed (*sod1*, *cat* and *hsp70*) were significantly affected by antibiotic treatment or subsequent recovery either in fish fed control or synbiotic diets (Fig. 8g–i).

The expression pattern of most of the genes was different for medicated control and synbiotic groups which in turn was different from the pattern observed for the respective nonmedicated groups throughout the experiment (Figs. 7 and 8). For example, most of the genes showed relative increase at S2 (immediately after antibiotic treatment) and S3 (after 3 weeks of recovery period) in control medicated group and in S3 and S4 (3 and 5 weeks of recovery period) in synbiotic medicated group differing to the pattern observed in respective nonmedicated groups.

Discussion

This study, using a longitudinal approach, showed that florfenicol, an antibiotic widely used in veterinary medicine, influenced growth performance, gut microbiota, and gene expression in Atlantic salmon. Concomitant feeding of a synbiotic diet containing FOS and *P. acidilactici* helped to maintain the gut microbial composition and influenced growth performance positively during antibiotic treatment. The synbiotic diet affected immune gene expressions differently than the standard commercial diet during antibiotic treatment and subsequent recovery.

Fish fed both the control and synbiotic medicated diets consumed relatively high florfenicol doses (43 and 46 mg/kg BW, respectively) in comparison to the recommended dose of 20–40 mg/kg BW for fish with salmonid rickettsial septicemia [61]. This could be attributed to the generally high feed intake [2, 71] observed in both control and synbiotic diet fed fish. Feed intake has been reported to change with various biological and environmental conditions including, life stage, diet, temperature, medication and disease [2, 28, 67, 71]. However, feed intake significantly reduced in the fish fed control diet during florfenicol treatment and those values were also lower compared to their nonmedicated counterparts. Similar observations were previously reported in studies with salmonids fed medicated diets and have been attributed to the low palatability of antibiotic-containing diets [28, 67]. On the other hand, a stable feed intake observed in fish fed synbiotic diets during florfenicol treatment with similar values with their nonmedicated counterparts may suggest that supplementation of synbiotic could have increased the acceptancy of feed by fish likely negating the low palatability issues with antibiotics. However, this warrants further studies. FCR was increased in both the control and synbiotic diet fed fish during the florfenicol treatment, aligning with the higher FCR observed in southern catfish, *Silurus meridionalis* subjected to the same antibiotic [75].

Florfenicol treatment negatively affected the SGR of fish fed both the control and synbiotic diets similar to that observed in short term (7 days) florfenicol treatment on southern catfish, [75] as well as zebrafish subjected to different antibiotics for longer duration [76]. The reduction in growth was less pronounced among the fish fed the synbiotic diet, suggesting a positive influence of the synbiotic diet in sustaining growth during the medication period. This observation may also be seen in relation to the general improved growth observed among the fish fed the synbiotic diet compared to the fish fed the commercial control diet in the nonmedicated group. As the composition of the control and synbiotic diets was identical, improved growth could be due to the enhanced feed intake in the fish fed synbiotic diet.

Two weeks of florfenicol treatment did not significantly affect the HSI in the fish fed both the control and synbiotic diet fed fish. HSI is used as an indicator of nutritional status and liver health and can be affected by various toxicants, pollutants, xenobiotics, antibiotics, and nutrition and dietary changes [33, 39, 46, 68, 69]. Variable results have been reported for antibiotic-induced responses in HSI depending on fish species, type of antibiotics and duration and dose of antibiotic usage [14, 46, 66, 68].

Effect of the synbiotic diet on intestinal microbiota during antibiotic treatment

Employing a longitudinal approach to assess intestinal microbiota through digesta collection via stripping enabled us to monitor the microbiota changes in same group of fish subjected to different dietary and antibiotic treatments, as well as during the subsequent recovery period. This approach could also have minimized inter-individual variations that otherwise could occur when sampling different group of fish at different sampling points.

Synbiotic intervention did not increase alpha diversity after antibiotic treatment or after 5 weeks of recovery period, in contrast to most reports on mammalian studies [17]. It remained relatively unchanged after 2 weeks of antibiotic treatment and decreased during the 5 weeks of recovery period in the fish fed the synbiotic diet. However, alpha diversity tended to increase in fish fed the control diet, during antibiotic treatment similar to the observation made for rainbow trout [55]. Synbiotic fed fish also showed lower alpha diversity compared to the fish fed the control diet after the recovery period. Continuous feeding of probiotic bacteria, *P. acidilactici* in combination with FOS increased the relative abundance of *P. acidilactici* in the gut, similar to observations in previous studies [1, 15]. Further, possible production of bacteriocins and pediocins by *P. acidilactici*, which can exert antagonistic effects towards a variety of bacteria, as reported previously [54, 57], may have influenced other bacterial taxa leading to the establishment of a less diverse microbial population in synbiotic diet fed fish along the trial period.

As evident from beta diversity analysis based on Bray–Curtis index, in fish fed synbiotic diet, microbiota before (S1) and after (S2) antibiotic treatment clustered together whereas in those fed control diet, they were clustered separately. This suggests a potential influence of the synbiotic diet in maintaining the intestinal microbial composition during the antibiotic treatment. The absence of similar separation in the nonmedicated control fish group, indicates that the observed pattern in microbial composition was related to antibiotic treatment. On the

other hand, the sustained pattern in microbial composition both in medicated and nonmedicated synbiotic group of fish could specifically be linked to the influence of the synbiotic diet rather than a general effect present across all dietary conditions. The fish fed the synbiotic diet had a distinct taxonomic composition with higher proportion of *Pediococcus* and *Lactobacillus* comprising around 66% of total abundance and they were relatively stable during the antibiotic treatment. On the other hand, in the fish fed control diet, *Lactobacillus* dominated (53%) and was also quite stable during the antibiotic treatment. Further, those fish had very minute levels of *Pediococcus*, 3% before and 0.1% after the antibiotic treatment. Therefore, the stability of highly abundant and favorable microbiota, *Pediococcus* [49] could possibly aid in maintaining the overall microbial composition in the synbiotic group upon antibiotic treatment.

The dispersion of microbiota from both diet groups after the 5-week recovery period, including the distinction in the S4 microbial composition among nonmedicated fish, suggests a divergence possibly influenced by environmental factors probably from the fluctuations in open sea rearing system. For fish receiving medication, this shift might also be associated with a delayed effect stemming from the antibiotic treatment, indicating a complex interplay between external environmental influences and the lasting impact of prior treatments on the gut microbiota composition. This effect seemed to be mainly caused by the large increase in *Pediococcus* (15%) and *Aliivibrio* (27%) respectively in the fish fed control and synbiotic diets as well as the reduction in *Lactobacillus* in both and alterations in the abundance of other genera changing the respective bacterial compositions.

Effect of the synbiotic diet on gut immune responses during antibiotic treatment

Florfenicol treatment (S1 to S2 time period) did not induce significant alterations in expression of pro- or anti-inflammatory cytokines, $CD3^+$ and $CD8^+$ T-cell markers or other immune- antioxidant- or stress-response gene markers analyzed in this study, either in fish fed the control or the synbiotic diet. The effect of antibiotics on immune parameters, including immune gene expression, can exhibit considerable variability depending on different host related factors and duration of the treatment, as reported for higher vertebrates [74] as well as for fish [24, 76]. For instance, oxytetracycline treatment for 6 weeks increased pro-inflammatory cytokines, but did not affect anti-inflammatory cytokines in zebrafish [76], while the same antibiotic treatment for 7 or 21 days did not modulate immune gene expression in turbot, *Scophthalmus maximus* L. [24]. Studies conducted on mice reported that antibiotic treatments up to 7 to 14 days increased immune gene expressions [40, 65] along with induction of microbial dysbiosis and intestinal inflammation. Although some alterations in microbial composition were observed in the present study, the persistent presence of predominant microbial genera such as *Lactobacillus* in the control group and *Lactobacillus* and *Pediococcus* in the synbiotic group, coupled with the absence of significant morphological signs of inflammation following florfenicol treatment (rather it has pacified inflammatory signs observed after pre-feeding), suggest that the impact of florfenicol on the Atlantic salmon intestine was relatively moderate and different from higher vertebrates.

Even though not significant, gene expression patterns were different in fish fed control and synbiotic diets. Expression of pro- and anti-inflammatory cytokines showed an increasing trend in control fed fish while it was constant or slightly reduced in synbiotic fed fish, upon antibiotic treatment. Also, cytokine expression levels in synbiotic group were lower compared to those of the control group. In the synbiotic group, expression levels of cytokines tended to increase after the 3 weeks of recovery period (S3). These differential expression patterns may be attributed to immune modulatory effects of the *P. acidilactici* and FOS combination in the synbiotic diet. Possible immune modulation by the synbiotic diet was also suggested for the groups of fish not subjected to antibiotic treatment. i.e., the fish fed the synbiotic diet showed elevated expression levels for most of the genes at S2 and S4 sampling time points which were also higher compared to the fish fed control diet. The observed modulation of immune responses raises questions about whether it signifies an enhancement in intestinal mucosal immune function or is simply indicative of the normal adaptation to dietary components. Further studies are needed to clarify this aspect and provide a deeper understanding.

Concomitant use of synbiotics supplementation along with antibiotic treatment reduced expression of pro-inflammatory cytokines by 2 weeks post antibiotic treatment and increased expression of the anti-inflammatory cytokine *il10*, 3 weeks post antibiotic treatment in mice, somewhat representing our study [27]. Most mammalian studies have applied probiotic or synbiotic interventions post antibiotic treatment, and have demonstrated positive impact on restoring microbial balance, mitigating gut barrier disruption, and reducing inflammation through immune modulation, along with alterations in nutrient, short-chain fatty acid, and bile acid metabolism [40, 44, 63]. In contrast to the significant changes induced by antibiotics in higher vertebrates, the findings of this study in Atlantic salmon revealed only moderate changes in performance, gut microbiota and health after 2 weeks of

florfenicol treatment. This could be attributed partly to the higher adaptivity of Atlantic salmon intestinal microbiota, which appears resilient to various dietary and environmental challenges without significantly impacting host health and performance, as reported in several studies [15, 34, 38, 70].

Conclusions

This study highlighted the negative impact of a two-week florfenicol treatment on feed intake and growth performance in Atlantic salmon with moderate influence on gut microbiota and gene expression. Concomitant use of a synbiotic diet containing FOS and *P. acidilactici* helped to maintain the gut microbial composition to some extent and affected the performance positively during the antibiotic treatment. The synbiotic diet influenced immune gene expressions differently than the standard commercial diet during antibiotic treatment and subsequent recovery. This study indicates the importance of nutritional interventions through synbiotic supplementation as a possible strategy for managing Atlantic salmon during antibiotic treatment.

Materials and methods

Experimental design, study variables and analytical procedures used to evaluate the effect of florfenicol in Atlantic salmon subjected to nutritional management through a synbiotic strategy are illustrated in the Fig. 1 and explained in the subsequent sections.

Feeding trial

A sea water feeding trial was conducted with post-smolt Atlantic salmon at AquaInnovo S.A., Chile (Aquaculture Technology Centre Patagonia) from 06/03/2019 to 04/06/2019, following the laws and guidelines from Chilean authority (SERNAPESCA) regulating the experimentation with live animals.

Three weeks before the start of the feeding trial, 970 Atlantic salmon were pit-tagged and acclimatized in fresh water until they were transferred to seawater at the start of the feeding trial. Pit-tagged fish were randomly assigned to 10 tanks with 97 fish each. Eight fish per tank were randomly assigned (adipose fin clip was used for further identification) and registered for faecal stripping for microbiota analysis throughout the experiment. The fish had an average initial weight of 108.6 ± S.E.M. 0.2 g. Feeds were formulated and produced by Biomar AS. A control diet (Ctr) was prepared based on standard grower feed recipes, and the synbioitic diet (Syn) was prepared by supplementing the control diet with fructo-oligosaccharide (FOS, 0.1%) and *P. acidilactici* (Bactocell, 0.03%), as nonmedicated diets. Composition of the control basal diet is presented in Table 3. Medicated feeds were prepared by coating respective control and synbiotic diets with 3500 ppm florfenicol in oil (CtrMed and SynMed diets respectively), according to the recommend dose of 20–40 mg /kg of BW for salmon with salmonid rickettsial septicemia [61]. Five tanks were allocated for each of control and synbiotic diets randomly distributed among the tanks. Fish were at first fed Ctr and Syn diets for 6 weeks pre-feeding period. Then the fish in three tanks from each of Ctr and Syn treatments were fed with respective medicated diets for a period of 2 weeks (medicated group, Med). After that, the fish were returned to the respective Ctr and Syn diets for a recovery period of 5 weeks. Fish in the remaining two tanks in each treatment were fed the respective control or synbiotic diets throughout the experimental period (NonMedicated group, NonMed). Samples were collected after the pre-feeding period (S1), after antibiotic treatment (S2), after 3 weeks (S3) and 5 weeks (S4) of recovery period, both from the medicated and nonmedicated groups. During the feeding trial period, seawater temperature averaged 12.3 ± 0.01 ℃, salinity of 31.7 ± 0.02‰ and oxygen of 88.5 ± 0.17 mg/L.

Percentage specific feeding rate (SFR) was calculated using the amount of feed intake per day and the weight of the fish in each tank during trial period. Performance of fish were evaluated based on SGR and FCR calculated using the standard method as detailed in our previous publications [33, 34]. SGR was analyzed based on the

Table 3 Composition of the control diet

Ingredients*	Composition %
Fish meal	15.00
Soy HP 46	5.00
Plant RM	36.90
Land animal protein	13.40
Wheat	9.00
Fish oil	5.60
Rapeseed oil	12.00
Vita-mineral premix	3.25
Antioxidants	0.47
Analyzed nutrients	
Moisture (%)	6.00
Energy—DE Salmon Std (MJ/kg)	19.40
Protein—crude (%)	44.86
Protein—DP Salmon (%)	39.01
Fat—crude (%)	23.73
Ash (%)	6.24

* The synbiotic diet was prepared by supplementing the control diet with FOS, 0.1% and Bactocell 0.03% to the basal diet

* Medicated feeds were prepared by coating respective control and synbiotic diets with 3500 ppm florfenicol in oil

DE, digestible energy; DP, digestible protein

weight gain of the 97 fish per tank, including n = 194 fish from nonmedicated group (randomly allocated 2 tanks each per control and synbiotic diets) and n = 291 fish form medicated group (randomly allocated 3 tanks each per control and synbiotic diets) at S1. At S2, n = 133 and 138 fish per Ctr and Syn diets (nonmedicated group), and n = 192 and 201 fish per CtrMed and SynMed diets (medicated group) were used removing outliers. At S3, n = 89 and 91 fish per Ctr and Syn diets (nonmedicated group), and n = 133 and 136 fish per CtrMed and SynMed diets (medicated group) were used removing the outliers. FCR was calculated per tank based on the increase in biomass and the consumption of feed.

Relative weight of liver (HSI) was calculated as HSI = (liver weight/body weigh) × 100 using 10 fish per tank, including n = 20 fish form nonmedicated group (2 tanks each per control and synbiotic diets) and n = 30 fish form medicated group (3 tanks each per control and synbiotic diets) at the time points S1, S2 and S3.

Samples were collected for histological evaluation and gene expression analysis from randomly sampled fish (number of fish used for respective analysis is given under each analysis). Digesta samples were collected by gentle fecal stripping from same set of pit-tagged fish under anesthesia for microbiota analysis at the S1, S2 and S4 sampling points. The weight and length of the fish were also registered. Distal intestinal tissue samples for both the histological and the gene expression analysis were collected from same sacrificed fish (also used for HSI calculation) at each of the S1, S2, S3, and S4 sampling points. For histological evaluation, tissues were fixed in 10% formalin. Tissue samples for gene expression analysis and digesta samples for microbiota analysis were preserved in RNAlater solution and stored at − 20 °C for further analysis.

Statistical analysis of performance data/HSI was performed using two-way analysis of variance (ANOVA) separately for nonmedicated and medicated groups using sampling time points and diet groups as factors, after checking the fulfillment of all the pertinent assumptions, normality of the distribution and homogeneity of variances. SGR was calculated using the averages of individual fish for diet group at a sampling point; SFR, FCR and HSI were calculated per each tank (n = 2 tanks per nonmedicated and n = 3 tanks per medicated groups). Pairwise comparisons were analyzed using Tukey's honestly significant difference (HSD) test, and $p < 0.05$ was considered as statistically significant.

Histological analysis

The distal intestine tissue sections were evaluated by Laboratorio Pathovet Lt da. Chile. After hematoxylin and eosin staining, tissue sections were assessed for histomorphological changes such as villi atrophy, loss of enterocyte supranuclear vacuolization, hyperemia and lamina propria hemorrhage. Histological evaluation was conducted on samples from 10 fish per tank, including n = 20 fish form nonmedicated group (2 tanks each per control and synbiotic diets) and n = 30 fish form medicated group (3 tanks each per control and synbiotic diets) at the time points S1, S2, S4. A lower number of fish were analyzed after the 3 weeks of recovery period (S3) including half of the above-mentioned numbers i.e., n = 10 per nonmedicated group and n = 15 per medicated group.

The degree of changes was graded using a scoring system with a scale of normal (0), moderate (1), marked (2) and severe (3). The percentage of fish belonging to each score was presented from the total number of fish examined. The histological evaluation was conducted randomly and blind, and assignment of individual samples to the treatments was obtained after the evaluation was completed. Statistical analysis of histological changes among the samples were analyzed for the nonmedicated and medicated groups separately using fifer package and extended packages in R. Both the fisher extract and chi-square test were done to find the significance among the feed groups, after converting histological scores into ordinal variables. Pairwise comparisons were analyzed using chi-square post hoc tests.

Microbiota analysis

DNA extraction

For analysis of the distal intestinal microbiota, digesta samples collected by stripping from pit-tagged fish were used. Digesta collected from same group of fish (n = 6) including n = 2 fish from each of the 3 tanks subjected to antibiotic treatment (medicated group) and n = 3 fish from each of the 2 tanks that were not subjected to antibiotic treatment (nonmedicated group) from both the control and synbiotic diet fed fish, were used. This enabled us to monitor the intestinal microbiota of the same group of individuals throughout the experiment; after pre-feeding period (S1), after antibiotic treatment (S2), and after 5 weeks of recovery period (S4). The intestinal microbiota of the fish which were not subjected to antibiotic treatment (nonmedicated group) were also analyzed at the same sampling time points. The DNA was extracted from respective digesta samples following the protocol of QIAamp Fast DNA Stool Kit (Qiagen, Crawley, UK) with some modifications as suggested by Knudsen et al. [31]. Samples were pre-processed with a bead-beating protocol of three times in the Fastprep at 6.5 m/s for 30 s with a mix of beads (120 mg acid-washed glass beads (150–212 µm) and 240 mg Zirconium oxide beads (1.4 mm). For quality control of the microbiota profiling protocol, along with the each of the DNA extraction batch, two

'blanks' (without any sampling materials) and two 'positive controls' i.e., mock (microbial community standard from Zymo-BIOMICS™, Zymo Research, California, USA) were included.

PCR amplification of V1-V2 region of the 16S rRNA gene

PCR amplification was carried out using 27F (5′ AGA GTTTGATCMTGGCTCAG 3′), and 338R-I (5′ GCW GCC TCCCGTAGGAGT 3′) and 338R-II (5′ GCWGCC ACCCGTAGGTGT 3′) to have about 300 bp amplicons according to the procedure established previously [15, 21]. PCRs were carried out in 25 µL reactions with 12.5 µL of Phusion® HighFidelity PCR Master Mix (Thermo Scientific, CA, USA); 1 µM of forward and reverse primers, and 1 µl template DNA. The PCR conditions were as follows: initial denaturation at 98 ℃ for 3 min followed by initial 10 cycles with denaturation at 98 ℃ for 30 s, annealing temperature decreasing from 63 ℃ to 53 ℃ for 30 s at each temperature and extension at 72 for 30 s; followed by 25 further cycles with denaturation at 98 ℃ for 30 s, annealing at 53 ℃ for 30 s, and extension at 72 ℃ for 30 s; followed by a final extension at 72 ℃ for 10 min. Negative PCR controls were included by replacing the template DNA with molecular grade water. PCR was performed in duplicate, pooled, and examined by 1.5% agarose gel electrophoresis.

Library preparation and sequencing

Library preparation of the products from amplicon PCR was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research) following the instructions from the producer and as described previously [15]. Briefly, PCR products were first enzymatically cleaned up followed by a PCR to add barcodes. Subsequently, the libraries were quantified by qPCR, pooled, and purified. A representative number of individual libraries were evaluated for DNA quality in Agilent Bioanalyzer 2100 system (Agilent Technologies, California, USA). The final pooled library was then denatured and diluted to 8 pM and sequenced on Illumina MiSeq platform with Miseq Reagent Kit v3 (600-cycle) (Illumina) to generate paired-end reads. 15% of PhiX control was added as an internal control.

Bioinformatics analysis of microbiota sequencing data

This was performed using QIIME2 version 2 [6, 11] as previously described [15]. The demultiplexed paired-ended reads were denoised, trimmed and quality filtered using the DADA2 algorithm [10] in QIIME2. Primer sequences were trimmed off (forward reads, first 20bps; reverse reads, first 18bps) and the reads were truncated at the position where the median Phred quality crashed (forward reads, at position 295 bp; reverse reads, at position 239 bp) and low-quality reads were filtered out. After merging the reads, chimeric sequences were removed. The taxonomy was assigned to ASVs table by a Scikitlearn Naive Bayes machine-learning classifier [5], after it was trained on the SILVA 132 99% ASVs [58] by trimming exclusively to include the regions of 16S rRNA gene amplified by the primers used in the current study. ASVs table was first filtered to remove ASVs assigned as chloroplast and mitochondria and then to remove ASVs that were without a phylum-level taxonomic assignment or appeared in only one biological sample. Low abundance ASVs with total abundance of less than 2 across all the samples were also filtered out. In total 11 ASVs were removed from digesta samples based on their presence in mocks, extraction blanks and negative PCR controls, and their negative correlation with bacterial DNA concentration obtained from qPCR analysis as described previously [15, 38]. The 11 ASVs removed from digesta samples belonged to several genera of family *Burkholderiaceae* including *Acidovorax* (1 ASV), *Curvibacter* (1 ASV), *Delftia* (1 ASV), *Pelomonas* (1 ASV), and *Schlegelella* (1 ASV), and several other genera *Cutibacterium* (1 ASV), *Brevibacillus* (1 ASV), *Candidatus* (1 ASV), *Bradyrhizobium* (1 ASV), *Litoreibacter* (1 ASV) and *Candidatus Berkiella* (1 ASV). After filtering, a total number of 5 654 ASVs were obtained. The ASVs filtered from the raw ASVs table were also removed from the representative sequences.

Further downstream analysis was performed separately for the digesta samples collected from the fish subjected to antibiotic treatment (medicated group, Med) and those were not subjected to antibiotic treatment (nonmedicated, NonMed). To compute alpha and beta diversity indices, the ASV table was rarified at 33 815 and 32137 reads respectively for Med and NonMed groups, in order to have an even number of reads across all the samples. Alpha diversity was calculated using observed species and Shannon`s diversity indices at ASV level using q2-diversity plugin in Qiime2. Alpha diversity data generated in qiime2 was used in R for further analysis using linear mixed effect model, LME with lme4 package [3]. Predictor variables in the model includes diet groups (Ctr and Syn in NonMed, CtrMed and SynMed in Med), sampling time (S1, S2, and S4) and their interaction, and the random effect, fish id, to account for repeated sampling from the same pit-tagged fish and tank id to account for tank effect. When the random factors explained zero variation they were excluded from the final model. For example, in Med group, fish id was excluded and NonMed group tank id was excluded. The homoscedasticity and normality of model residuals were visually assessed by inspecting diagnostic plots generated by the R package ggResidpanel [23]. The statistical significance of fixed predictors was estimated with emmeans package

[35] using LME model object and further confirmed by analyzing the statistical significance of fixed predictors with Type III ANOVA with Satterthwaite's method using anova () function in R. Statistically significant differences were calculated by specifying conditional contrast for the diet group (differences between the diet group at a sampling time point) and sampling time point (difference between the sampling time points in a diet group) with emmeans package using LME model object. Kenward-Roger approximation procedure was used as degree of freedom method and Holm-Bonferroni method was used as p-value adjustment method. Differences were considered significant when the $p < 0.05$.

Beta diversity was evaluated using Bray–Curtis and weighted Unifrac diversity matrices at ASV level. Beta diversity distance matrix files generated in qiime2 was used in vegan package [51] in R to perform PERMANOVA test with 1000 permutations (adonis2 function). Effect of diet group, sampling time point and their interaction were evaluated including fish id as a blocking factor (strata) to control sampling from the same individuals. As the order of variables matter in adonis function, we used the order of variables, first diet, then sampling time, for both the analysis. Principal coordinate ordination analysis, PCoA of the beta-diversity indices was performed using MicrobiomeAnalyst package [13, 16]. Multiple pairwise comparisons among all the 15 possible comparisons (2 types of diets, control and synbiotic, and 3 sampling points, S1, S2 and S4 were included in each of the Med and NonMed groups separately) were analyzed using pairwiseAdonis package [43] using fish id as a blocking factor (strata). Differences were considered significant when the $p < 0.05$.

The abundant taxa among the treatments, and graphical presentations of the data was performed using the MicrobiomeAnalyst package.

Gene expression analysis

Gene expression analysis was performed using the distal intestine samples collected at S1, S2, S3 and S4 sampling time points. n = 6 samples were used from Ctr, Syn, CtrMed and SynMed diets at each sampling time points (S1, S2, S3, and S4) consisting of total of 96 samples per gene. Gene expression profiling was carried out using the real time PCR (qPCR) assays following the MIQE guidelines [8]. Total RNA was extracted using Precellys homogenizer homogenizer, Trizol® reagent and further purified with PureLink (Thermo Fisher Scientific) with an on-column DNase treatment. RNA integrity was checked by 1% agarose gel electrophoresis (Agilent Technologies, Santa Clara, USA), and RNA quantity and RNA purity were measured using both Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA) and NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Synthesis of cDNA was performed using 1.0 µg total RNA from all the samples using a Superscript™ IV VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) and obtained cDNA was diluted 1:10 before used for qPCR assay.

The qPCR assays were performed using the LightCycler 96 (Roche Applied Science, Basel, Switzerland). 10-µL reaction volume including 2 µL of PCR-grade water, 2 µL diluted cDNA template, 5 µL LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) and 0.5 µL (10 µM) of each forward and reverse primer. Samples were run in duplicates along with a no-reverse-transcription control and a no-template control for each gene. qPCR program include an enzyme activation step at 95 ℃ for 5 min, and 40 cycles of 95 ℃ for 10 s, 55–63 ℃ (depending on the gene) for 10 s and 72 ℃ for 15 s, and a melting curve analysis at the end. Quantification cycle (Cq) values were determined using the second derivative method. Three reference genes including glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase II (*rnapoli*), and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) were selected based on to their stability across and within the treatments as described by Kortner et al. [32]. The expression of target genes was normalized to the geometric mean of the 3 reference genes. The mean normalized expression of the target genes was calculated from raw Cq values. The immune related genes analyzed in the study were selected from the gene panel used in our group, and the primer pair sequences, efficiency, amplicon size and annealing temperature for the selected genes can be found in the publication of Li et al. [37]. Primers for *lysozyme C II* were obtained from Yada et al. [73].

LME with lme4 package was used to analyze gene expression changes separately in medicated (Med) and nonmedicated (NonMed) groups. Predictor variables in the model includes diet (Ctr, Syn in NonMed, CtrMed and SynMed in Med), sampling time point (S1, S2, and S3 and S4) and their interaction, and the random effect, tank id to account for tank effect. The homoscedasticity and normality of model residuals were visually assessed by inspecting diagnostic plots generated by the R package ggResidpanel and log transformed gene expression values were used most of the time. The statistical significance of fixed predictors was estimated with emmeans package using LME model object and further confirmed by analyzing the statistical significance of fixed predictors by Type III ANOVA with Satterthwaite's method using anova () function in R. Statistically significant differences were calculated by specifying conditional contrast for the diet group (differences between diets at a sampling time point) and sampling time point (differences between the

sampling time points in a diet) with emmeans package using LME model object. Kenward-Roger approximation procedure was used as degree of freedom method and Holm-Bonferroni method was used as p-value adjustment method. Differences were considered significant when the $p < 0.05$. The gene expression plots were generated using R packages, ggplot2 and the extension packages (http://www.cookbook-r.com/Graphs/).

Abbreviations

FOS	Fructo-oligosaccharides
SGR	Specific growth rate
SFR	Specific feeding rate
FCR	Feed conversation ratio
HIS	Hepatosomatic index
ASV	Amplicon sequence variant
LME	Linear mixed effect model
SEM	Standard error of mean
DF	Degree of freedom
F	F-ratio
P	P value
BW	Body weight
UniFrac	Unique fraction
PCoA	Principal coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance
R^2	Eta-squared
il1β	Interleukin 1β
il17a	Interleukin 17a
il8	Interleukin 8
ifnγ	Interferon γ
il10	Interleukin 10
tgfβ	Transforming growth factor β
cd3γδ	Cluster of differentiation 3 γδ
cd8β	Cluster of differentiation 8β
myd88	Myeloid differentiation primary response gene 88
mhc1	Major histocompatibility complex, class 1
muc2	Mucin 2
sod1	Superoxide dismutase 1
hsp70	Heat shock protein 70
cat	Catalase
DE	Digestible energy
DP	Digestible protein
ANOVA	Analysis of variance
HSD	Honestly significant difference
gapdh	Glyceraldehyde-3-phosphate dehydrogenase
rnapoli	RNA polymerase II
hprt1	Hypoxanthine phosphoribosyltransferase 1
SRA	Sequence read archive

Supplementary Information

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Supplementary material 1. Figure S1. The hepatosomatic index (HSI). Figure 1a: Results for fish not subjected to antibiotic treatment (NonMed). Figure 1b: results for medicated fish (Med). HSI presented as an average per tank. Error bars represent S.E.M. Fish were fed either a commercial control diet or a synbiotic diet for 6 weeks pre-feeding period (S1) and fish in 3 out of 5 tanks from each treatment were fed with medicated diet coated with florfenicol for 2 weeks (S2). Then those fish were refed with respective control and synbiotic diets for 3 (S3) weeks of recovery period. The fish not subjected to antibiotic treatment were fed with control or synbiotic diets throughout the experimental period and sampled at the same sampling time points as medicated group. See the materials and methods section for number of fish used per group at each time points. Different letters among values indicate statistically significant differences ($p < 0.05$) in each treatment among the sampling points. Values sharing the same letters are not statistically significant. Any significant differences between the diet groups at a sampling point are reported in the text.

Supplementary material 2. Table S1: LME analysis of the relationship between relative gene expression and diet, sampling time, and their interaction.

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Author contributions
Project leadership and management: TMK and TF. Experiment design: TMK and TF. Bioinformatics analyses: AD and YL. Data interpretation and writing of the original draft of the manuscript: AD. Manuscript development: AD, YL, ÅK, TF and TMK. All the authors have read, revised, and approved the manuscript.

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Availability of data and materials
16S rRNA sequencing data are publicly available at the NCBI Sequence Read Archive (SRA), under the Bioproject PRJNA679207.

Declarations

Ethics approval and consent to participate
All experiments involving Atlantic salmon were conducted in agreement with the guidelines from SERNAPESCA, Chile.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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