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Involvement of dietary salt in shaping bacterial communities in European sea bass (*Dicentrarchus labrax*)

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Bacteria associated with the digestive tract of multicellular organisms have been shown to play a major role in their hosts' functioning. In fish, it has been proposed that food fermentation occurs inside the pyloric ceca, pouch like organs found in their digestive tract. However, this notion remains controversial. Furthermore, changes in pyloric cecal bacterial populations under different diets have yet to be demonstrated in fish. In this study, we explore the changes occurring in the bacterial community residing in the pyloric ceca of carnivorous fish fed different diets, which were shown to induce different growth rates. Our results revealed that different diets do indeed induce distinct bacterial compositions within the pyloric ceca. We found that, when salt was added to a low fish meal diet, the bacterial changes were accompanied by a significant enhancement in weight gain, hinting at a possible involvement of the bacterial community in energy harvest.

Pyloric ceca are finger-like pouches attached to the proximal intestine of many fish species. They are quite different from their counterparts in birds and mammals with respect to location, presence or absence, and numbers¹. In 345 B.C., Aristotle described these appendages and suggested three hypotheses for their function: "to store up the food," "to putrify it up," or "to concoct it" (i.e. storage, fermentation, and absorption)². These hypotheses were revisited by Buddington and Diamond^{3,4}, and the functions of enzymatic digestion and absorption within fish pyloric ceca were confirmed. However, these authors rejected the notion of a storage function because of the relatively short food-retention time. Buddington and Diamond also rejected the fermentative function based on another study reporting a lack of specific resident microflora in the cecum⁵. However, a subsequent microscopy study showed the existence of bacteria at the tips of the microvilli as well as between adjacent microvilli in the pyloric ceca of Arctic charr (*Salvelinus alpinus* L.)⁶. More recently, it was revealed that pyloric ceca, stomach and intestine have similar bacterial compositions in juvenile farmed Atlantic salmon⁷. However, whether the ceca in fish have a fermentative function linked to their associated microbiota remains unknown.

The relationship between diet and cecum size/number in fish also differs from that found in mammals and birds. In the latter, ceca are small in carnivores and relatively larger in herbivores and frugivores which have a high-fiber diet suitable for fermentation in that region⁸. However, ceca tend to be better developed in carnivorous fish⁹, leading to the conclusion that ceca play a more important role in these organisms. In mammals, gut morphology has a strong impact on bacterial community composition, and bacterial diversity is influenced by host diet, increasing from carnivorous to omnivorous to herbivorous diets¹⁰. Therefore, we sought to characterize cecal bacterial communities in carnivorous fish during the course of a feeding trial with diets containing different macronutrient contents.

In freshwater-adapted fish, the passive outward flux of ions such as Na⁺ and Cl⁻ from the fish to the external medium must be overcome by active uptake of ions (e.g. Na⁺, Cl⁻, K⁺, and Ca²⁺) from the water and/or from the diet¹¹. A large percentage of the dietary salt in commercial feeds for carnivorous fish originates from the fish meal component of the diet¹². Some studies have shown that dietary salt supplementation has a positive effect on fish growth performance under freshwater conditions¹³⁻¹⁷. For example, adding salt to the diet of Asian sea bass *Lates calcarifer* at a level of up to 4% led to better feed utilization coupled with lower feed costs¹⁶. A diet without fish meal but supplemented with 3% NaCl significantly increased the specific growth rate and feed conversion ratio of



Table 1 | Composition and salt content of the diets and corresponding body weight gain after a 6-week feeding trial

	Control	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	(HF)	(HF)	(HF)	(MF)	(LF)	(LF + 4% NaCl)
Fish meal (%)	50	50	50	30	10	10
Fat (%)	16	18	20	16	16	16
NaCl (%)	—	—	—	—	—	3
Protein (%)	45	45	45	45	45	45
Fiber (%)	1.8	1.7	1.7	1.8	2.3	2.1
Salt content (%)	2.77	3.35	3.57	2.40	1.71	3.98
Specific growth rate (%)	2.06 ± 0.12 ^a	1.99 ± 0.06 ^a	2.10 ± 0.10 ^a	1.51 ± 0.03 ^b	0.89 ± 0.07 ^c	1.40 ± 0.02 ^b

Values of body weight gain are expressed as mean ± SD. Groups not sharing a superscript letter within the row indicate significant differences in values ($P < 0.05$, by Tukey's test). The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).

a tilapia hybrid¹³. The regulatory mechanism of dietary salt supplementation involves digestive enzyme activities, such as alkaline phosphatase, lactase, and leucine amino acid peptidase in the pyloric caeca and plasma osmolality¹⁶. However, we still do not know how dietary salt addition affects the intestinal bacterial community residing in fish.

In the present study, ARISA (automated ribosomal intergenic spacer analysis), a molecular ribotype technique, was applied to investigate the cecal microbiota of carnivorous fish using European sea bass (*Dicentrarchus labrax*) as the model. Being euryhaline, this species can withstand a wide range of salinities. They are found in the littoral zone and occasionally in rivers. Their distribution is the Mediterranean as well as the Eastern Atlantic: the coast from Norway to Morocco and Senegal.

They feed mainly on shrimps, mollusks, and fishes. Juveniles feed on invertebrates, taking increasingly more fish with age while adults are piscivorous. Since under natural conditions they consume fish, their natural diet has a low level of salt - probably around 0.5%¹⁸.

The goal of the present study was to explore the effects of dietary salt supplementation in a low fishmeal diet as well as various levels of fat and fish meal in the diet on the cecal bacterial community during the course of a feeding trial conducted with *D. labrax* kept in freshwater.

Results

Effect of the experimental diets on growth. The experimental diets differed in their contents of fish meal or fat, but they contained either the same level of fish meal or the same level of fat as the control diet (Table 1). Survival was around 95% with no significant differences among groups. Sea bass fed the 10% fish meal diet (no. 4) showed the lowest average body weight gain (Table 1). However, diet no. 5, which had the exact same composition as no. 4 but with an addition of 3% NaCl, significantly enhanced weight gain, with values comparable to that of fish fed a 30% fish meal diet (no. 3). No clear trend was observed between the increase in fat content and changes in weight gain (Table 1).

Changes in cecal bacterial communities in the different dietary groups over time. To explore the dynamic change in cecal bacterial communities over time in the different dietary groups, we assessed the similarity of the bacterial communities in each dietary group at each sampling point to its previous time point (Fig. 1). All of the groups also exhibited a change over time compared to the original community (Fig. S1). This was true also for dietary group no. 1, which was fed the control diet during the period of acclimatization and in the subsequent feeding trial. The bacterial community of groups (control, diet 1, diet 2) that were fed 50% fish meal, stabilized after two weeks, as exhibited by the increase in similarity at the final time points, indicating what is considered a more moderate change in the bacterial community. These were close to the typical similarity values found between the bacterial communities of fish

within the same group or time point, averaging around 0.6 (Table 2), as seen in the similarity values of each diet in the 6 week time point, and suggested in other studies examining fish gut microbial communities¹⁹. On the other hand, the groups fed a lower fish meal level diet (diet 3, 4, & 5) continued to significantly change even 6 weeks after the beginning of the experiment as revealed by their low similarity to the previous time points (Fig. 1). The number of OTUs remained within the same range in all dietary groups except for group no. 4, which showed a significant gradual increase and group no. 1, which showed a significant decrease over

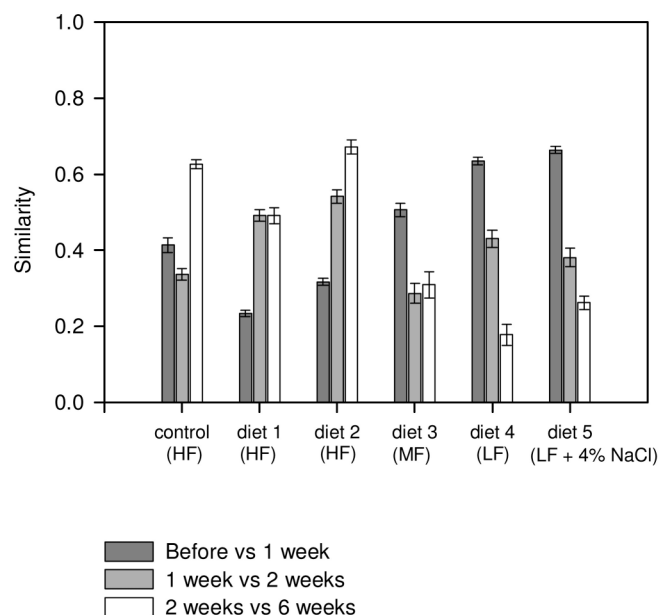


Figure 1 | Changes in bacterial community throughout the experiment.

The bacterial community of samples from each time point of each diet was analyzed and compared to its previous time point to assess the degree of change/similarity that occurred between the time points. The darkest bars represent the average similarity comparison between the microbiota found before the beginning of the experiment (during the acclimation period) to 1 week after the beginning of the experiment. The medium grey bars represent the average similarity comparison between the microbiota 2 weeks after the beginning of the experiment to the week before (week 1). The white bars represent the community comparison between the 6 weeks and 2 weeks samples. The similarity between the different time points was obtained using the Bray-Curtis index. Values closer to 0 indicate low similarity, meaning high degree of difference in bacterial composition between the two time point while values closer to 1, which denote a higher similarity, implying that little change occurred between two time points. The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).



Table 2 | Bray-Curtis similarity values between the microbial community of each group 6 weeks into the experiment with Bonferroni-corrected *P*-values

	Control (HF)	diet 1 (HF)	diet 2 (HF)	diet 3 (MF)	diet 4 (LF)	diet 5 (LF + 4% NaCl)
control	0.626852	<i>2.30E-05</i>	<i>8.40E-14</i>	<i>< 2e-16</i>	<i>< 2e-16</i>	<i>< 2e-16</i>
diet 1	0.532747	0.665898	<i>0.0004</i>	<i>1.40E-12</i>	<i>< 2e-16</i>	<i>< 2e-16</i>
diet 2	0.471354	0.438428	0.642312	<i>0.0023</i>	<i>< 2e-16</i>	<i>1.20E-10</i>
diet 3	0.416285	0.507282	0.224025	0.603222	<i>< 2e-16</i>	<i>0.0504</i>
diet 4	0.054754	0.048877	0.070174	0.063257	0.438433	<i>< 2e-16</i>
diet 5	0.37491	0.30976	0.360032	0.260121	0.087737	0.514951

The lower left corner of the table, in bold, denotes the *R* values representing the similarity between the groups, with values close to 0 denoting high difference between the groups and values close to 1 indicating that the groups are more similar in terms of bacterial composition. The upper right corner shows the respective *P*-values of each comparison (significant values are shown in italics). The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).

the course of the experiment (Table 3). When the Shannon diversity index was measured, only diets 4 and 5 exhibited significant changes over time, with a trend toward increased diversity (Table 4).

Effects of the diets on cecal bacterial communities. We performed a sample accumulation curve for each dietary group at the 6 week time point of the experiment. This test produced saturated curves, indicating an adequate OTU coverage for each groups (Fig. 2). The analysis also revealed that a significantly higher number of total possible OTUs existed for diets 4 and 5, which contained 10% fish meal complemented with plant protein and had a higher fiber content than the other diets (Table 1), coinciding with the higher diversity and OTU number per samples seen in these groups (Tables 3 and 4).

We analyzed the bacterial communities using NMDS plots at the final time point of the experiment based on the OTU profile generated by the ARISA (Fig. 3). Cecal bacterial communities seemed to cluster together according to the diets, as seen in the NMDS analysis (Fig. 3) and similarity values showing significant differences between the groups (Table 2). Diet 4 exhibited the biggest distance with all the other groups both in the NMDS and ANOSIM. This clustering could not be biased by the bacteria residing in the water or biofilters as each group was assigned to different tanks and biofilters, and ANOSIM did not reveal any clustering of samples according to biofilter (Table 5).

Although diets 4 and 5 had the same contents of fish meal and fat and differed only in the supplementation of 3% NaCl, they clustered significantly differently from each other. The bacterial community of the diet containing only 10% fish meal and no salt (no. 4) was significantly different from the community found in samples from diet no. 5, which clustered closer together with all of the other diets (Fig. 3).

Effect of dietary ingredients on cecal bacterial communities. The shift in cecal bacterial communities in response to supplementation with 3% NaCl in the low fish meal diet (diet no. 4) suggested that the salt parameter in the diet makes a dominant contribution to the composition of pyloric cecal bacteria. We further explored the reason for the increase in similarity of diet no. 5 to all other diets containing high fish meal content.

We examined the correlation between fat, fish meal, and salt levels in the diets, to the distance of cecal bacterial communities of each

group to the community in the control group. The strongest correlation of the change in similarities was with the contents of dietary salt and fish meal, whereas this correlation was lower for the fat ingredient, yet still statistically significant (Table 6). The correlations between the change in similarity values and the concentration of dietary salt and fish meal were similar. When we examined the salt content of the different diets, we found that they are correlated with the ratio of fish meal in the diet, further more the salt content in diet no. 4 was lowest, whereas the value in diet no. 5 was similar to that in the diets containing 50% fish meal further indicating that the salt originating from the fish meal in the diets has a dominant impact on cecal bacterial communities (Table 1).

We counted the number of shared OTUs at the final time point of the feeding trial in dietary groups 4 and 5 and all other diets possessing higher fish meal levels and therefore higher salt content (Table 7). Dietary group no. 4 shared only two OTUs with the dietary group no. 2 which contained 50% fish meal and 20% fat. Group no. 5 shared a significantly higher number of OTUs with all of the other groups, regardless of their different dietary contents, except diet no. 4, highlighting the strong impact of salt on the composition of pyloric cecal bacteria.

Discussion

Evidence of a possible involvement of the pyloric cecum in synthesis and oxidation of lipids in rainbow trout has been described, where it was demonstrated that synthesis and oxidation rates fluctuate after feeding²⁰. This, along with the fact that bacteria isolated from fish were also reported to produce various lipids²¹, suggests that cecal microbiota may be partly involved in lipid metabolism in fish. Nevertheless, the question of whether pyloric ceca function in food fermentation remained unresolved²². Here we used a molecular ribotyping technique to determine whether pyloric ceca harbor bacteria responsive to diet, as this could suggest the occurrence of a fermentative process on the ingested food within the ceca. This in turn would imply that this organ may have fermentative role in fish and could subsequently be verified by direct measurements. During the length of the experiment, all of the groups exhibited a change over time, diverging from their starting point, including the control group, which was kept on the same diet before and during the experiment. This indicates that the shift in community composition was not only induced by the diets and is possibly the result of bacterial community

Table 3 | Average number and \pm SD of OTUs in each dietary group during the feeding trial

	Control (HF)	Diet 1 (HF)	Diet 2 (HF)	Diet 3 (MF)	Diet 4 (LF)	Diet 5 (LF + 4% NaCl)
1 week	38.0 \pm 9.7 ^a	44.6 \pm 10.8 ^{ab}	41.8 \pm 4.0 ^a	52.2 \pm 10.8 ^a	51.1 \pm 6.1 ^b	61.3 \pm 11.5 ^a
2 weeks	43.9 \pm 12.1 ^a	59.4 \pm 15.7 ^a	45.5 \pm 18.4 ^a	59.0 \pm 11.5 ^a	48.0 \pm 9.5 ^b	51.3 \pm 12.1 ^a
6 weeks	47.4 \pm 10.1 ^a	40.4 \pm 5.7 ^b	42.8 \pm 3.8 ^a	49.6 \pm 5.8 ^a	78.1 \pm 12.3 ^a	65.1 \pm 18.4 ^a

Different superscript letters within a column (same diet different sampling point) indicate the Bonferroni corrected significant differences between the sampling time points using Tukey's test (*P*-values $<$ 0.05). The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).


Table 4 | Shannon diversity values \pm SD for each dietary group during the feeding trial

	Control (HF)	Diet 1 (HF)	Diet 2 (HF)	Diet 3 (MF)	Diet 4 (LF)	Diet 5 (LF + 4% NaCl)
1 week	2.82 \pm 0.27 ^b	2.87 \pm 0.24 ^{ab}	2.96 \pm 0.11 ^a	3.08 \pm 0.18 ^a	3.03 \pm 0.09 ^b	3.23 \pm 0.17 ^b
2 weeks	2.95 \pm 0.18 ^{ab}	3.17 \pm 0.24 ^a	2.82 \pm 0.33 ^a	3.27 \pm 0.29 ^a	3.10 \pm 0.23 ^b	3.16 \pm 0.34 ^b
6 weeks	3.14 \pm 0.17 ^a	2.87 \pm 0.20 ^b	2.96 \pm 0.11 ^a	3.12 \pm 0.17 ^a	3.53 \pm 0.25 ^a	3.51 \pm 0.22 ^a

Different superscript letters within a column (same diet different sampling point) indicate the Bonferroni corrected significant differences between the sampling time points using Tukey's test (P values $<$ 0.05). The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).

maturation related to fish growth. However, the community might respond to other environmental shifts during the length of the experiment not taken into account by our analyses which might account for the intervariation seen.

Although diets 4 and 5 had the same contents of fish meal and fat and differed only in the supplementation of 3% NaCl, they clustered significantly differently from each other. The bacterial community of the diet containing only 10% fish meal and no salt (no. 4) was significantly different from the community found in samples from diet no. 5, which clustered closer together with all of the other diets (Fig. 3) also seen in their similarity values (Table 2). Hence, supplementation of 3% NaCl to the diet containing 10% fish meal resulted in a bacterial community that was more similar to those found in the fish fed higher levels of fish meal diets (30% and 50%).

We speculated that the dietary ratio of fish meal affects the salt concentration in the diets, as a large percentage of the dietary salt usually originates from the fish meal component of the diet¹². This, in turn, might induce the cecal bacterial communities to be more similar to each other in the salt-enriched and fish meal-containing dietary groups. Indeed, the salt content of the different diets, was highly correlated with the ratio of fish meal in the diet. Thus, the salt content in diet no. 4 was lowest, whereas the value in diet no. 5 was similar to that in the diet containing 50% fish meal (Table 1).

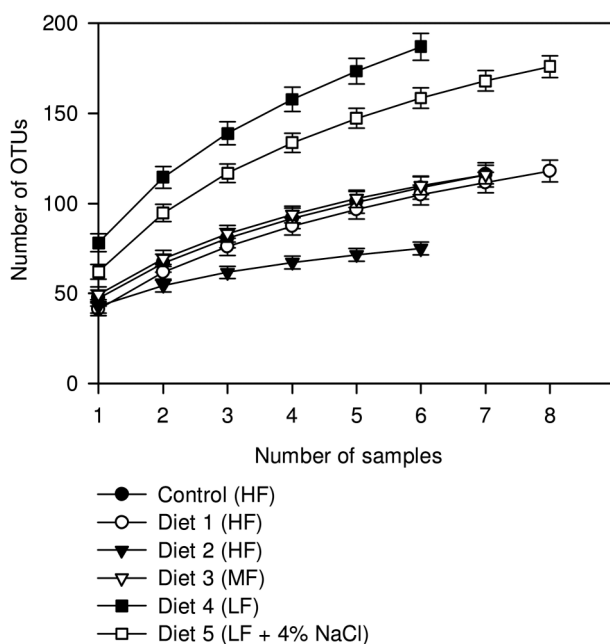


Figure 2 | Rarefaction analysis for each diet at the 6-week time point. Sample-based rarefaction curves show an increase in OTU numbers for each diet as a function of the number of individuals sampled. Each curve represents one dietary group and its corresponding rarefaction plot. The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).

The connection between the dietary salt content resulting from the ratio of fish meal in the diets (Table 1) and the effect on the bacterial community was also obvious in the highly significant correlation between the similarity each of the groups exhibited to the control group and their salt content (Table 6). Furthermore, dietary group no. 5 shared OTUs with all other dietary groups, as opposed to group no. 4, which shared only two OTUs with group no. 2 (Table 7). These findings support the notion that salt has a dominant effect on the composition of fish pyloric cecal bacteria. Interestingly, the bacterial shift in group no. 5, whose diet was supplemented with 3% salt, was also accompanied by improved weight gain compared to dietary group no. 4, even though the contents of protein and fat were similar in these two groups. This may be also attributed to the slightly higher fiber levels in the diet 4 composition, however, fiber did not correlate strongly with weight gain. Diet 1 and 2 had a higher salt content than the control group however no significant increase in size was observed, this might hint at an upper limit to the salt effect, as previously reported¹³.

Several studies on the microbiota of mice and human have associated body weight gain with a microbial component^{23–28}. When microbes were transplanted from obese mice to mice raised in a sterile environment, the latter gained significantly more fat^{23,27}. Thus,

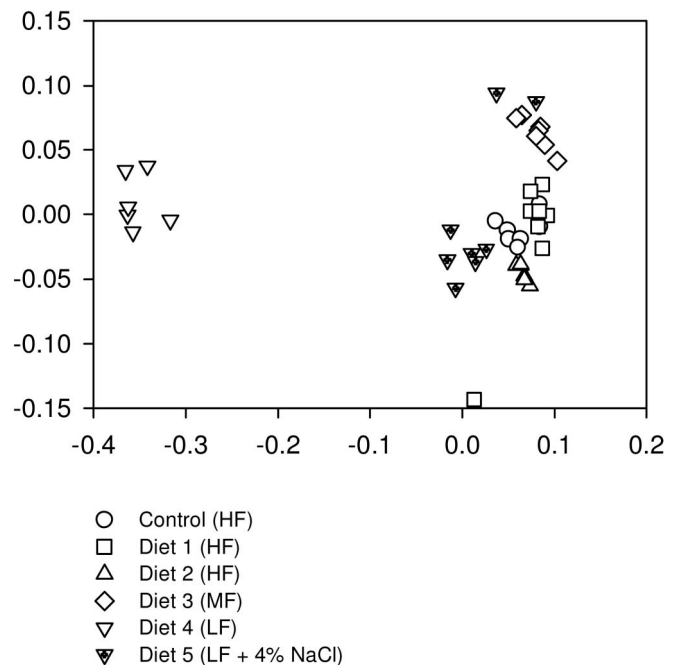


Figure 3 | Non-metric multidimensional scaling plot of all dietary groups at the final time point of the trial. Multivariate analysis uses the whole community OTU composition to infer similarity between the samples. The analysis was performed at the 6-week time point using the Bray-Curtis metric. In the resulting plot, samples carrying more similar bacterial communities cluster closer together. The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).



Table 5 | ANOSIM between the fish grouped according to the three biofilters used during the experiment (Filter 1–3). Data are from the end point of the trial and were analyzed based on the Bray-Curtis metric

	Filter.1	Filter.2	Filter.3
Filter.1		<i>0.7057</i>	<i>0.8311</i>
Filter.2	-0.0286		<i>0.2971</i>
Filter.3	-0.0444	0.0153	

The lower left corner of the table, in bold, denotes the *R* values representing the similarity between the groups, with values close to 0 denoting no difference between the groups and values close to 1 indicating that the groups are separate in terms of bacterial composition. The upper right corner shows the respective *P* values of each comparison (in italics).

the composition of the microbial community in the gut clearly affects the amount of energy extracted from the diet^{27,28}. It is interesting to speculate that the shift in bacterial communities and the weight gain observed in the present study are somehow connected; however, this has yet to be experimentally verified.

Hence, salt supplementation in fish diets is a dominant factor in determining the composition of the cecal microbiota in European sea bass and may also play a potential role in other carnivorous fish as well. These findings raise new questions regarding the role of cecal bacteria in the host fish physiological parameters and their ability to harvest energy from feed and possible ways of altering them.

Methods

Animals and rearing facilities. The experimental procedures used in this study were approved by the Animal Policy and Welfare Committee of the Agricultural Research Organization (ARO) approval number IL-241/10, Volcani Research Center, and were in accordance with the guidelines of the Israel Council on Animal Care.

European sea bass (*Dicentrarchus labrax*) were brought to the laboratory from a commercial hatchery (Maagan Michael, Israel) in brackish water (10 ppt), and housed in cages placed in a large (6,000 l) concrete pond. After a week of acclimatization, they were transferred to the experimental tanks and slowly acclimated to fresh water (< 3 ppt) over a period of 1 week.

The tanks were set up indoors and equipped with recirculating systems. The light source was natural photoperiod enhanced with fluorescent light, providing a light intensity of 1,200 lx during the day. The water was heated and maintained at $24 \pm 2^\circ\text{C}$ using submersible aquarium heaters.

At the beginning of the experiment, 50 fish were counted, bulk weighed, and housed in each tank after the very small or large fish deviating from the average weight (approximately 10 g) had been removed. There was no significant difference in fish weight among the experimental tanks at the start of the experiment ($P > 0.05$).

Diets. Six isonitrogenous diets with various inclusion levels of fish meal and fat (main contributor: a blend of mixed vegetable and fish oil) were formulated and prepared in the form of sinking extruded pellets, 3 mm in diameter, by Raanan Feeds Ltd. (Ashrat, Israel) (Table 1). Two of the experimental diets (nos. 1 and 2) were formulated to contain 50% fish meal and increasing fat levels; the other three experimental diets (nos. 3 to 5) contained 16% fat and decreasing amounts of fish meal. The salt-enriched diet (no. 5) was prepared by adding 3% NaCl to the pellet containing 10% fish meal. These diets were tested with a control diet, which was also used during the acclimatization period for all the groups and into the feeding trial as a control group. All diets contained the same percentage of protein. At the lower fish meal inclusion level, the protein content was supplied with defatted soybean meal and soy protein concentrate.

To quantify salt content in the diets, NaCl (> 99%) was purchased from Bio-Lab Ltd. (Jerusalem, Israel) and used as the standard. The chemical was weighed and dissolved in distilled water to give serial concentrations. The values were read in triplicate with a HI 8733 Conductivity meter (HANNA instruments, Sarmeola di Rubano, Italy). The standard curve was calibrated using a linear least-squares regression equation derived from the values: $y = 1741x + 0.117$ (x = salt content; y = salinity value). The corresponding correlation coefficient was 0.999 when the

Table 6 | Linear Pearson correlation coefficient between the degree of community similarity between the samples in each diet to the control diet and the OTU number in each diet, compared to the dietary levels of fish meal, fat and salinity

	Similarity to the control diet		OTU number	
	Pearson (R)	<i>P</i> -value	Pearson (R)	<i>P</i> -value
Fish meal	0.726	<i>7.78E-07</i>	-0.81423	<i>5.04E-10</i>
Fat	0.547	<i>0.0007</i>	-0.48072	<i>0.002251</i>
Salt	0.716	<i>1.33E-06</i>	-0.6455	<i>1.21E-05</i>

The Pearson correlation coefficient values were obtained by comparing the Bray-Curtis similarity of each samples from all the groups to the control group samples, and the degree of correlation was assessed by comparing these values to the concentration of fish meal, salt and fat in the feed. The correlation between the number of OTUs in each diet and the composition values of the feed was also assessed. Positive significant correlations mean a strong association exists between either the similarity with the control group or the number of OTUs in the groups, with one of the feed parameters. The correlation values are indicated in bold in columns designated "Pearson (R)" and their respective *P* values are shown next in italic.

range of salt concentrations was 0 to 0.6% (w/v), suggesting that the meter was suitable for detection purposes. For each diet sample, 3g of diet was dissolved in 30 ml of distilled water overnight in triplicate. Corresponding salinity value for each solution was assessed using the meter. Based on these values, salt contents in the diets were calculated using the linear equation described above.

Feeding trial and sampling. The experimental setup consisted of three systems, each containing six 250-l tanks and a central main biofilter of 350 l. The experimental feed was given in triplicate for each diet. Each of the diets was represented in each of the three systems used to assess the influence of biofilter on intestinal bacterial community. The fish were fed *ad libitum* to apparent satiation twice daily (08:30 and 14:00 h) for 6 weeks. Food consumption and mortality in each tank were registered daily. Fish in each tank were batch weighed after an overnight fast at the end of the trial.

Prior to the experiment, nine fish were sacrificed and their pyloric ceca were dissected using sterile instruments. After dissection, the entire ceca, including content and mucosa was removed and the connective tissue cleaned; the sample was then ground, frozen and stored at -80°C for further analysis. Sampling was done following a 24 hour fasting period.

At three time points during the trial (weeks 1, 2, and 6) three fish from each tank were randomly selected and their ceca were collected and stored as described above.

DNA extraction. Bacterial DNA was isolated using the protocol described by Roeslers et al.²⁹ with some modifications. The content of sodium dodecyl sulfate (SDS) for cell lysis was reduced to 5%, and the concentration of lysozyme (Sigma, St. Louis, MO) was changed to a final concentration of 2 mg/ml. In addition, a longer incubation was performed at 37°C for 60 min. Following this step, the solution was sequentially extracted with TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA), saturated phenol, phenol-chloroform (1:1 v/v), and chloroform-isoamyl alcohol (24:1 v/v). Finally, DNA in the aqueous phase was precipitated with 0.1 volume 3 M sodium acetate (pH 5.2) and 0.7 volume isopropanol. Before quantification for subsequent PCR, the DNA solution was incubated with RNase OneTM (10 U/ μl) (Promega, Madison, WI) at 37°C for 2 h. The concentration of DNA in the solution was measured using a Nanovue spectrophotometer (Danyel Biotech, Ltd., Rehovot, Israel) and it was stored at -20°C for further analysis. Only samples that resulted in high yield quality DNA were used for subsequent analyses.

Community fingerprinting using ARISA. ARISA, developed by Fisher and Triplett³⁰, is an automated, culture-independent molecular fingerprinting technique. Due to its high resolution and reproducibility, it has been widely used to study a variety of habitats, including soil, aquatic environments, and vertebrate gut bacteria^{31,32}. Here, the technique was used to characterize cecal bacterial communities during the feeding trial. All reaction templates were normalized to the same DNA concentration of 50 ng/ μl for ARISA. PCR was performed with 0.75 U of DNA polymerase (My TaqTM, Biorline, Taunton, MA), 1.5 μl $5\times$ PCR buffer, and 1.5 pmol primers. Universal primers ITS-F/ITS-R consisted of 5'-GTCGTAACAA-

Table 7 | Number of shared OTUs between the low fish meal dietary groups (diets 4 and 5) and all other diets at week 6

	Control (HF)	Diet 1 (HF)	Diet 2 (HF)	Diet 3 (MF)	Diet 4 (LF)	Diet 5 (LF +4% NaCl)
Diet 4 (LF)	0	0	2	0	18	0
Diet 5 (LF +4% NaCl)	4	2	5	3	0	7

Only OTUs that were shared by all the samples in each dietary group and of group 4 and 5 were included in this analysis. For diets 4 and 5, only OTUs shared by all samples were included. The abbreviations inside the parentheses refer to the fish meal concentration in each diet; HF, high fish meal (50%), MF, medium fish meal (30%), LF, low fish meal (10%).



GGTAGCCGTA-3' (forward) and 5'-GCCAAGGCATCCAC-3' (reverse - TET-fluorescent dye labeled).

Reactions were prepared in duplicate. Negative controls, containing no template, were also prepared to verify lack of contamination. The reaction was performed as follows: preliminary denaturation, 3 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C; a final elongation step of 5 min at 72°C, using a MultiGene gradient thermocycler (Labnet International Inc.). All PCR products were observed by agarose gel electrophoresis to verify successful amplification and to rule out contamination.

Fragment analysis. The fluorescently labeled PCR products were further analyzed using the ABI PRISM 3100 Genetic Analyzer. The labeled fragments were separated on a capillary sequencer, and an internal size standard, a custom-made marker—ROX labeled 250–1150 was used in each capillary (Bioventures, Murfreesboro, US).

Analysis of fingerprinting data. Raw data generated by the ABI PRISM 3100 Genetic Analyzer was initially analyzed using GeneMarker (SoftGenetics, State College, PA). After performing accurate size calling using this software, the data was exported to Microsoft Excel for further analysis. In Excel, all operational taxonomic units (OTUs) were binned as previously described^{33,34} and intensities were summed for each bin. Then the relative intensity for each binned OTU in a certain sample was calculated, and binned OTUs that contributed less than 0.1% to the total intensity of the sample were excluded from further analysis. Duplicates were compared, and only OTUs that appeared in both duplicates were used and their new relative intensities were calculated. Finally, the averaged values for each sample were normalized to reflect relative intensity values.

All data were then exported to Paleontological Statistics (PAST) software, a statistical data analysis package³⁵. Similarity values were calculated as the average similarity between all possible sample pairs using the Bray-Curtis index, which takes sample taxon richness and abundance into account³⁶. For diversity analysis, total OTUs and Shannon index (entropy), which takes into account the number of species present and their relative importance within the assemblage, were calculated, and a rarefaction test was performed using the software. Non-metric multidimensional scaling (NMDS) was performed, based on the Bray-Curtis similarity index, using the same software. Data were presented as mean \pm SD. The significance of the mean difference between the control and experimental diets was analyzed by Jmp program (SAS) using Tukey's test. Significance was set at a probability level of 0.05 with Bonferroni corrections.

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Author contributions

H.S., S.H. & I.M. designed the research; H.S. & I.M. performed the research, H.S., E.J., S.H. & I.M. analyzed the data; all authors interpreted the results and wrote, reviewed and approved the manuscript.

Additional information

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