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Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar)* and Chinook salmon (*Oncorhynchus tshawytscha*) but not in pink salmon (*O. gorbuscha*)

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ABSTRACT

To improve sustainability in the aquaculture industry plant meals are increasingly used to replace fish meal in fish feed. Solvent-extracted soybean meal (SBM) is an attractive protein source for fish feed because of its high protein content, favorable amino acid profile, and low cost. In Atlantic salmon (*Salmo salar*), SBM at low levels causes soybean meal-induced enteritis (SBMIE). Few studies have been done with SBM in Pacific salmon, and none of those have included intestinal inflammation analysis. To gain more insight into salmonid responses to SBM, we assessed and compared the effects of SBM on intestinal morphology, inflammation and microbiome composition of Chinook salmon (*Oncorhynchus tshawytscha*), pink salmon (*O. gorbuscha*) and Atlantic salmon (*Salmo salar*).

Atlantic, Chinook and pink salmon were fed for three weeks on a diet with 20% inclusion of SBM, or a control diet with fish meal. After one week on the SBM diet, Atlantic and Chinook salmon showed increased submucosa thickness in the distal intestine compared to the fish fed on the fishmeal diet. Intestinal inflammation in these species increased over time, with a further increase in submucosa thickness coincident with an infiltration of eosinophilic granular and mononuclear leucocytes. After 3 weeks on the SBM diet, intestinal inflammation was most severe in Chinook salmon. In contrast, pink salmon only showed a slight increase in submucosa thickness after three weeks on the SBM diet, and no significant increase in inflammatory cell infiltrate. Sequence-based analysis of the intestinal microbiome showed a significant difference in overall microbiome composition between species, but did not show an effect of the SBM diet on microbiome diversity or composition in any of the three salmon species. In conclusion, SBM-fed Chinook salmon were more susceptible to SBMIE than Atlantic salmon whereas pink salmon were not susceptible to SBMIE at the levels of SBM tested.

1. Introduction

Fish meal is an important source for protein in aquaculture feeds, especially for carnivorous species like salmon. Most fish meal is sourced from wild oil-rich fish, which may not be sustainable for those stocks with limited abundance. Therefore alternative sources of protein such as plant meals are being considered. Despite the widespread availability of crop-based plant meals, these protein sources can contain anti-nutritional factors such as fibers, indigestible sugars, and chemicals that adversely affect feed intake, palatability and nutrient digestibility (Krogdahl et al., 2010). In addition, some anti-nutritional factors are inflammatory with the potential to elicit enteritis. Furthermore, in some aquaculture species, plant-based diets may induce changes in intestinal microbiota composition (Desai et al., 2012; Green et al., 2013; Navarrete et al., 2013), which can alter intestinal health including the feed digestibility and intestinal immunity (Nayak, 2010).

Solvent-extracted soybean meal (SBM) is an attractive protein source for fish feed because of its high protein content, favorable amino acid profile, and low cost. In Atlantic salmon (*Salmo salar*), SBM can be included in the diet at low levels without significant negative effects on feed intake or fish growth (Krogdahl et al., 2003; Romarheim et al., 2013). However, a 20% inclusion rate causes shortening of the mucosal

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folds, thickening of the lamina propria and submucosa, and disappearance of supranuclear vacuoles in the enterocytes of the distal intestine, accompanied by an infiltration of inflammatory cells; a process known as soybean meal-induced enteritis (SBMIE) (Baeverfjord and Krogdahl, 1996; Urán et al., 2009). Recovery from SBMIE is spontaneous in some species whereas in Atlantic salmon, recovery occurs after the fish are returned to a fishmeal-based diet (Baeverfjord and Krogdahl, 1996; Urán et al., 2008). The mechanism of SBMIE is not well understood, but saponins may play a role (Knudsen et al., 2008; Krogdahl et al., 2015).

Few studies have examined the effects of SBM in diets for Pacific salmon. Inclusion of most soy products in diets for Chinook salmon (*Oncorhynchus tshawytscha*) severely reduced feed intake (Bureau et al., 1998; Fowler, 1980; Hajen et al., 1993), and the products were deemed unacceptable with no further analyses undertaken. However, most of these studies used either soybean products containing high levels of saponins, or solvent-extracted soybean meal at relatively high inclusion levels and none included analyses of intestinal morphology and inflammation. To gain more insight into salmonid responses to SBM, we compared Chinook and pink salmon (*O. gorbuscha*) with Atlantic salmon, a well-established SBMIE model organism, fed an experimental soybean meal diet. The objectives of the study were to assess the effects of SBM on intestinal morphology and inflammation and to investigate the effect of an SBM diet on the intestinal microbiome.

2. Materials and methods

2.1. Fish husbandry

Juvenile Atlantic salmon were obtained from a commercial hatchery on Vancouver Island and held at the Pacific Biological Station (PBS) in Nanaimo, BC, Canada. Pink salmon were obtained as swim-up fry from Quinsam River Hatchery on Vancouver Island, BC and were reared at the PBS. Chinook salmon were obtained from a commercial hatchery on Vancouver Island, BC and held at the Centre for Aquaculture & Environmental Research (CAER) in West Vancouver, BC. One week before the start of the experiment, fish were stocked in their experimental tanks at 50 fish per tank, 4 tanks per species. Pink salmon (initial mean weight of 166 g) and Atlantic salmon (535 g) were maintained in 850 L and 1900 L tanks respectively, and Chinook salmon (140 g) were maintained in 650 L tanks. All tanks were supplied with flow-through sea water with mean salinity at PBS of 29.6 ppt (range 28.0–29.9 ppt) and at CAER of 30.2 ppt (range 27.9–31.0 ppt) and an ambient temperature at PBS of 9.4 °C (range 9.1–9.8 °C) and at CAER of 9.0 °C (range 8.7–9.8 °C) under natural photoperiod. These trials were conducted during February and March 2016 and approved by the Pacific Regional Animal Care Committee (AUP #15-015), according to Canadian Council of Animal Care guidelines.

2.2. Experimental diets and feeding experiment

Two diets were produced: a control diet containing fish meal (FM) and a test diet containing 200 g/kg solvent-extracted soybean meal (SBM). These diets were formulated to be isonitrogenous and isolipidic (Table 1). Diets were produced by blending dry ingredients in a mixer (Model M-802, The Hobart Manufacturing Co., Troy, Ohio, USA) for 15 min with a portion of the oil added to the mash. Hot water (ca. 85 °C) was added to the mash at 10% by weight, further mixed for 15 min and passed through a pellet mill (Model CL3, California Pellet Mill Co, San Francisco, California, USA). The pellets were spread onto screens, sieved to remove fines and dried with gentle heating (30–40 °C) until moisture content was approximately 8%. The pelleted diets were then top-coated with fish oil and stored in plastic bags in a humidity-controlled room at 4–5 °C. Two pellet sizes were produced: 3 mm for pink and Chinook salmon, and 4 mm for Atlantic salmon. For each species, fish in two tanks received the FM control diet while those in the remaining two tanks received the SBM test diet. All fish were fed a daily ration at 1% biomass, adjusted weekly, for 3 weeks. Pink and Atlantic salmon were fed half their ration by hand in the morning, and the remainder on automated feeders. Chinook salmon were fed by hand equally in the morning and afternoon.

Table 1
Formulations of fish meal (FM) and solvent-extracted soybean meal (SBM) diets used in this study.

Ingredients and composition	FM diet (g/kg)	SBM diet (g/kg)
Fishmeal	500.0	378.8
Soybean meal	0.0	200.0
Whole wheat flour	298.0	211.4
Fish oil	190.0	197.8
Vitamin/mineral premix	2.0	2.0
Permapell	10.0	10.0
Crude protein	372.4	372.4
Lipid	230.5	230.5

2.3. Sampling

Four fish were haphazardly sampled from each tank for histology at weeks 1, 2, and 3. Each fish was euthanized with an overdose of tricaine methane sulphonate (TMS, 400 mg/L). A 2.5 cm section of the distal intestine was removed, contents rinsed out with 10% neutral buffered formalin (NBF), and the tissue fixed in NBF for 48 h. At week 3, two additional fish per tank were euthanized and sampled for intestinal microbiome analysis. Each fish was wiped with ethanol, and the combined mid- and distal intestine including digesta was aseptically removed and flash-frozen in liquid nitrogen.

2.4. Histology

2.4.1. Slide preparation

For samples collected in weeks 1, 2 and 3, eight fish per diet per species were processed further for histology. Each NBF-fixed intestine sample was cut into 5 equal-sized pieces, dehydrated in an alcohol gradient, cleared in two changes of xylene and sequentially embedded into a single paraffin block. For each fish, 5 μm-thick cross-sections of all five pieces of intestine were mounted onto a glass slide, stained with haematoxylin and eosin and sealed under a coverslip.

2.4.2. Quantitative and semi-quantitative measurements

Histological features of the intestine were viewed at a total magnification of 125 × and images captured using a QImaging digital Camera (QImaging, Surrey, BC, Canada). The thickness of the submucosa (SM), i.e. the distance between the base of the villus and the stratum compactum, was measured at four points on the intestinal wall where these features were clearly visible, preferably at 0, 90, 180 and 270 degrees as observed in the microscopic field of view of each intestinal cross-section. Twenty such measurements were used to calculate the mean SM per fish.

For semi-quantitative measurements, an Inflammation Score (IS) of 0 to 4 was assigned to each intestinal cross-section according to the following criteria:

0: Normal background leucocyte infiltrate
1: Minor increase in infiltrate – primarily by eosinophilic granular leucocytes (EGL)
2: Mild increase in infiltrate – EGL and/or mononuclear leucocytes
3: Moderate increase in infiltrate – EGL and mononuclear leucocytes
4: Heavy increase in infiltrate – EGL and mononuclear leucocytes.

For each fish, the individual cross-sectional scores were used to

generate a composite IS. For each fish, the median IS was subsequently used for statistical comparisons.

2.5. Intestinal microbiome analysis

The microbiome of the combined mid-and distal intestine including digesta was analyzed for 4 fish per species per diet (2 per tank) (Microbiome Insights, Vancouver, BC, Canada). Briefly, intestine samples were homogenized and DNA isolated using the MoBio PowerSoil Extraction kit. Approximately 20 ng of purified DNA was amplified by PCR using dual bar-coded primers targeting the V4 region of the 16S rRNA gene (Kozich et al., 2013). Normalized library concentrations of 1–2 ng/μl (as per the specifications of the Sequal Prep normalization kit) were used. A final library concentration of 8 pM (10% phiX) was loaded onto the flow cell (as per Kozich et al., 2013) and subjected to 2 × 250 paired-end sequencing on a MiSeq (Illumina), producing 632 k/mm^2 clusters passing filter, an average Q30 score of 79.40%, and an overall sequencing error rate of 1.88%. Resulting quality-filtered Fastq files were clustered into 97% similarity operational taxonomic units (OTUs) using MOTHUR (www.mothur.org). Overall error rates used for downstream analyses were minimized according to methods described in Kozich et al. (2013). High quality reads were classified using Greengenes (v. 13_8) and OTU abundances summarized into Bray-Curtis dissimilarities (Bray and Curtis, 1957). For the latter, the metaMDS algorithm of the vegan package (R v. 2.4.1) was used with random starts to find a stable solution.

2.6. Statistics

Histology data were analyzed in GraphPad Prism v5.0. The submucosa thickness data failed normality tests (D'Agostino & Pearson omnibus or Shapiro-Wilk), and Inflammation Score data were non-continuous. Thus all data were analyzed using non-parametric tests to determine significant differences over time (Kruskal-Wallis with Dunn's post-hoc test where applicable) or between diets (Mann-Whitney). Differences were considered statistically significant when $p < 0.05$. For the microbiome analysis, differences between treatment groups were determined using ANOVA on the distance matrices using the adonis function in R package vegan (Oksanen et al., 2016, R Core Team, 2016) and graphically represented by a non-metric multidimensional scaling (NMDS) ordination. Diversity of the microbiome was determined using the number of OTUs and the Shannon and Simpson diversity indices, and analyzed using Kruskal-Wallis tests in GraphPad Prism v5.0b. General linear models using DESeq2 (Love et al., 2014) were used to determine the statistical significance of differences in abundance of individual OTUs between diets, or between species after controlling for diet.

3. Results

3.1. Histology

Inflammation of the intestine was evident in Atlantic and Chinook salmon after feeding on the SBM diets (Fig. 1 and Table 2). There was a significant ($p = 0.010$) increase in mean SM thickness in Atlantic salmon fed on the SBM diet compared with controls after one week, and this further increased in week 3 ($p = 0.0002$) to 147% of the SM thickness observed in FM-fed Atlantic salmon (Fig. 1). In Chinook salmon, an increase in SM thickness was observed at all three timepoints ($p = 0.0002$, $p = 0.0047$ and $p = 0.0012$ in weeks 1, 2 and 3 respectively), and after three weeks the SM in SBM-fed Chinook salmon was 244% thicker than the SM in FM-fed fish. In contrast, in SBM-fed pink salmon, a small but significant increase in mean submucosa thickness of 129% was observed only after 3 weeks ($p = 0.014$). Accompanying the increase in submucosa thickness, a significantly increased infiltration of inflammatory cells was observed in SBM-fed Atlantic salmon in week 2 compared to controls ($p = 0.032$; Table 2), and in SBM-fed Chinook salmon in weeks 1, 2 and 3 ($p = 0.0168$, 0.0046 and 0.0044). There was no evidence of changed inflammatory cell infiltration in pink salmon.

3.2. Microbiome analysis

A total of 184,628 high-quality reads clustered into 9458 operational taxonomic units (OTUs), 15% of which were singletons, and the 10 most abundant OTUs contain 30% of all sequences. Six of the 24 samples had fewer than 500 reads and were removed from further analysis. Twelve phyla were recognized from OTU aggregates to contain at least 1% of all sequences (Fig. 2). Together, these 12 phyla accounted for 92.6% of all sequences. In all but one sample, the phylum Proteobacteria was the most abundant (35–55% of all sequences). The phylum Tenericutes was found mostly in the Chinook salmon, but with high individual variation, ranging from 0.2% to 90%. Almost all sequences (99.8%) classified as Tenericutes belonged to the most abundant OTU (OTU1), and were further classified as family Mycoplasmataceae. NMDS ordination revealed that microbiome profiles grouped by species, most notably for Chinook salmon, but not by diet (Fig. 3). This was confirmed by a diet x species ANOVA on the distance matrices, which only showed a significant species effect ($p = 0.005$).

No effect of species or diet on microbiome diversity was evident from analysis of OTU numbers, the Shannon index or the Simpson index (data not shown). General linear models found no evidence that OTUs were significantly more or less abundant in the SBM diet compared to controls ($p > 0.05$; data not shown). The abundance of three OTUs was significantly lower (16- to 32-fold) in Atlantic salmon compared to Chinook salmon: one belonging to the genus *Bacillus*, one belonging to the genus *Sporanaerobacter*, and one belonging to the family Bacillaceae ($p < 0.05$).

4. Discussion

In this study, a diet composed of solvent-extracted SBM incorporated at 20% resulted in significant alterations to the intestinal mucosa consistent with inflammation, and these changes were most pronounced in Chinook salmon and less so in Atlantic salmon. Changes to the intestine of pink salmon were minor. Chinook salmon are sensitive to dietary SBM products and when fed full-fat SBM diets showed poor appetite, high mortality and reduced growth at inclusion levels above 12.7% (Fowler, 1980; Hajen et al., 1993). Bureau et al. (1998) concluded from feeding trials in Chinook salmon and rainbow trout that the feed deterrent in soybean products was present in the alcohol extract, with soy saponins being the most likely candidates. That may explain their finding that Chinook salmon more readily accepted a diet containing methanol-extracted SBM at an inclusion level of 38% than one containing full fat SBM, and although the fish fed on methanol-extracted SBM still showed increased mortality and reduced growth compared to controls, the effects were less severe than those in fish fed full fat SBM. In the earlier work, the effects of SBM on intestinal morphology were not investigated. Studies in Atlantic salmon with diet formulations comparable to those used here have shown that 20% incorporation of solvent-extracted SBM does not reduce feed intake, but does cause soybean meal-induced enteritis (SBMIE) (Krogdahl et al., 2003; Romarheim et al., 2013; Urán et al., 2009).

The duration of the current study was insufficient to examine diet effects on growth or mortality. Digested feed was evident in the intestine in the majority of the fish when tissue samples were collected (data not shown) suggesting that fish in none of the three species rejected the SBM diet, which supported observations made during manual feeding. There was evidence for SBMIE in the SBM-fed Atlantic salmon and Chinook salmon after 1 week of feeding, increasing in severity with time. This corresponds to previous studies reporting an onset of SBMIE symptoms at about 5 days, with rapid progression of severity within

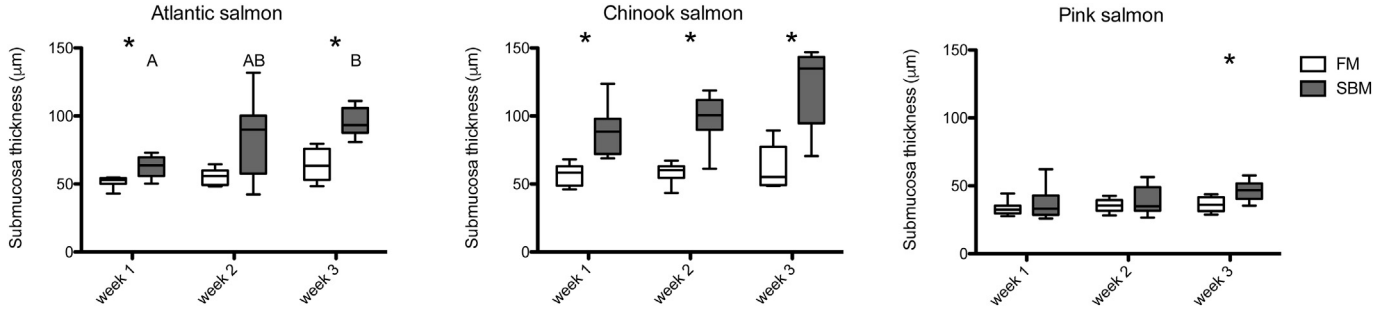

Fig. 1. The effect of a soybean meal diet on thickness of the intestinal submucosa in Atlantic, Chinook and pink salmon. Data are summarized in box plots in which the inner horizontal line is the median, the lower and upper edges of the box are 25th and 75th percentiles and the whiskers are minimum and maximum values. Uppercase letters indicate statistical significance between weeks for the soybean meal (SBM) diet (weekly differences were not statistically significant for the fishmeal (FM) diet). Asterisks indicate statistical significance between FM and SBM diets within a week.

21 days (Marjara et al., 2012; Urán et al., 2009). At week 3, the submucosa in Atlantic salmon had increased in thickness by about 50% compared to controls and in Chinook salmon, the increase was almost 100%. The extent of leucocyte infiltration in the submucosa at week 3 was also relatively higher in Chinook salmon than in Atlantic salmon. These results confirm the previous reports that Chinook salmon are highly sensitive to soybean meal. Interestingly, pink salmon were minimally affected by the SBM diet, only showing a slight increase in submucosa thickness at the end of the study, without evidence of an increased leucocyte infiltration. Further research is required to better understand species-specific responses to diets containing SBM.

Our ability to effectively analyze species and diet effects on the salmonid intestinal microbiota was limited by small sample size. Previous studies have used sample sizes comparable to the numbers used in the current study (Gajardo et al., 2016), and in some studies, material from individual fish was pooled (Standen et al., 2015). A challenge in future studies will be to establish sample size criteria that permit discrimination among dietary and species effects given elevated individual variability. Nevertheless, our data suggested that the principle feature of the intestinal microbial community that discriminated among species was the increased presence in Chinook salmon of OTU1 (Tenericutes), relative to that in Atlantic and pink salmon. Environmental factors can influence gut microbiota (Dehler et al., 2017), and the Chinook salmon were held at a separate facility from the pink and Atlantic salmon, suggesting that the apparent species difference was actually a difference based on trial location. Supporting this, Tenericutes have previously been described from the Atlantic salmon intestinal microbiota (Abid et al., 2013; Green et al., 2013). Also, in other experiments performed at PBS and at CAER we found that Tenericutes were the dominant phylum (up to 98% of all sequences) in both Chinook and Atlantic salmon (M. Booman, unpublished observations).

Overall, the most highly represented phyla were Proteobacteria, Firmicutes and Bacteroidetes, which corresponds well to other studies of the intestinal microbiota of salmon and other teleost species (Gajardo et al., 2016; Gajardo et al., 2017; Gatesoupe et al., 2016; Ingerslev et al., 2014; Standen et al., 2015; Wong et al., 2013; Zarkasi et al., 2014). Some studies have shown that marine diets favor Proteobacteria, while Firmicutes show a higher relative abundance in fish fed plant diets (Desai et al., 2012; Gajardo et al., 2017; Ingerslev et al., 2014) and in fish with SBMIE (Reveco et al., 2014). These studies showed that lactic acid bacteria (LAB) were increased when soybean meal was added to the diet. In our study, LAB made up a small percentage of OTUs: 0.33% both in FM-fed controls and SBM-fed fish and diet effects were not significant in any species (data not shown). In contrast and consistent with the studies cited above, Proteobacteria were the dominant members of the gut microbial community regardless of diet or fish species. Therefore this study adds to a confusing and often contradictory pool of knowledge concerning the role of plant diets on the microbiome of the fish intestine. Not all studies find a significant effect of plant diets on microbiota composition or diversity (Cai et al., 2012; Wang et al., 2016; Wong et al., 2013), and there is little agreement on whether diversity is increased or decreased, or which taxa differ in relative abundance (Desai et al., 2012; Green et al., 2013; Mansfield et al., 2010; Rhodes et al., 2016). This discrepancy between studies may in part be a reflection of the differences between the more commonly used culture-based methods and the more recent culture-independent methods. Other important factors may be the large inter-individual variation in microbiome composition, as seen in the current study and reported by others (Star et al., 2013), or the variation between digesta-associated and mucosa-associated microbiota (Gajardo et al., 2016, Gajardo et al., 2017).

In conclusion, the current study showed that SBM-fed Chinook salmon are more susceptible to SBMIE than are Atlantic salmon, which can explain the problems with growth and mortality in Chinook salmon fed SBM. Pink salmon were not susceptible to SBMIE at the levels tested in this study. The gut microbiome, although differing between species, was not shown in this study to be related to the presence of soybean meal in the diet.

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Table 2
Inflammation scores in salmon during feeding trial with fishmeal (FM) or soybean meal (SBM) diets. Data are medians with interquartile range in brackets. $N = 8$ except for week 1 pink FM diet where $n = 7$. p-Values show statistical significance (Mann-Whitney test) between diets; no significant differences were found between time points.

Week	Atlantic			Chinook			Pink		
	FM	SBM	p-Value	FM	SBM	p-Value	FM	SBM	p-Value
1	2 (1–2)	2 (2–2)	0.4275	1 (1–1)	2 (1.25–2)	0.0168	1 (1–1)	1 (1–2)	0.6279
2	1 (1–2)	2 (2–2.75)	0.0324	1 (1–1)	2 (2–2.75)	0.0046	1 (1–1)	1 (1–1)	na[a]
3	2 (1–2)	2 (2–2)	0.0522	1 (1–2)	2.5 (2–3)	0.0044	1 (1–1)	1 (1–1)	na[a]

[a] No statistical analysis since variance was 0 in one or both treatments.

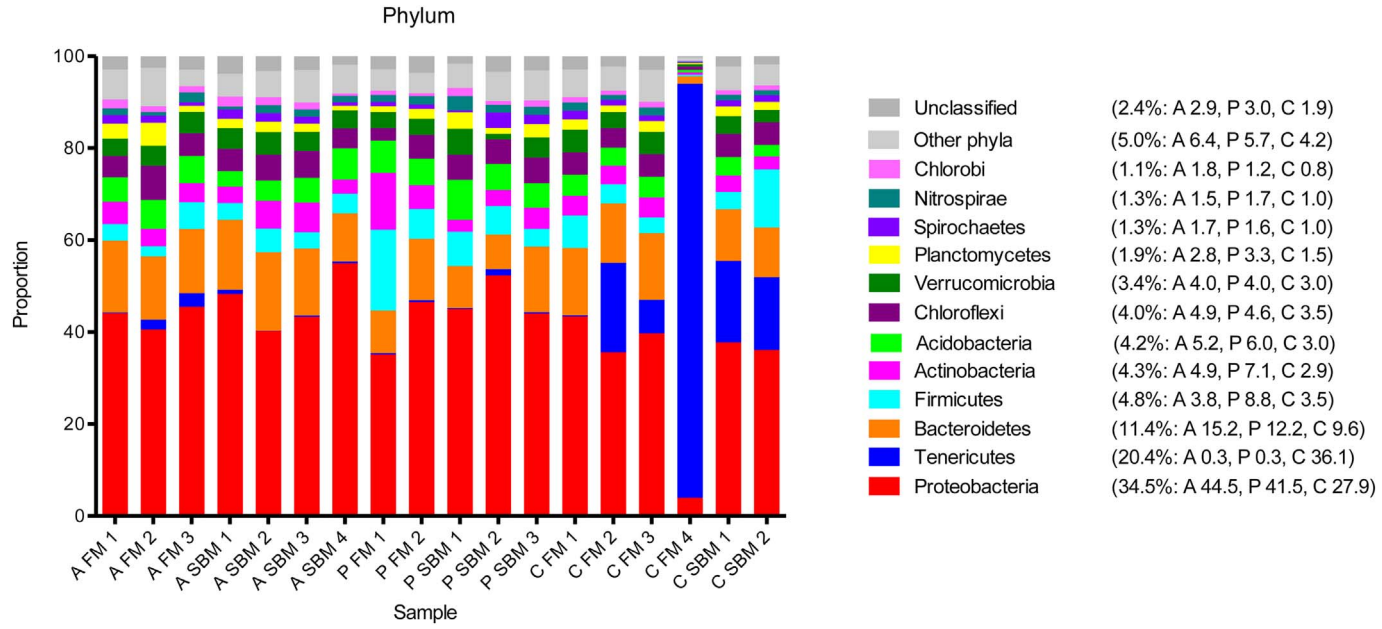

Fig. 2. Intestinal microbiome composition based on sequence analysis of the 16S ribosomal RNA gene. The relative proportion of the most highly represented phyla (> 1% of all sequences) is plotted for each sample. The relative proportion (%) of each phylum over all samples per salmon species (A: Atlantic salmon; P: pink salmon; C: Chinook salmon) is indicated in brackets beside each phylum name.

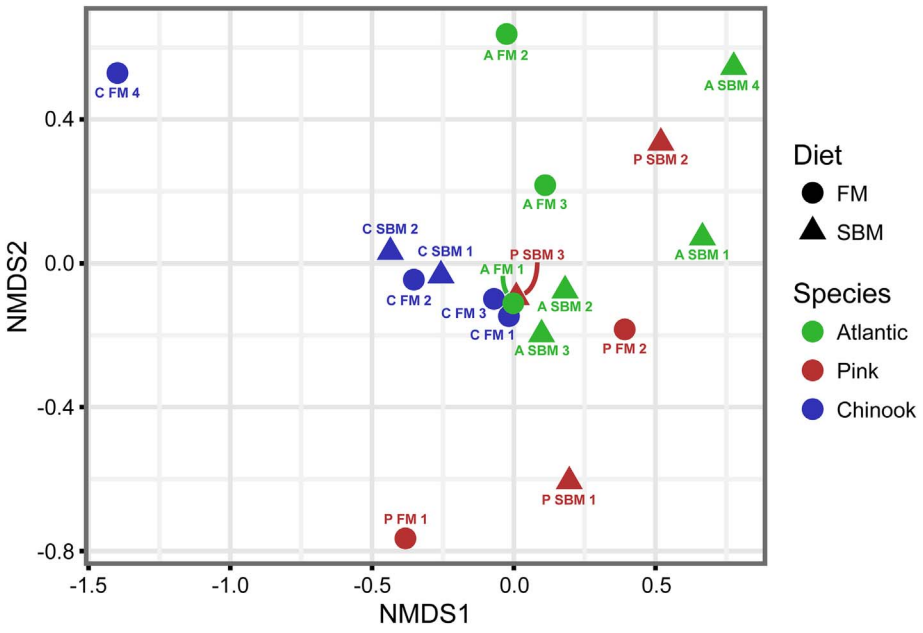

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination plot displaying the effect of soybean meal (SBM) and fish meal (FM) diets on composition of the intestinal microbiome. The NMDS plot displays relationships among microbial operational taxonomic units (OTU) based on Bray-Curtis dissimilarities (NMDS plot stress = 0.12), and reveals OTU clustering by host species but not by diet.

intestinal morphology. MB was supported by funding from the Natural Sciences and Engineering Research Council of Canada (Strategic Grant 447374).

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