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Probiotic and Pathogen *Ex-vivo* Exposure of Atlantic Salmon (*Salmo Salar L.*) Intestine from Fish Fed Four Different Protein Sources

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Abstract

The present study addressed the adherence of *Carnobacterium divergens* and *Aeromonas salmonicida* subsp. *salmonicida* to the intestinal lining of Atlantic salmon (*Salmo salar* L.) using an *ex vivo* method–intestinal sac-following feeding with pea protein concentrate, extracted sunflower or feather meal at 200 g/kg inclusion level. Control diet was a 450 g/kg fishmeal diet. The experimental feeds were fed to two groups each for seven weeks at EWOS Innovations' facilities in Lønningdal, Norway. *Ex vivo* intestinal challenge was carried out at Institute for Marine Research, Bergen. Excised intestines of salmon from all feeding groups were exposed to a probiotic, *C. divergens* or a pathogen, *A. salmonicida* either alone or in combination and control samples were exposed to sterile saline solution.

Exposure to *A. salmonicida* caused severe damage to the intestinal ultrastructure of the mid intestine, but after exposure to *C. divergens*, sterile saline solution or any of the combination treatments, morphology remained mostly unaltered indicating an alleviating effect of the probiotic. Feather meal intensified the damaging effect of exposure to *A. salmonicida* and there were otherwise no effect of diet on the morphology.

qPCR analysis of adhered *C. divergens* and *A. salmonicida* showed that although the pathogen has a higher adherence efficiency, *C. divergens* was more efficient at displacing the pathogen if allowed to adhere to the mucosal lining first indicating that the probiotic should be present prior to the pathogen for optimal effect. There were no dietary effects on bacterial adherence.

The present study shows that use of some commercially available alternative feed ingredients may not affect the probiotic abilities of *C. divergens* or make the fish more susceptible to disease through intestinal invasion.

Keywords: Salmon; Intestine; *Carnobacterium; Aeromonas;* Microscopy; qPCR

Introduction

Fish possess an indigenous intestinal microbiota which it is under constant challenge from non-commensal bacterial populations [1-3]. Several investigations have shown that *Carnobacterium* spp. are a natural part of the gut microbiota in salmonids [4-15] and that they display antimicrobial abilities and *in vitro* growth inhibition of several fish pathogens including *Aeromonas salmonicida* spp. *salmonicida* (*A. salmonicida*) [16,17] a well-known fish pathogen of salmonids [18]. Carnobacteria has been suggested as probiotics [19] but a favorable criterion of a probiotic bacterium is its ability to adhere to and grow in the mucus or on the enterocyte surface and is an important criteria when evaluating the use of probiotics in endothermic animals as well as in fish [20-24].

Previous studies has shown that gastrointestinal (GI) tract in fish is one of the major infection routes for *A. salmonicida* [12,22,23] and other pathogens [25-27]. Furthermore, some studies have shown that exposing fish intestine to *Carnobacterium* ssp. and a pathogen bacteria result in alleviation to some degree of the potentially damaging effect of the pathogen bacteria [24-26]. This is however difficult to measure *in vivo* and during the last few years the *ex vivo* intestinal sack method has been used in several studies to evaluate possible histological and bacteriological changes in the fish intestine after exposure to high levels of lactic acid bacteria (LAB) and pathogens [28-32]. In the present study, the *ex vivo* method was applied to circumvent the uncertainty of an *in vivo* experiment because it has proved useful in evaluating bacteriological effects on intestinal tissue under controlled experimental conditions [33]. This method has been developed according to the EU recommendation to reduce the number of *in vivo* experiments and the number of fish used (Revision of the EU directive for the protection of animals used for scientific purposes [Directive 86/609/EEC]; 08th September 2010). However, the method has limitation by the viability of the tissue once it has been removed from the host. Therefore only one hour of incubation has been used. Prolonged incubation; > one hour following excision of the tissue may result in natural degradation making the negative effects as result of bacterial exposure indiscernible. Due to the short term durability of the *ex vivo* method, results generated should only be considered a snapshot of the whole story, and although will not replace *in vivo* experiments, may contribute to reduce the number of fish in subsequent *in vivo* trials. The present study used live bacteria as previous studies have shown epithelial damage and bacterial adherence as a result of exposure to live bacteria [34-37],

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higher bacterial translocation than using heat-inactivated bacteria [23] and enhanced stimulation of phagocytotic activity [38].

Fishmeal (FM) has become a limited feed ingredient and the dependency on marine protein is alleviated using alternative feed ingredients. Alternative terrestrial proteins, especially plant based raw materials may contain anti-nutritional factors (ANF) which have shown to have potential negative effects for the intestinal morphology in Atlantic salmon [29,39]. Dietary alterations have shown to influence the indigenous intestinal microbiota in fish [27-30] as well as modulate changes in the intestinal mucosal integrity [28,29]. Although there is information available on the influence of dietary manipulations on the endogenous intestinal microbiota in fish, few studies have investigated the importance of diet on the susceptibility to pathogenic bacteria [26,31] and less information is available on the modulation of the gut microbiota using animal proteins or oils in the diet [27,32].

The purpose of the present study was therefore to investigate whether *C. divergens* originally isolated from the digestive tract of Atlantic salmon (*Salmo salar* L.) [4] could exclude and displace *A. salmonicida* in the Atlantic salmon intestine by using the intestinal sack method; a method used in some previous studies [26,31,33,34]. The intestinal sac method was chosen for its simplicity and superior efficiency over *in vivo* methods [31], however by using this method one should bear in mind that it is restricted to short term experiments as the viability of intestinal tissue is limited once it is excised from the host. This was addressed in two parts; assessment of the effect of *ex vivo* exposure of the intestinal to; a) saline, b) *C. divergens*, c) *A. salmonicida*, d) first to *C. divergens* thereafter to *A. salmonicida*, and finally, e) first to *A. salmonicida* thereafter to *C. divergens*. Moreover, the present study addresses the effect of dietary; a) fishmeal, b) pea protein concentrate, c) extracted sunflower and d) feather meal on intestinal morphology.

Materials and Methods

Preparation of experimental diets

One control and three experimental diets were produced at EWOS Innovation's feed production plant in Dirdal, Norway. Feed formulation is shown in Table 1. The control was a fishmeal (FM; 450 g/kg) and fish oil (FO; 260 g/kg) based diet. The test diets were similar to the control in terms of FO but had 200 g/kg of the FM replaced with pea protein concentrate (PPC), extracted sunflower (ESF) or hydrolyzed feather meal (FeM). The diets were formulated to keep the energy and protein ratio constant and fulfill the minimal nutritional requirements for the Atlantic salmon [35]. The feeds however were not balanced according to amino acid profile or amount of energy.

	Experimental diets			
Ingredients (g kg^{-1})	FM	PPC	ESF	FeM
Fishmeal*	20.00			
Pea Protein Concentrate¥		20.00		
Extracted SF ‡			20.00	
Feather meal‼				20.00
Fishmeal*	25.00	25.00	25.00	25.00
Wheat Gluten	10.00	10.00	10.00	10.00
Wheat grain	17.14	17.14	17.14	17.14
EWOS premix	1.86	1.86	1.86	1.86
Fish oi‼Δ	26.00	26.00	26.00	26.00

FM: fishmeal, ESF: extracted sunflower; FeM: feather meal; PPC: pea protein concentrate
¥ AgriMarin, Stavanger, Norway
‡ Unknown
‼ Ge-Pro, Germany
Δ Fiskerens Fiskeindustri, Skagen, Denmark

Table 1: Diet formulations and chemical composition of the dietary treatments.

Fish and rearing conditions

Sixty unvaccinated, sea-water adapted Atlantic salmon with initial mean weight of 328 ± 68 grams was used. The fish were tagged using a passive integrated transponder (PIT) for identification and allocated into eight tanks at EWOS Innovation research facility in Lønningdal, Norway. During the four week acclimatization period, the fish were fed a commercial feed (EWOS Opal 50, EWOS, Norway) to satiation twice a day. Temperature (mean 8°C) and salinity (mean 32‰) were measured daily, while dissolved oxygen (DO) remained above 77% for the duration of the trial. Post acclimatization, the fish were fed the trial diets for seven weeks and thereafter transferred to challenge facilities at the Institute of Marine Research (Bergen, Norway). After the transfer, the fish were fed for two additional weeks for acclimatization in order to reduce stress related effects prior to *ex vivo* challenge experiment. No mortalities were recorded for the duration of the trial.

Bacterial suspensions

Carnobacterium divergens Lab01 cultivated from a pure cell culture was used as an indigenous probiotic bacterium in the present study. The bacterium was originally isolated from the distal intestine (DI) of juvenile Atlantic salmon fed a commercial diet [4]. The bacterium has been identified on the basis of 16S rDNA sequence analysis and amplified fragment length polymorphism (AFLP™) fingerprinting [10]. The pathogen used was *Aeromonas salmonicida* ssp. *salmonicida* strain VI-88/09/03175 (culture collection, Central Veterinary Laboratory, Oslo, Norway), and is pathogenic to salmonids [36]. Both bacteria were cultured in tryptic soy broth added 5% glucose for 48 hours at 12°C. Exposure dose was measured by plate counts of viable colony forming units (CFU) and the exposure dose for *C. divergens* was 3.2×10^7 CFU ml^{-1} and 8.6×10^6 CFU ml^{-1} for *A. salmonicida*.

Ex vivo intestinal exposure

Ex vivo exposure of the intestines to the bacterial strains was performed using the intestinal sac method as previously described in several studies [12,25,26,31,34,37] with some modifications. Prior to the *ex vivo* challenge fish were starved for 24 hours and sacrificed with a blow to the head. Briefly, the entire intestine, from behind of the last pyloric caeca to the anus was removed aseptically and flushed three times using sterile physiological saline (0.9‰) to remove allochthonous (non-adherent) bacteria. The distal end was closed tight using a cotton string before filling with the appropriate treatment solution (Table 2). In the control group, the intestine was exposed only to sterile saline solution. Intestines exposed to saline or bacteria were incubated in Falcon tubes containing saline for one hour at 10°C. In two treatments exposed to *C. divergens* and *A. salmonicida*; treatment 4 and *A. salmonicida* and *C. divergens*; treatment 5 the intestines were first exposed to bacteria for 30 min. cut open, emptied and flushed 3 times by saline, and thereafter exposed to the 2nd bacteria. Intestines from four individual fish per dietary groups were subjected to each of the treatments described in Table 2. All intestines were flushed three times prior to and post incubation using three ml saline with every rinse to ensure that only the autochthonous bacteria were sampled. In order to obtain enough samples for each analysis, samples for histological analysis were taken from pyloric intestine (PI) and samples for autochthonous bacteria were taken from DI.

		Treatment 1			Treatment 2		
	nr	Exposure bacteria	Duration	Rinse	Exposure bacteria	Duration	Rinse
Single treatments	1	Saline	60 min	Yes		-	-
	2	*Carnobacterium divergens* 3.2×10^{7} CFU ml^{-1}	60 min	Yes		-	-
	3	*Aeromonas salmonicida* 8.6×10^{6} CFU ml^{-1}	60 min	Yes		-	-
Double Treatment	4	*Carnobacterium divergens* 3.2×10^{7} CFU ml^{-1}	30 min	Yes	*Aeromonas salmonicida* 8.6×10^{6} CFU ml^{-1}	30 min	Yes
	5	*Aeromonas salmonicida* 8.6×10^{6} CFU ml^{-1}	30 min	Yes	*Carnobacterium divergens* 3.2×10^{7} CFU ml^{-1}	30 min	Yes

CFU–Colony forming units

Table 2: Experimental treatment overview over exposure solution and duration applied to Atlantic salmon intestine *ex vivo*.

Histology sampling and image analysis of proximal intestine (PI)

Samples of PI from each diet and treatment groups were immediately fixed in McDowell's fixative [38] and prepared for transmission electron microscopy (TEM) analysis as described elsewhere [39]. TEM samples were washed twice in buffer (1% Sørensen's buffer) then post fixated in OsO_4. After a series of dehydration steps (70%-100% ethanol), the sample was incubated in propylene oxide before embedded in epoxy resin and polymerized for 48 hours at 60°C. TEM samples were sectioned 1 µm and stained using uranyl acetate as described elsewhere [40]. Ten random micrographs were taken from two individuals from each diet and treatment groups. The impacts of diet and treatment were monitored in terms of status of mitochondria (healthy [P1] or unhealthy [P12]), mitochondrial anchorage (P2), edema (P3), vacuolization (P4), presence of rodlet cells (P5), bacteria-like particles (P6), inter-epithelial lipid storage (P7), terminal web (P8), intraepithelial leukocytes (IEL) (P9), damaged microvilli (P10) and the presence of cell debris in the lumen (P11).

Intestinal microbiological analysis of distal intestine (DI)

Sampling of autochthonous (adhered) bacteria was carried out as previously described [37] following exposure to either saline or bacteria. DI's were placed in separate sterile Seward® Stomacher bags and added 2 ml saline. The homogenates were immediately transferred to Nunc tubes and flash frozen in liquid nitrogen. Homogenized intestinal samples were thawed on ice and DNA extracted as previously described [15] using 1 ml phosphate buffered saline to wash the samples.

The primers used in the present study have been used in a previous study [15]. Primer pair one (Fw: CTCAACCGDGGASGGT; Rv: TCCCCAGGCGGAGTG) was designed to capture a cluster of microbes from families *Bacillaceae, Planococcaceae, Staphylococcaceae* within order *Bacillales* and families *Carnobacteriaceae*, and *Enterococcaceae* within order *Lactobacillales*, including Carnobacteria, and is referred to as Bacilli. Primer pair two (Fw: CTGGGCGTAAAGCGCAT; Rv: TTAACGCGTTAGMTCCGAAAG) was designed to detect *Vibrionaceae* and *Aeromonadaceae*. The qPCR analyses were carried out in a 15 µl reaction mixture consisting of 0.37 µl primer solution (0.25 µM of each), 6.25 µl SYBR Green qPCR master mix 2x (Applied Biosystems), 5 µl DNA template and enough MilliQ water to bring the total reaction volume to 15 µl. The parameters were conducted as follows: initial denaturation of the DNA template at 94°C for 10 min; amplification of the DNA template for 40 cycles where each cycle consisted of denaturation at 94°C for 30 sec, annealing for 30 sec, and elongation at 72°C for 1 min. Annealing temperature was set to 60°C for analysis of *A. salmonicida*, and 58°C for analysis of *C. divergens*.

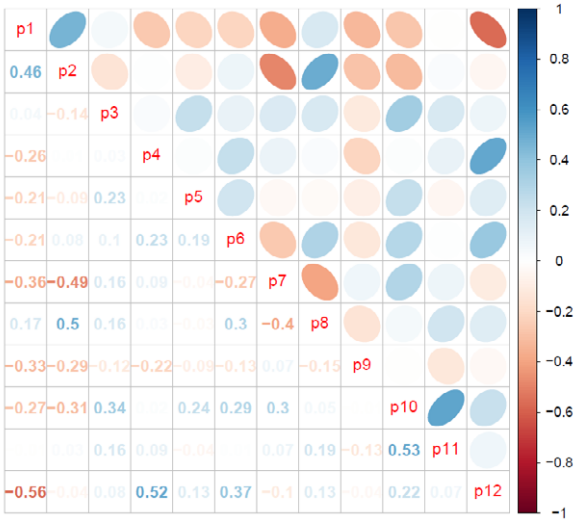

Figure 1: Correlogram based on the Spearman rank correlation between the different TEM parameters measured. Correlation coefficients are given in the lower triangle. Color denotes the strength of the correlation. If the correlation circle tilt to the left the correlation is negative, and if it tilts to the right the correlation is positive. When close to round, the correlation is close to neutral. The parameter for which the micrographs were scored for are: mitochondria (healthy [P1] or unhealthy [P12]), mitochondrial anchorage (P2), edema (P3), vacuolization (P4), presence of rodlet cells (P5), bacteria-like particles (P6), inter-epithelial lipid storage (P7), terminal web (P8), intraepithelial leukocytes (IEL) (P9), damaged microvilli (P10) and the presence of cell debris in the lumen (P11).

Following the amplification a melt curve analysis was carried out for 60 min at 0.5°C increments. All qPCR assays were performed using the StepOne Real-Time PCR System (Applied Biosystems) in 96-well plates and the threshold value was set at 53,700 fluorescent units as determined by the non-template control.

Statistical analysis

A Spearman rank correlation analysis was carried out to evaluate the correlation coefficients between the scores of the TEM micrographs on a scale from 0 to 1 (Figure 1). Electron microscopic scores were also analyzed using multilevel binomial model with an observation level random effect to evaluate the additive or interactive effect of treatment and diet. A multilevel model was necessary to acknowledge the fact that several individuals were examined from each replicate tank. P-values for this model are not defined because the actual degrees of freedom are unknown. Instead fixed effect of diet and treatment and their interaction was fitted with the help of an R-package (blme) and F-values estimated for the main effects and the interaction for each parameter P1-12.

Generally large F-values exceeding 2.5 is considered significant. Based on the fitted statistical model, the expected percentage of micrographs with the condition with 95% confidence interval was analyzed and is shown in Figure 2.

Effects of treatments and diets on the number of autochthonous *C. divergens* and *A. salmonicida* were analyzed using general linear models. Since the bacteria counts are high, the normal distribution could be used as an approximation of the Poisson distribution. Due to the wide range of counts, all counts were transformed to logarithms before analysis (1 was added to all counts before logging to avoid taking a logarithm of zero). Likelihood tests were run on nested models of diet and treatment to evaluate effect of diet, treatment and the interaction between these. All statistical analyses were carried out with the R language [41].

Results

Intestinal histology of PI

The Spearman rank correlation (Figure 1) of the TEM micrographs show that there is a strong positive correlation coefficient between the damaged microvilli and presence of cell debris in the lumen (0.53), consistent with the effects of exposure of the mucosal lining to *A. salmonicida*. There was also a strong correlation between the prevalence of mitochondria with an unhealthy appearance and increased vacuolization (0.52). A strong negative correlation was observed between the prevalence of mitochondria with a healthy and an unhealthy appearance in the micrographs (-0.56).

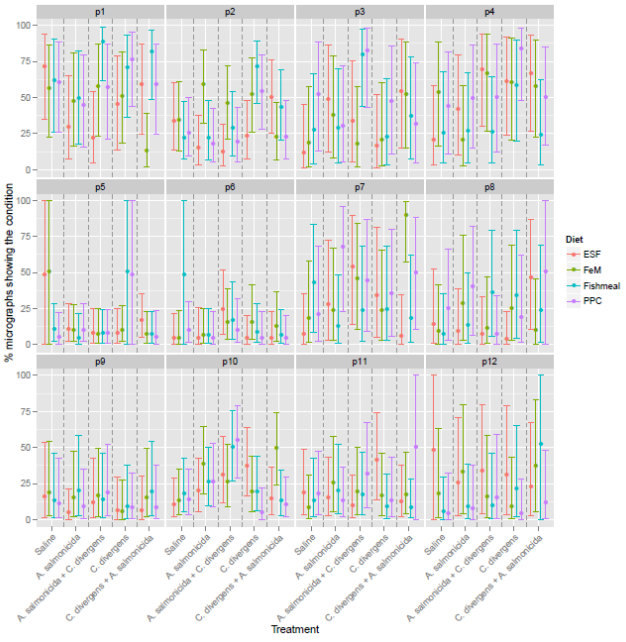

Figure 2: Expected % of 10 micrographs per fish showing the specific condition with 95% confidence intervals as indicated by the fitted statistical model. Subplots denote conditions p1-p12 (denoted in strip text). Colors represent background diets. The parameter for which the micrographs were scored for are: mitochondria (healthy [P1] or unhealthy [P12]), mitochondrial anchorage (P2), edema (P3), vacuolization (P4), presence of rodlet cells (P5), bacteria-like particles (P6), inter-epithelial lipid storage (P7), terminal web (P8), intraepithelial leukocytes (IEL) (P9), damaged microvilli (P10) and the presence of cell debris in the lumen (P11).

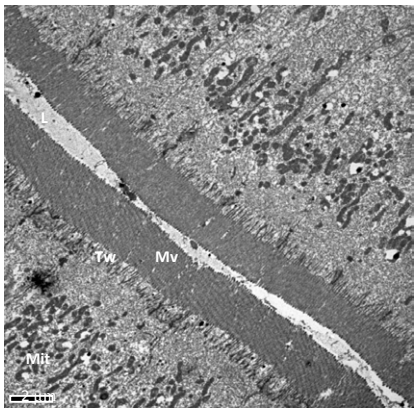

Figure 3: Transmission electron micrograph of tissue from fish fed feather meal and exposed to saline. The micrograph shows undamaged microvilli, enterocytes and mitochondria.
L: Lumen; Tw: Terminal web; Mv: Microvilli; Mit: Mitochondria

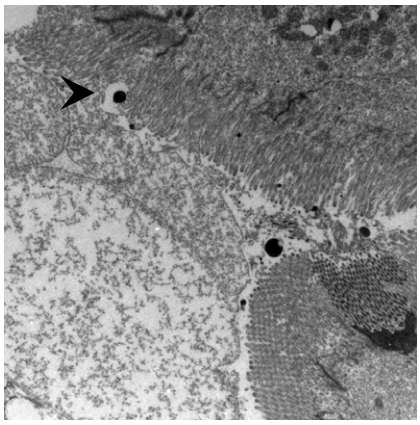

Figure 4: Presence of bacteria-like cells (arrowhead) in the midst of the microvilli of fish fed ESF and exposed to *A. salmonicida* for 60 minutes.

Control samples from PI of each dietary group exposed to saline showed normal appearance of enterocytes. The enterocytes had normal undamaged microvilli and intact apical tight junctions, indicating that diet did not significantly affect intestinal histomorphology.

Effect of exposure to *C. divergens* on intestinal histology

Following exposure to *C. divergens* (Figure 2) TEM showed an apparent improvement of the intestinal structure. Generally there were lower frequency of intra-epithelial leucocytes (IEL's), lower frequency of debris in the lumen and a higher frequency of healthy looking mitochondria. Following use of FeM (Figure 3), PPC and ESF intestinal structure appeared normal and did not statistically differ from the FM control group.

Effect of exposure to *A. salmonicida* on intestinal histology

Intestinal tissue exposed to *A. salmonicida* showed sign of damage: disrupted microvilli, damaged enterocytes and cell components in the form of debris in the lumen. These detrimental changes were observed in intestine from fish fed FM, PPC and ESF (Figure 2) from low frequencies as no more than 3 micrographs from each individual showed signs of tissue damage. In fish fed FeM however the detrimental

changes were observed in medium frequencies as up to 7 micrographs per individual showed tissue damage (Figure 2). Bacteria-like structures were observed between the microvilli (Figure 4). Following feeding with ESF and exposure to *A. salmonicida* an aggregation of rodlet cells was observed (Figure 5) which were not observed in any of the other groups.

Effect of exposure to *C. divergens* prior to *A. salmonicida* on the intestinal structure

Intestines exposed to *C. divergens* prior to *A. salmonicida* generally showed similar appearance of intestinal structure to that of the control groups (exposed to saline) (Figure 2). Fish fed FeM prior to exposure to the bacterial strains, however, showed excess lipid vacuoles (Figure 6).

Effect of exposure to *A. salmonicida* prior to *C. divergens* on the intestinal structure

Intestines exposed to *A. salmonicida* prior to *C. divergens* showed a general increase in tissue edema and vacuolization (Figure 2) which was observed in all dietary groups. There was also an apparent decrease in the prevalence of healthy mitochondria in fish fed FeM compared

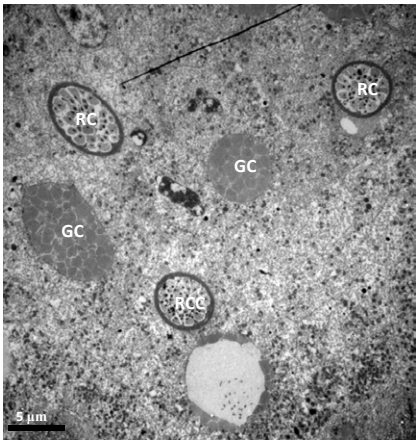

Figure 5: Aggregation of rodlet cells in tissue from fish fed extracted sunflower and exposed to *A. salmonicida*. Transmission electron micrograph shows rodlet cells in close proximity. Rodlet cells are recognized by their characteristic thick outer sheath and inner rodlets.
Rc: Rodlet cell; Gc: Goblet cell

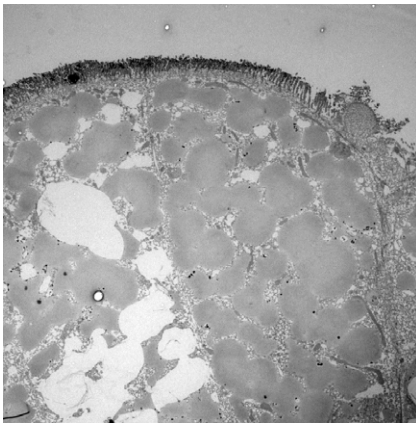

Figure 6: Excess lipid storage in fish fed FeM and exposed to *A. salmonicida* for a period of 60 minutes.

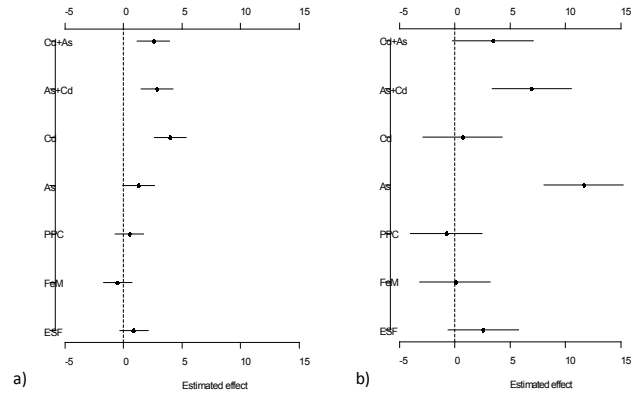

Figure 7: Estimated effects of diet and pathogen treatment on attachment of a) *Bacilli* and b) *Vibrionaceae* in comparison to the fishmeal control with saline (denoted by the dashed line at zero) from the general linear model. The dots denote the estimated mean effect and the lines 95% confidence intervals (CI). Effects with 95% CI not touching the zero line are considered statistically significant at P<0.05.

ESF: Sunflower meal; FeM: Feather meal; PPC: Pea protein concentrate; A.s: *Aeromonas salmonicida*; C.d: *Carnobacterium divergens*

	Diet	Treatment	Interaction
P1	1,88	0,99	1,40
P2	0,73	**3,61**	2,49
P3	0,62	1,76	1,21
P4	1,26	2,18	0,61
P5	0,29	0,10	0,33
P6	0,34	1,94	0,51
P7	0,93	0,81	1,76
P8	0,25	0,61	1,05
P9	0,19	0,93	0,43
P10	0,23	**3,86**	1,76
P11	0,20	0,32	0,70
P12	1,97	0,35	0,37

Table 3: F-values for the fixed effects of diet, treatment and interaction of both from scoring of intestinal micrographs. F=2.5 is considered significant.

fish fed FM, PPC and ESF. Lipid storage and vacuolization of the enterocytes increased in fish fed ESF, PPC and FeM increased compared to the FM control group. There were also an apparent increase in the prevalence of damaged microvilli in fish fed FM and PPC compared to fish fed ESF and FeM.

Intestinal microbiota

By exposing intestinal tissue to saline; effect of diet on endogenous levels of *C. divergens* and *A. salmonicida* was investigated. Results show that endogenous levels of *C. divergens* and *A. salmonicida* were not significantly affected by diet compared to fish feed FM (Figure 7a and 7b).

Adherence of *C. divergens* to the distal intestine

Exposure to either *C. divergens* alone (treatment 2; Table 2) or the two combination treatments, (treatment 4 and 5; Table 2) revealed significantly increased adherence of *C. divergens* compared to the saline exposed control group (Figure 7a). When intestine was exposed to *A. salmonicida* (treatment 3; Table 2), adherence of *C. divergens* was not different from control group.

Diet did not significantly affect the adherence of *C. divergens* and there was no interaction between exposure treatment and diet (Table 3).

Adherence of *A. salmonicida* to the intestine

Ex vivo exposure to *A. salmonicida*, and *A. salmonicida* prior to *C. divergens*, resulted in a significant increase in adherent *A. salmonicida* compared to fish fed FM and exposed to saline (Figure 7b). Levels of adherent *A. salmonicida* increased numerically but the effect remained non-significant following exposure to *C. divergence* prior to *A. salmonicida* indicating a hindrance in adherence of the pathogen by the probiotic compared to the increase in *A. salmonicida* following exposure to *A. salmonicida* and *A. salmonicida* prior to *C. divergens*. Diet did not significantly affect the adherence of *A. salmonicida* and there was no interaction between exposure treatment and diet (Table 3).

Discussion

Based on the results of the present study it is apparent that both *C. divergens* and *A. salmonicida* have an inherent capability to adhere to the DI of Atlantic salmon. For *C. divergens* these result are in accordance to Ringø who reported that *C. divergens* was able to colonize the gut of early developing turbot (*Scophthalmus maximus* L.) [48] and Jöborn et al. which reported colonization of *Carnobacterium* sp. strain K1 in rainbow trout (*Oncorhynchus mykiss* Walbaum) fingerlings [5]. Carnobacteria are reported to be a natural part of the endogenous microbiota in several fish species [41-49]. The number of adhered *A. salmonicida* however was almost three-fold higher that of *C. divergens*. The mechanism involved to give the pathogen such advantage in adhering to the mucosal lining is unknown. Host specificity may be involved; however, as both bacteria used in the present study originally were isolated from Atlantic salmon, this is unlikely. A more likely explanation may be that *A. salmonicida* is an opportunistic bacterium which in order to enhance its own adherence may displace the endogenous autochthonous bacteria. Displacement of the endogenous gut microbiota has previously been shown in Arctic charr (*Salvelinus alpinus* L.) following *in vivo* challenge with *A. salmonicida* [27]. In order to clarify the mechanism involved in the improved adherence of *A. salmonicida* to fish mucosal lining, this topic merits further studies.

The results of the combination treatment show that the level of *C. divergens* was similar in both treatments, but the levels were lower than when the intestine was exposed to *C. divergens* alone. In the case where *C. divergens* was allowed to adhere prior to *A. salmonicida* the results suggest that *A. salmonicida* is able to displace to some degree the adherent *C. divergens*. Furthermore, as the levels of *A. salmonicida* were lower than when the pathogen was exposed alone these results indicate that *C. divergens* is able to exclude *A. salmonicida* from binding sites in the mucosal lining. Following the treatment where *A. salmonicida* were exposed prior to *C. divergens*, the level of adherent *A. salmonicida* was lower than singular exposure, indicating a displacement of the pathogen by the probiotic bacteria. The mechanism for this ability however is unknown. Probiotics are known to have mechanisms which hinder pathogen bacteria from attaching and even prevent them from invading [50] however it is yet unknown which mechanism probiotic bacteria uses to displace pathogens, or if this is up-regulated in the presence of pathogenic bacteria.

During the last decade, several studies have been published about the importance of probiotics in protection against disease through stimulation of the immune system [51-54]. In the present study exposure of *A. salmonicida* prior to *C. divergens* resulted in the presence of IEL's within the enterocytes lying parallel to the lamina propria. These cells were not observed when intestine was first exposed to *C. divergens* prior to *A. salmonicida*. These results suggest that adherence of *C. divergens* to the mucus prior to the appearance of *A. salmonicida* and most likely had a prohibitive effect against the pathogen and prevented attachment to the mucosal lining and probably blocking stimulation of the immune response. Evaluation of the intestinal immune response in studies using the intestinal sack method may shed light on the role of the immune system in the prevention of adherence of pathogen bacteria and merits further investigations.

Several studies have evaluated the effect of co-incubating a probiotic and pathogen bacteria on intestinal tissue of Atlantic salmon [24,33] and beluga (*Huso huso*) [31]. Ringø and colleagues reported undamaged microvilli and abundant goblet cells and leucocytes after exposing Atlantic salmon intestine to *A. salmonicida* prior to *C. divergens* indicating an alleviation of the potential intestinal damage caused by the pathogen [24]. Furthermore, Salinas et al. reported an alleviation of the damages caused by the presence of *A. salmonicida* following pre-treatment with *Lactobacillus delbrueckii* ssp. *lactis* in Atlantic salmon [33]. Following pre-treatment with *Leuconostoc mesenteroides* and subsequent exposure to *Staphylococcus aureus* no damage was observed in the intestinal tissue from beluga (*Huso huso*) indicating a protective ability of *L. mesenteroides* [31]. In the present study, pre-treatment with *C. divergens* resulted in less severe damage (observed as less prevalence of damaged microvilli and less luminal debris) by *A. salmonicida* compared to tissue only exposed to the pathogen alone indicating an alleviating effect of the pathogen by the probiotic. As *C. divergens* is accepted as part of the endogenous intestinal microbiota in Atlantic salmon, these results suggest that the bacterium may play an important role in the protection against *A. salmonicida*.

Ringø et al. reported intact Atlantic salmon intestine; proximal part following exposure to *C. divergens* at 6×10^6 bacteria ml^{-1} [24]. Similarly, Kristiansen et al. reported undamaged intestinal structure following feeding with prebiotics and exposure to *C. divergens* [37] and Løvmo Martinsen et al. displayed that *C. maltaromaticum* did not cause changes in the intestinal structure following exposure to Atlantic cod (*Gadus morhua* L.) intestine [26]. In the present study, exposure of the PI of Atlantic salmon to *C. divergens* did not cause damage to the mucosal structure hence supporting previously reported results [24].

Probiotics are able to attach to and pass through the intestinal wall without causing damage to the structural integrity [33,37] indicating a non-destructive mode of entrance. *A. salmonicida* may cause damage to the intestinal structure after exposure [55,56]. Damaged microvilli, increased excitation of enterocytes observed as increased luminal debris and increased occurrence of dense chromatin are all observed following the presence of the pathogen in the salmon intestine suggesting that the mode of action for the bacteria to invade the tissue is by disrupting the integrity of the intestinal wall [57, present study]. Furthermore as exposure to *A. salmonicida* resulted in the presence of bacteria-like structures observed between the microvilli, these results support the suggestion that the PI can be used as an entrance site for pathogens in Atlantic salmon. The mechanism by which *A. salmonicida* has the ability to gain entrance through the intestinal mucosa is attributed to its extracellular products known to be important for its virulence and pathogenicity [58]. It is also likely that the potent exotoxins released by *A. salmonicida* can affect surrounding microbiota as well as host tissues. Ringø et al. reported a significant reduction in the autochthonous bacteria following exposure to *A. salmonicida* indicating an ability to exclude the endogenous bacteria likely through the production of exo- and endotoxins [27]. The results of the present study are consistent with that previously reported investigating the effect of *A. salmonicida* exposed *ex vivo* to Atlantic salmon intestine [12,24,33].

Dietary components are known to influence both intestinal microbiota and intestinal structural integrity [28,29,39]. Observations of the PI from fish fed FeM and exposure to *A. salmonicida* revealed an intensification of the damage caused by the pathogen. It is uncertain why the use this alternative raw material caused this. Use of FeM and exposure to saline revealed no structural changes. A recent paper from a related study revealed that although no morphological changes were observed using light microscopy using FeM, the feed ingredient caused increased organosomatic index as well as increased brush-border membrane associated leucine aminopeptidase (BBM-LAP) [15] which may compromise the enterocytes making them more susceptible to damage by opportunistic pathogen bacteria. A possible interaction between use of alternative feed ingredients and the presence of opportunistic pathogen bacteria merits further investigation to avoid rendering the host susceptible to disease by intestinal invasion.

In the present study ESF seem to facilitate increased, albeit insignificant, adherence of both *C. divergens* and *A. salmonicida*, which may be a result of disorganized microvilli providing more binding sites. Following observation of inflammatory response in the DI in Atlantic salmon fed soybean meal Krogdahl et al. suggested that the enteritis might affect the integrity of the epithelial barrier resulting in increased susceptibility to pathogenic infection [59]. As there were no apparent changes in the intestinal structure following inclusion of ESF to the diet the reason for the increased adherence may be yet unknown and effect of diet on the binding mechanism of bacteria merits further study.

Conclusion

The present study investigates the effect of alternative protein sources on the adherence of a probiotic, *C. divergens* and a pathogen, *A. salmonicida* bacteria to the mucosal lining of Atlantic salmon intestine using *ex vivo* methodology. In conclusion this study has shown that inclusion of pea protein concentrate, extracted sunflower and feather meal will not significantly affect the adherence of the bacteria to the intestinal lining. Furthermore none of the chosen protein sources resulted in changes in the intestinal structure following exposure to saline or to the probiotic. It was however evident that use of feather meal may intensify the damage caused by *A. salmonicida* to the mucosal lining. As some differences in adherence was observed following a 30 minute exposure and a 60 minute exposure future studies undertaken to use the intestinal sac method to evaluate adherence of bacteria should consider also adding a bacterial control group where the intestine is first exposed to 30 minutes with bacteria followed by 30 minutes with saline.

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