1 ***Probiotic and pathogen ex vivo exposure of Atlantic salmon (Salmo salar L.) intestine***
2 ***from fish fed four different protein sources.***
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26 **Abstract**

27 The present study addressed the adherence of *Carnobacterium divergens* and *Aeromonas*
28 *salmonicida* subsp. *salmonicida* to the intestinal lining of Atlantic salmon (*Salmo salar* L.)
29 using an *ex vivo* method – intestinal sac - following feeding with pea protein concentrate,
30 extracted sunflower or feather meal at 200 g/kg inclusion level. Control diet was a 450g/kg
31 fishmeal diet. The experimental feeds were fed to two groups each for seven weeks at
32 EWOS Innovations' facilities in Lønningdal, Norway. *Ex vivo* intestinal challenge was
33 carried out at Institute for Marine Research, Bergen. Excised intestines of salmon from all
34 feeding groups were exposed to a probiotic, *C. divergens* or a pathogen, *A. salmonicida*
35 either alone or in combination and control samples were exposed to sterile saline solution.
36 Exposure to *A. salmonicida* caused severe damage to the intestinal ultrastructure of the
37 mid intestine, but after exposure to *C. divergens*, sterile saline solution or any of the
38 combination treatments, morphology remained mostly unaltered indicating an alleviating
39 effect of the probiotic. Feather meal intensified the damaging effect of exposure to *A.*
40 *salmonicida* and there were otherwise no effect of diet on the morphology.
41 qPCR analysis of adhered *C. divergens* and *A. salmonicida* showed that although the
42 pathogen has a higher adherence efficiency, *C. divergens* was more efficient at displacing
43 the pathogen if allowed to adhere to the mucosal lining first indicating that the probiotic
44 should be present prior to the pathogen for optimal effect. There were no dietary effects on
45 bacterial adherence.
46 The present study shows that use of some commercially available alternative feed
47 ingredients may not affect the probiotic abilities of *C. divergens* or make the fish more
48 susceptible to disease through intestinal invasion.

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50

51 **Introduction**

52 Fish possess an indigenous intestinal microbiota which it is under constant challenge from
53 non-commensal bacterial populations [1, 2, 3]. Several investigations have shown that
54 *Carnobacterium* spp. are a natural part of the gut microbiota in salmonids [4-15] and that
55 they display antimicrobial abilities and *in vitro* growth inhibition of several fish pathogens
56 including *Aeromonas salmonicida* spp. *salmonicida* (*A. salmonicida*) [16,17] a well-
57 known fish pathogen of salmonids [18]. Carnobacteria has been suggested as probiotics
58 [19] but a favorable criterion of a probiotic bacterium is its ability to adhere to and grow in
59 the mucus or on the enterocyte surface and is an important criteria when evaluating the use
60 of probiotics in endothermic animals as well as in fish [20,21,42-44].
61 Previous studies has shown that gastrointestinal (GI) tract in fish is one of the major
62 infection routes for *A. salmonicida* [12, 22, 23] and other pathogens [24, 32, 45, 46].
63 Furthermore, some studies have shown that exposing fish intestine to *Carnobacterium* ssp.
64 and a pathogen bacteria result in alleviation to some degree of the potentially damaging
65 effect of the pathogen bacteria [24-26]. This is however difficult to measure *in vivo* and
66 during the last few years the *ex vivo* intestinal sack method has been used in several studies
67 to evaluate possible histological and bacteriological changes in the fish intestine after
68 exposure to high levels of lactic acid bacteria (LAB) and pathogens [24-26,37]. In the
69 present study, the *ex vivo* method was applied to circumvent the uncertainty of an *in vivo*
70 experiment because it has proved useful in evaluating bacteriological effects on intestinal
71 tissue under controlled experimental conditions [33]. This method has been developed
72 according to the EU recommendation to reduce the number of *in vivo* experiments and the
73 number of fish used (Revision of the EU directive for the protection of animals used for
74 scientific purposes [Directive 86/609/EEC]; 08th September 2010). However, the method
75 has limitation by the viability of the tissue once it has been removed from the host.

76 Therefore only one hour of incubation has been used. Prolonged incubation; > one hour
77 following excision of the tissue may result in natural degradation making the negative
78 effects as result of bacterial exposure indiscernible. Due to the short term durability of the
79 *ex vivo* method, results generated should only be considered a snapshot of the whole story,
80 and although will not replace *in vivo* experiments, may contribute to reduce the number of
81 fish in subsequent *in vivo* trials. The present study used live bacteria as previous studies
82 have shown epithelial damage and bacterial adherence as a result of exposure to live
83 bacteria [12,24,31,33,37], higher bacterial translocation than using heat-inactivated
84 bacteria [23] and enhanced stimulation of phagocytotic activity [47].
85 Fishmeal (FM) has become a limited feed ingredient and the dependency on marine
86 protein is alleviated using alternative feed ingredients. Alternative terrestrial proteins,
87 especially plant based raw materials may contain anti-nutritional factors (ANF) which
88 have shown to have potential negative effects for the intestinal morphology in Atlantic
89 salmon [29, 39]. Dietary alterations have shown to influence the indigenous intestinal
90 microbiota in fish [27-30] as well as modulate changes in the intestinal mucosal integrity
91 [28, 29]. Although there is information available on the influence of dietary manipulations
92 on the endogenous intestinal microbiota in fish, few studies have investigated the
93 importance of diet on the susceptibility to pathogenic bacteria [26,31] and less information
94 is available on the modulation of the gut microbiota using animal proteins or oils in the
95 diet [27,32].
96 The purpose of the present study was therefore to investigate whether *C. divergens*
97 originally isolated from the digestive tract of Atlantic salmon (*Salmo salar* L.) [4] could
98 exclude and displace *A. salmonicida* in the Atlantic salmon intestine by using the
99 intestinal sack method; a method used in some previous studies [26, 31, 33, 34]. The
100 intestinal sac method was chosen for its simplicity and superior efficiency over *in vivo*

101 methods [31], however by using this method one should bear in mind that it is restricted to
102 short term experiments as the viability of intestinal tissue is limited once it is excised from
103 the host. This was addressed in two parts; assessment of the effect of *ex vivo* exposure of
104 the intestinal to; a) saline, b) *C. divergens*, c) *A. salmonicida*, d) first to *C. divergens*
105 thereafter to *A. salmonicida*, and finally, e) first to *A. salmonicida* thereafter to *C.*
106 *divergens*. Moreover, the present study addresses the effect of dietary; a) fishmeal, b) pea
107 protein concentrate, c) extracted sunflower and d) feather meal on intestinal morphology.
108

109 Materials and methods

110 Preparation of experimental diets

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

119 Fish and rearing conditions

120 Sixty unvaccinated, sea-water adapted Atlantic salmon with initial mean weight of 328 ±
121 68 grams was used. The fish were tagged using a passive integrated transponder (PIT) for
122 identification and allocated into eight tanks at EWOS Innovation research facility in
123 Lønningdal, Norway. During the four week acclimatization period, the fish were fed a
124 commercial feed (EWOS Opal 50, EWOS, Norway) to satiation twice a day. Temperature
125 (mean 8°C) and salinity (mean 32 ‰) were measured daily, while dissolved oxygen (DO)

126 remained above 77% for the duration of the trial. Post acclimatization, the fish were fed
127 the trial diets for seven weeks and thereafter transferred to challenge facilities at the
128 Institute of Marine Research (Bergen, Norway). After the transfer, the fish were fed for
129 two additional weeks for acclimatization in order to reduce stress related effects prior to *ex*
130 *vivo* challenge experiment. No mortalities were recorded for the duration of the trial.

131 **Bacterial suspensions**

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

143 **Ex vivo intestinal exposure**

144 *Ex vivo* exposure of the intestines to the bacterial strains was performed using the intestinal
145 sac method as previously described in several studies [12, 25, 26, 31, 34, 37] with some
146 modifications. Prior to the *ex vivo* challenge fish were starved for 24 hours and sacrificed
147 with a blow to the head. Briefly, the entire intestine, from behind of the last pyloric caeca
148 to the anus was removed aseptically and flushed three times using sterile physiological
149 saline (0.9‰) to remove allochthonous (non-adherent) bacteria. The distal end was closed
150 tight using a cotton string before filling with the appropriate treatment solution (Table 2).

151 In the control group, the intestine was exposed only to sterile saline solution. Intestines
152 exposed to saline or bacteria were incubated in Falcon tubes containing saline for one hour
153 at 10°C. In two treatments exposed to *C. divergens* and *A. salmonicida*; treatment 4 and *A.*
154 *salmonicida* and *C. divergens*; treatment 5 the intestines were first exposed to bacteria for
155 30 min. cut open, emptied and flushed 3 times by saline, and thereafter exposed to the 2nd
156 bacteria. Intestines from four individual fish per dietary groups were subjected to each of
157 the treatments described in Table 2. All intestines were flushed three times prior to and
158 post incubation using three ml saline with every rinse to ensure that only the
159 autochthonous bacteria were sampled. In order to obtain enough samples for each analysis,
160 samples for histological analysis were taken from pyloric intestine (PI) and samples for
161 autochthonous bacteria were taken from DI.

162 **Histology sampling and image analysis of proximal intestine (PI)**

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

176 **Intestinal microbiological analysis of distal intestine (DI)**

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

183 The primers used in the present study have been used in a previous study [15]. Primer pair
184 one (Fw: CTCAACCGDGGASGGT ; Rv: TCCCCAGGCGGAGTG) was designed to
185 capture a cluster of microbes from families *Bacillaceae, Planococcaceae,*
186 *Staphylococcaceae* within order *Bacillales* and families *Carnobacteriaceae*, and
187 *Enterococcaceae* within order *Lactobacillales*, including Carnobacteria, and is referred to
188 as Bacilli. Primer pair two (Fw: CTGGGCGTAAAGCGCAT; Rv:
189 TTAACGCGTTAGMTCCGAAAG) was designed to detect *Vibrionaceae* and
190 *Aeromonadaceae*. The qPCR analyses were carried out in a 15 µl reaction mixture
191 consisting of 0.37 µl primer solution (0.25 µM of each), 6.25 µl SYBR Green qPCR
192 master mix 2x (Applied Biosystems), 5 µl DNA template and enough MilliQ water to
193 bring the total reaction volume to 15 µl. The parameters were conducted as follows: initial
194 denaturation of the DNA template at 94°C for 10 min; amplification of the DNA template
195 for 40 cycles where each cycle consisted of denaturation at 94°C for 30 sec, annealing for
196 30 sec, and elongation at 72°C for 1 min. Annealing temperature was set to 60°C for
197 analysis of *A. salmonicida*, and 58°C for analysis of *C. divergens*. Following the
198 amplification a melt curve analysis was carried out for 60 min at 0.5°C increments. All
199 qPCR assays were performed using the StepOne Real-Time PCR System (Applied

200 Biosystems) in 96-well plates and the threshold value was set at 53,700 fluorescent units as
201 determined by the non-template control.

202 **Statistical analysis**

203 A Spearman rank correlation analysis was carried out to evaluate the correlation
204 coefficients between the scores of the TEM micrographs on a scale from 0 to 1 (Fig.1).
205 Electron microscopic scores were also analyzed using multilevel binomial model with an
206 observation level random effect to evaluate the additive or interactive effect of treatment
207 and diet. A multilevel model was necessary to acknowledge the fact that several
208 individuals were examined from each replicate tank. P-values for this model are not
209 defined because the actual degrees of freedom are unknown. Instead fixed effect of diet
210 and treatment and their interaction was fitted with the help of an R-package (blme) and F-
211 values estimated for the main effects and the interaction for each parameter P1-12.
212 Generally large F-values exceeding 2.5 is considered significant. Based on the fitted
213 statistical model, the expected percentage of micrographs with the condition with 95%
214 confidence interval was analyzed and is shown in figure 2.

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

223 ## Results

224 ### Intestinal histology of PI

225 The Spearman rank correlation (Fig. 1) of the TEM micrographs show that there is a
226 strong positive correlation coefficient between the damaged microvilli and presence of cell
227 debris in the lumen (0.53), consistent with the effects of exposure of the mucosal lining to
228 *A. salmonicida*. There was also a strong correlation between the prevalence of
229 mitochondria with an unhealthy appearance and increased vacuolization (0.52). A strong
230 negative correlation was observed between the prevalence of mitochondria with a healthy
231 and an unhealthy appearance in the micrographs (-0.56).
232 Control samples from PI of each dietary group exposed to saline showed normal
233 appearance of enterocytes. The enterocytes had normal undamaged microvilli and intact
234 apical tight junctions, indicating that diet did not significantly affect intestinal
235 histomorphology.

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

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

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

243 Intestinal tissue exposed to *A. salmonicida* showed sign of damage: disrupted microvilli,
244 damaged enterocytes and cell components in the form of debris in the lumen. These
245 detrimental changes were observed in intestine from fish fed FM, PPC and ESF (Fig. 2)
246 from low frequencies as no more than 3 micrographs from each individual showed signs of
247 tissue damage. In fish fed FeM however the detrimental changes were observed in medium
248 frequencies as up to 7 micrographs per individual showed tissue damage (Fig. 2). Bacteria-
249 like structures were observed between the microvilli (Fig. 4). Following feeding with ESF

250 and exposure to *A. salmonicida* an aggregation of rodlet cells was observed (Fig. 5) which
251 were not observed in any of the other groups.

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

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

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

258 Intestines exposed to *A. salmonicida* prior to *C. divergens* showed a general increase in
259 tissue edema and vacuolization (Fig. 2) which was observed in all dietary groups. There
260 was also an apparent decrease in the prevalence of healthy mitochondria in fish fed FeM
261 compared fish fed FM, PPC and ESF. Lipid storage and vacuolization of the enterocytes
262 increased in fish fed ESF, PPC and FeM increased compared to the FM control group.
263 There were also an apparent increase in the prevalence of damaged microvilli in fish fed
264 FM and PPC compared to fish fed ESF and FeM.

265 Intestinal microbiota

266 By exposing intestinal tissue to saline; effect of diet on endogenous levels of *C. divergens*
267 and *A. salmonicida* was investigated. Results show that endogenous levels of *C. divergens*
268 and *A. salmonicida* were not significantly affected by diet compared to fish feed FM (Fig.
269 7a, b)

270 **Adherence of C. divergens to the distal intestine**

271 Exposure to either *C. divergens* alone (treatment 2; Table 2) or the two combination
272 treatments, (treatment 4 and 5; Table 2) revealed significantly increased adherence of *C.*
273 *divergens* compared to the saline exposed control group (Figure 7a). When intestine was

274 exposed to *A. salmonicida* (treatment 3; Table 2), adherence of *C. divergens* was not
275 different from control group.
276 Diet did not significantly affect the adherence of *C. divergens* and there was no interaction
277 between exposure treatment and diet (Table 3).

278 **Adherence of A. salmonicida to the intestine**

279 *Ex vivo* exposure to *A. salmonicida*, and *A. salmonicida* prior to *C. divergens*, resulted in a
280 significant increase in adherent *A. salmonicida* compared to fish fed FM and exposed to
281 saline (Figure 7b). Levels of adherent *A. salmonicida* increased numerically but the effect
282 remained non-significant following exposure to *C. divergence* prior to *A. salmonicida*
283 indicating a hindrance in adherence of the pathogen by the probiotic compared to the
284 increase in *A. salmonicida* following exposure to *A. salmonicida* and *A. salmonicida* prior
285 to *C. divergens*. Diet did not significantly affect the adherence of *A. salmonicida* and there
286 was no interaction between exposure treatment and diet (Table 3).
287

288 ## Discussion

289 Based on the results of the present study it is apparent that both *C. divergens* and *A.*
290 *salmonicida* have an inherent capability to adhere to the DI of Atlantic salmon. For *C.*
291 *divergens* these result are in accordance to Ringø who reported that *C. divergens* was able
292 to colonize the gut of early developing turbot (*Scophthalmus maximus* L.) [48] and Jöborn
293 *et al*. which reported colonization of *Carnobacterium* sp. strain K1 in rainbow trout
294 (*Oncorhynchus mykiss* Walbaum) fingerlings [5]. Carnobacteria are reported to be a
295 natural part of the endogenous microbiota in several fish species [3,4,7,14,29,49]. The
296 number of adhered *A. salmonicida* however was almost three-fold higher that of *C.*
297 *divergens*. The mechanism involved to give the pathogen such advantage in adhering to
298 the mucosal lining is unknown. Host specificity may be involved; however, as both

299 bacteria used in the present study originally were isolated from Atlantic salmon, this is
300 unlikely. A more likely explanation may be that *A. salmonicida* is an opportunistic
301 bacterium which in order to enhance its own adherence may displace the endogenous
302 autochthonous bacteria. Displacement of the endogenous gut microbiota has previously
303 been shown in Arctic charr (*Salvelinus alpinus* L.) following *in vivo* challenge with *A.*
304 *salmonicida* [27]. In order to clarify the mechanism involved in the improved adherence of
305 *A. salmonicida* to fish mucosal lining, this topic merits further studies.
306 The results of the combination treatment show that the level of *C. divergens* was similar in
307 both treatments, but the levels were lower than when the intestine was exposed to *C.*
308 *divergens* alone. In the case where *C. divergens* was allowed to adhere prior to *A.*
309 *salmonicida* the results suggest that *A. salmonicida* is able to displace to some degree the
310 adherent *C. divergens*. Furthermore, as the levels of *A. salmonicida* were lower than when
311 the pathogen was exposed alone these results indicate that *C. divergens* is able to exclude
312 *A. salmonicida* from binding sites in the mucosal lining. Following the treatment where *A.*
313 *salmonicida* were exposed prior to *C. divergens*, the level of adherent *A. salmonicida* was
314 lower than singular exposure, indicating a displacement of the pathogen by the probiotic
315 bacteria. The mechanism for this ability however is unknown. Probiotics are known to
316 have mechanisms which hinder pathogen bacteria from attaching and even prevent them
317 from invading [50] however it is yet unknown which mechanism probiotic bacteria uses to
318 displace pathogens, or if this is up-regulated in the presence of pathogenic bacteria.
319 During the last decade, several studies have been published about the importance of
320 probiotics in protection against disease through stimulation of the immune system [25,51-
321 54]. In the present study exposure of *A. salmonicida* prior to *C. divergens* resulted in the
322 presence of IEL's within the enterocytes lying parallel to the lamina propria. These cells
323 were not observed when intestine was first exposed to *C. divergens* prior to *A.*

324 *salmonicida*. These results suggest that adherence of *C. divergens* to the mucus prior to the
325 appearance of *A. salmonicida* and most likely had a prohibitive effect against the pathogen
326 and prevented attachment to the mucosal lining and probably blocking stimulation of the
327 immune response. Evaluation of the intestinal immune response in studies using the
328 intestinal sack method may shed light on the role of the immune system in the prevention
329 of adherence of pathogen bacteria and merits further investigations.
330 Several studies have evaluated the effect of co-incubating a probiotic and pathogen
331 bacteria on intestinal tissue of Atlantic salmon [24, 33] and beluga (*Huso huso*) [31].
332 Ringø and colleagues reported undamaged microvilli and abundant goblet cells and
333 leucocytes after exposing Atlantic salmon intestine to *A. salmonicida* prior to *C. divergens*
334 indicating an alleviation of the potential intestinal damage caused by the pathogen [24].
335 Furthermore, Salinas *et al*. reported an alleviation of the damages caused by the presence
336 of *A. salmonicida* following pre-treatment with *Lactobacillus delbrueckii* ssp. *lactis* in
337 Atlantic salmon [33]. Following pre-treatment with *Leuconostoc mesenteroides* and
338 subsequent exposure to *Staphylococcus aureus* no damage was observed in the intestinal
339 tissue from beluga (*Huso huso*) indicating a protective ability of *L. mesenteroides* [31]. In
340 the present study, pre-treatment with *C. divergens* resulted in less severe damage
341 (observed as less prevalence of damaged microvilli and less luminal debris) by *A.*
342 *salmonicida* compared to tissue only exposed to the pathogen alone indicating an
343 alleviating effect of the pathogen by the probiotic. As *C. divergens* is accepted as part of
344 the endogenous intestinal microbiota in Atlantic salmon, these results suggest that the
345 bacterium may play an important role in the protection against *A. salmonicida*.
346 Ringø *et al* reported intact Atlantic salmon intestine; proximal part following exposure to
347 *C. divergens* at $6x10^6$ bacteria ml^{-1}[24]. Similarly, Kristiansen *et al* reported undamaged
348 intestinal structure following feeding with prebiotics and exposure to *C. divergens* [37] and

349 Løvmo Martinsen *et al*. displayed that *C. maltaromaticum* did not cause changes in the
350 intestinal structure following exposure to Atlantic cod (*Gadus morhua* L.) intestine [26]. In
351 the present study, exposure of the PI of Atlantic salmon to *C. divergens* did not cause
352 damage to the mucosal structure hence supporting previously reported results [24].
353 Probiotics are able to attach to and pass through the intestinal wall without causing damage
354 to the structural integrity [33, 37] indicating a non-destructive mode of entrance. *A.*
355 *salmonicida* may cause damage to the intestinal structure after exposure [55, 56].
356 Damaged microvilli, increased excitation of enterocytes observed as increased luminal
357 debris and increased occurrence of dense chromatin are all observed following the
358 presence of the pathogen in the salmon intestine suggesting that the mode of action for the
359 bacteria to invade the tissue is by disrupting the integrity of the intestinal wall [57, present
360 study]. Furthermore as exposure to *A. salmonicida* resulted in the presence of bacteria-like
361 structures observed between the microvilli, these results support the suggestion that the PI
362 can be used as an entrance site for pathogens in Atlantic salmon. The mechanism by
363 which *A. salmonicida* has the ability to gain entrance through the intestinal mucosa is
364 attributed to its extracellular products known to be important for its virulence and
365 pathogenicity [58]. It is also likely that the potent exotoxins released by *A. salmonicida*
366 can affect surrounding microbiota as well as host tissues. Ringø *et al*. reported a significant
367 reduction in the autochthonous bacteria following exposure to *A. salmonicida* indicating an
368 ability to exclude the endogenous bacteria likely through the production of exo- and
369 endotoxins [27]. The results of the present study are consistent with that previously
370 reported investigating the effect of *A. salmonicida* exposed *ex vivo* to Atlantic salmon
371 intestine [12, 24, 33].
372 Dietary components are known to influence both intestinal microbiota and intestinal
373 structural integrity [28, 29, 39]. Observations of the PI from fish fed FeM and exposure to

374 *A. salmonicida* revealed an intensification of the damage caused by the pathogen. It is
375 uncertain why the use this alternative raw material caused this. Use of FeM and exposure
376 to saline revealed no structural changes. A recent paper from a related study revealed that
377 although no morphological changes were observed using light microscopy using FeM, the
378 feed ingredient caused increased organosomatic index as well as increased brush-border
379 membrane associated leucine aminopeptidase (BBM-LAP) [15] which may compromise
380 the enterocytes making them more susceptible to damage by opportunistic pathogen
381 bacteria. A possible interaction between use of alternative feed ingredients and the
382 presence of opportunistic pathogen bacteria merits further investigation to avoid rendering
383 the host susceptible to disease by intestinal invasion.
384 In the present study ESF seem to facilitate increased, albeit insignificant, adherence of
385 both *C. divergens* and *A. salmonicida*, which may be a result of disorganized microvilli
386 providing more binding sites. Following observation of inflammatory response in the DI in
387 Atlantic salmon fed soybean meal Krogdahl *et al*. suggested that the enteritis might affect
388 the integrity of the epithelial barrier resulting in increased susceptibility to pathogenic
389 infection [59]. As there were no apparent changes in the intestinal structure following
390 inclusion of ESF to the diet the reason for the increased adherence may be yet unknown
391 and effect of diet on the binding mechanism of bacteria merits further study.
392
393 **Conclusive remarks**

394 The present study investigates the effect of alternative protein sources on the adherence of
395 a probiotic, *C. divergens* and a pathogen, *A. salmonicida* bacteria to the mucosal lining of
396 Atlantic salmon intestine using *ex vivo* methodology. In conclusion this study has shown
397 that inclusion of pea protein concentrate, extracted sunflower and feather meal will not
398 significantly affect the adherence of the bacteria to the intestinal lining. Furthermore none
399 of the chosen protein sources resulted in changes in the intestinal structure following

400 exposure to saline or to the probiotic. It was however evident that use of feather meal may
401 intensify the damage caused by *A. salmonicida* to the mucosal lining. As some differences
402 in adherence was observed following a 30 minute exposure and a 60 minute exposure
403 future studies undertaken to use the intestinal sac method to evaluate adherence of bacteria
404 should consider also adding a bacterial control group where the intestine is first exposed to
405 30 minutes with bacteria followed by 30 minutes with saline.
406
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415

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631 **Table legends**

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

633 FM: fishmeal, ESF: extracted sunflower; FeM: feather meal; PPC: pea protein concentrate

634 ¥ AgriMarin, Stavanger, Norway

635 ‡ Unknown

636 ‼ Ge-Pro, Germany

637 ∆ Fiskerens Fiskeindustri, Skagen, Denmark

638

639 Table 2. Experimental treatment overview over exposure solution and duration applied to Atlantic
640 salmon intestine *ex vivo*.
641

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

657 Table1

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[lΔ]	26.00	26.00	26.00	26.00

658

659 Table 2.

		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 x 10^7 CFU ml^{-1}	60 min	Yes		-	-
	3	*Aeromonas salmonicida* 8,6 x 10^6 CFU ml^{-1}	60 min	Yes		-	-
Double Treatment	4	*Carnobacterium divergens* 3,2 x 10^7 CFU ml^{-1}	30 min	Yes	*Aeromonas salmonicida* 8,6 x 10^6 CFU ml^{-1}	30 min	Yes
	5	*Aeromonas salmonicida* 8,6 x 10^6 CFU ml^{-1}	30 min	Yes	*Carnobacterium divergens* 3,2 x 10^7 CFU ml^{-1}	30 min	Yes

660 CFU – Colony forming units

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666 Table 3.

	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

693 **Figures legends**

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

703

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

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713
714 Figure 1. Transmission electron micrograph of tissue from fish fed feather meal and exposed to
715 saline. The micrograph shows undamaged microvilli, enterocytes and mitochondria.
716 L: Lumen; Tw: Terminal web; Mv: Microvilli; Mit: Mitochondria

717

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

720

721 Figure 5. Aggregation of rodlet cells in tissue from fish fed extracted sunflower and exposed to *A.*
722 *salmonicida*. Transmission electron micrograph shows rodlet cells in close proximity. Rodlet cells
723 are recognized by their characteristic thick outer sheath and inner rodlets.
724 Rc: Rodlet cell; Gc: Goblet cell
725

726 Figure 6. Excess lipid storage in fish fed FeM and exposed to *A. salmonicida* for a period of 60
727 minutes.
728

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

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

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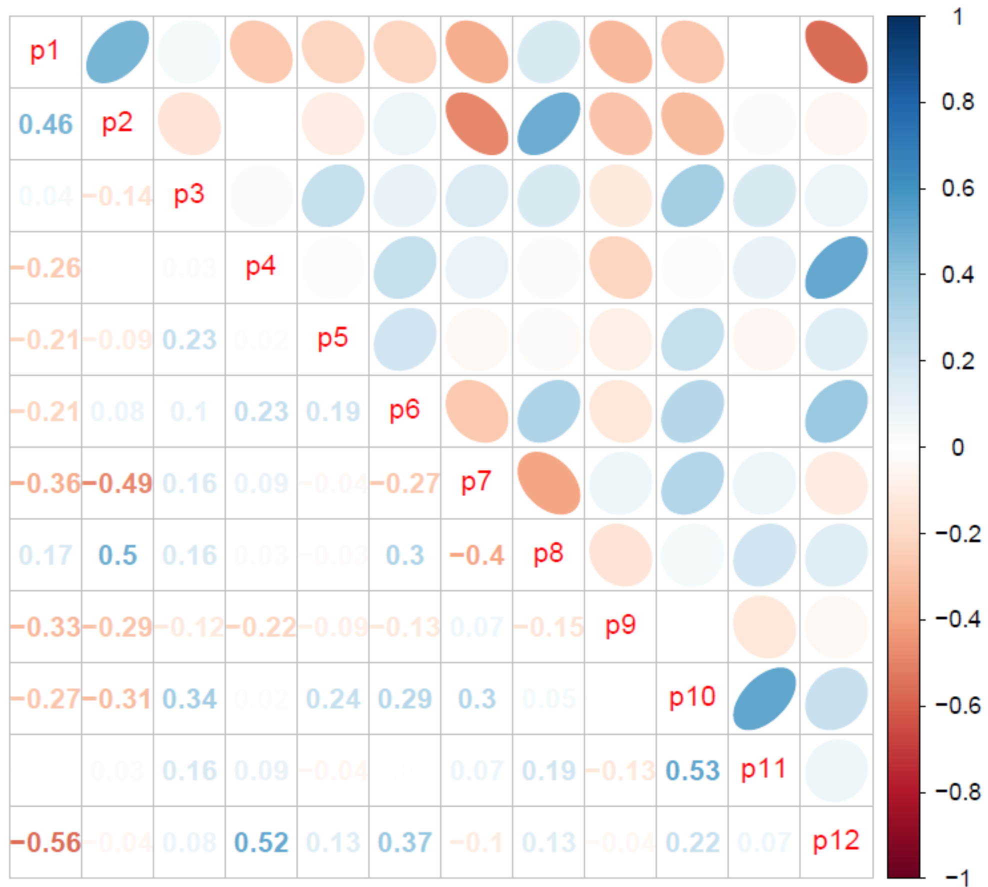
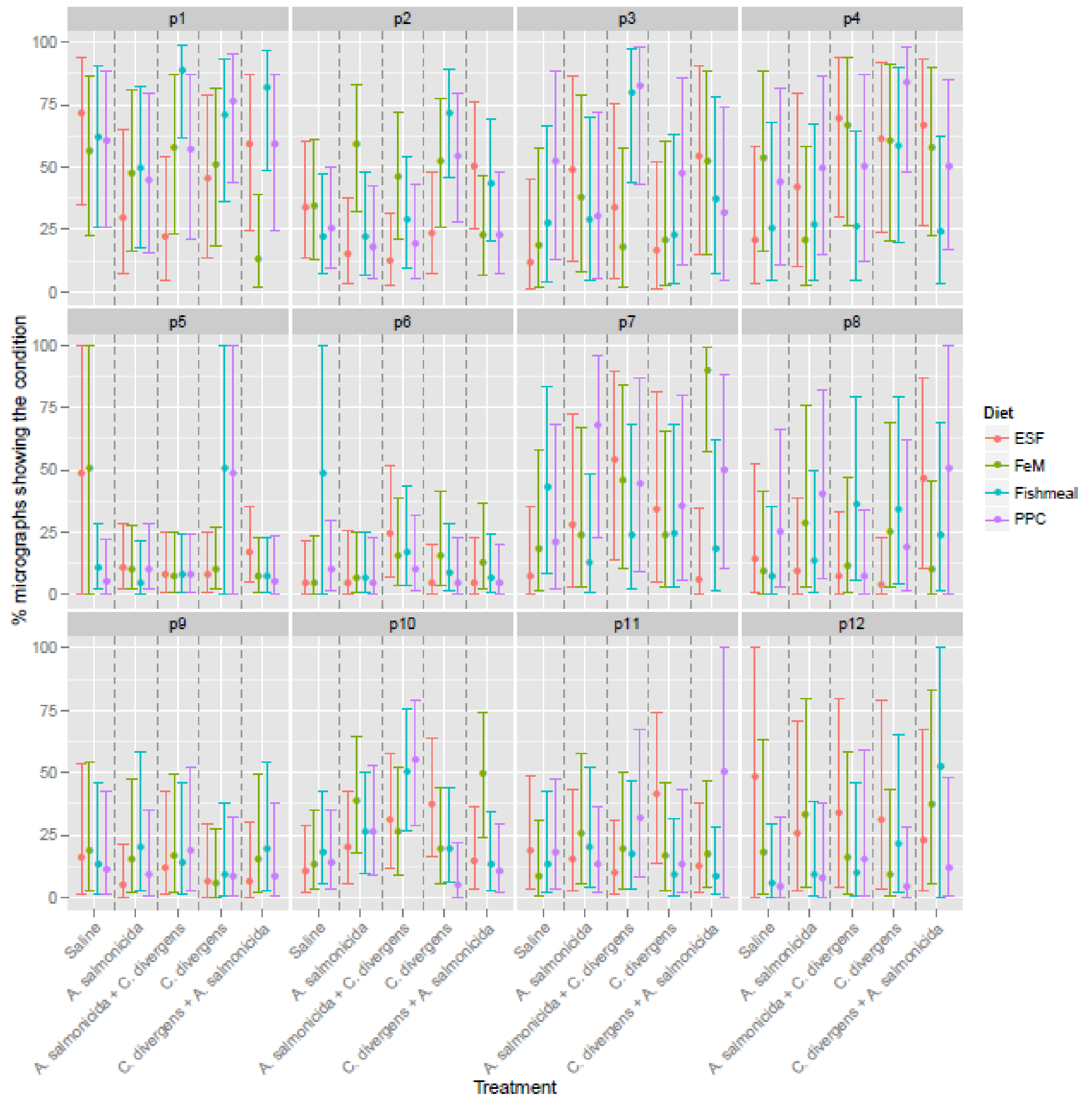
Figure 1.



772 **Figure 2.**



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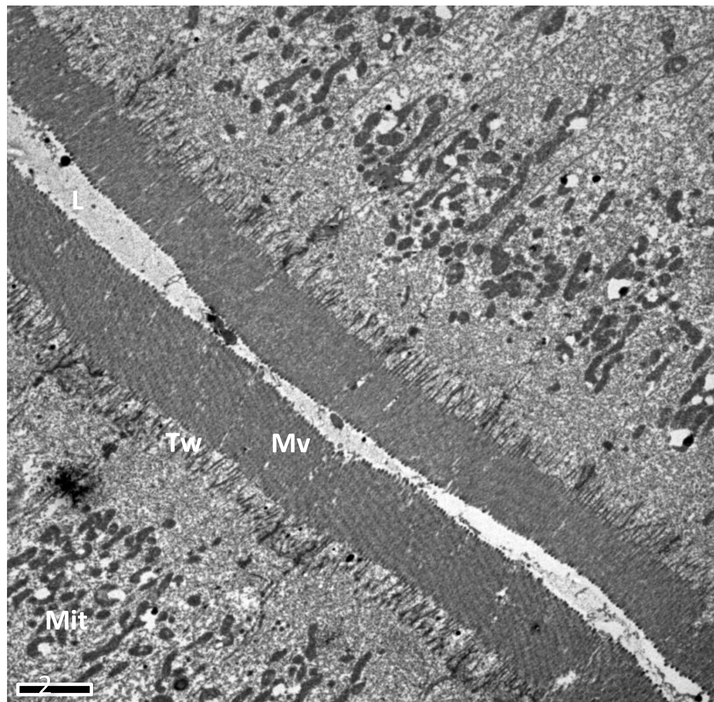
781 **Figure 3.**



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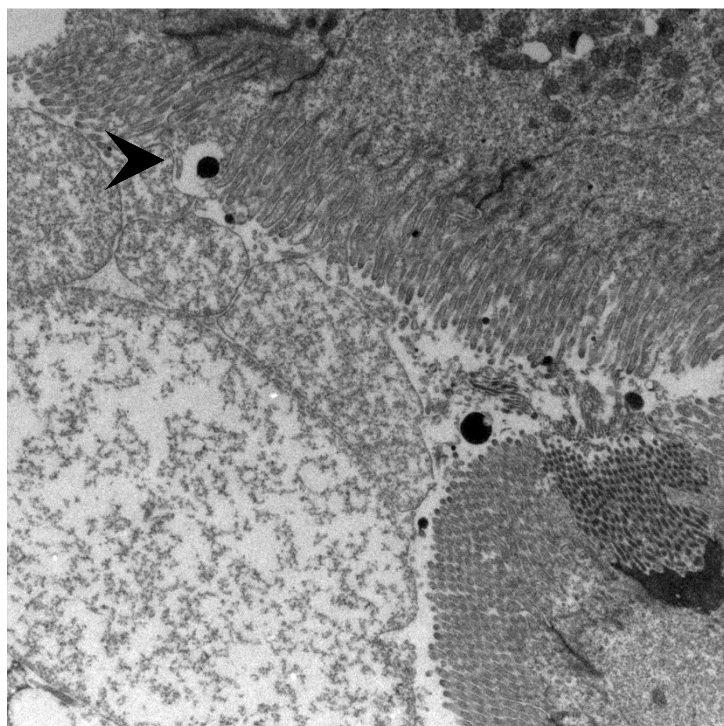
783 **Figure 4**



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792 **Figure 5.**
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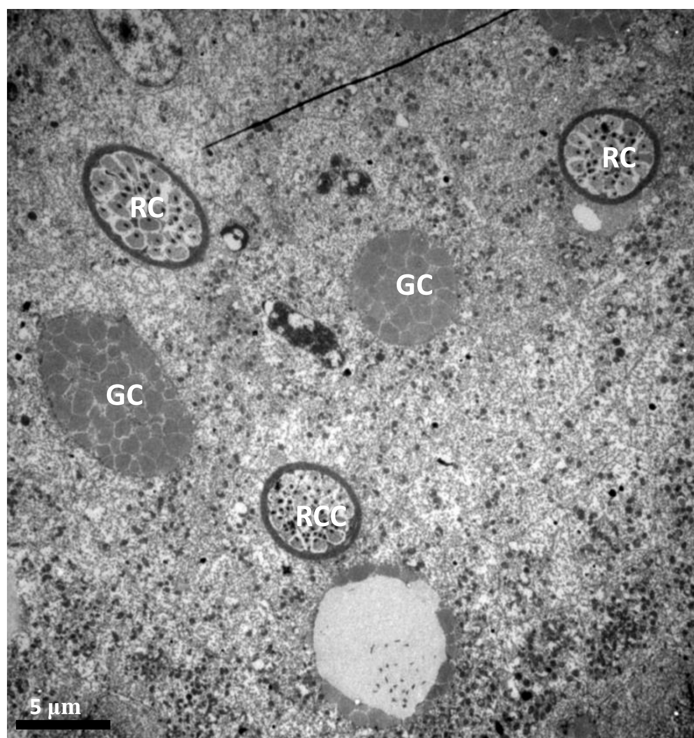

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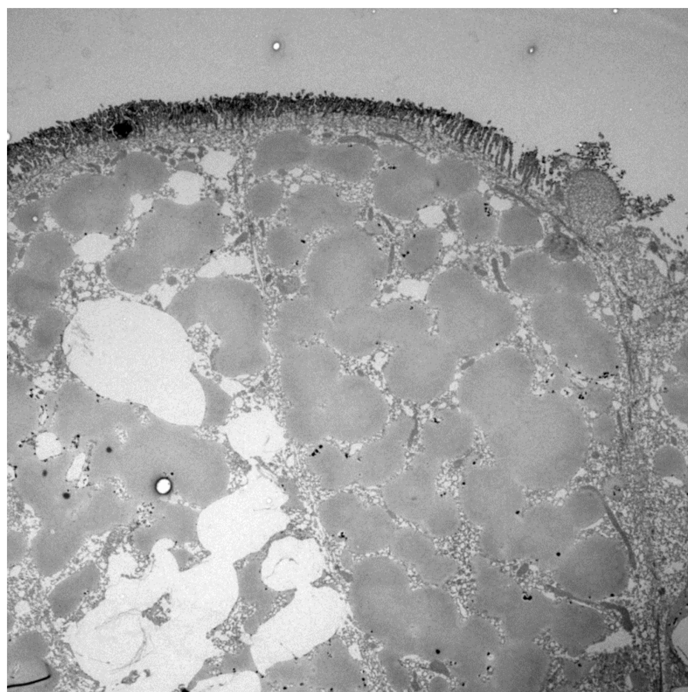
795 **Figure 6**



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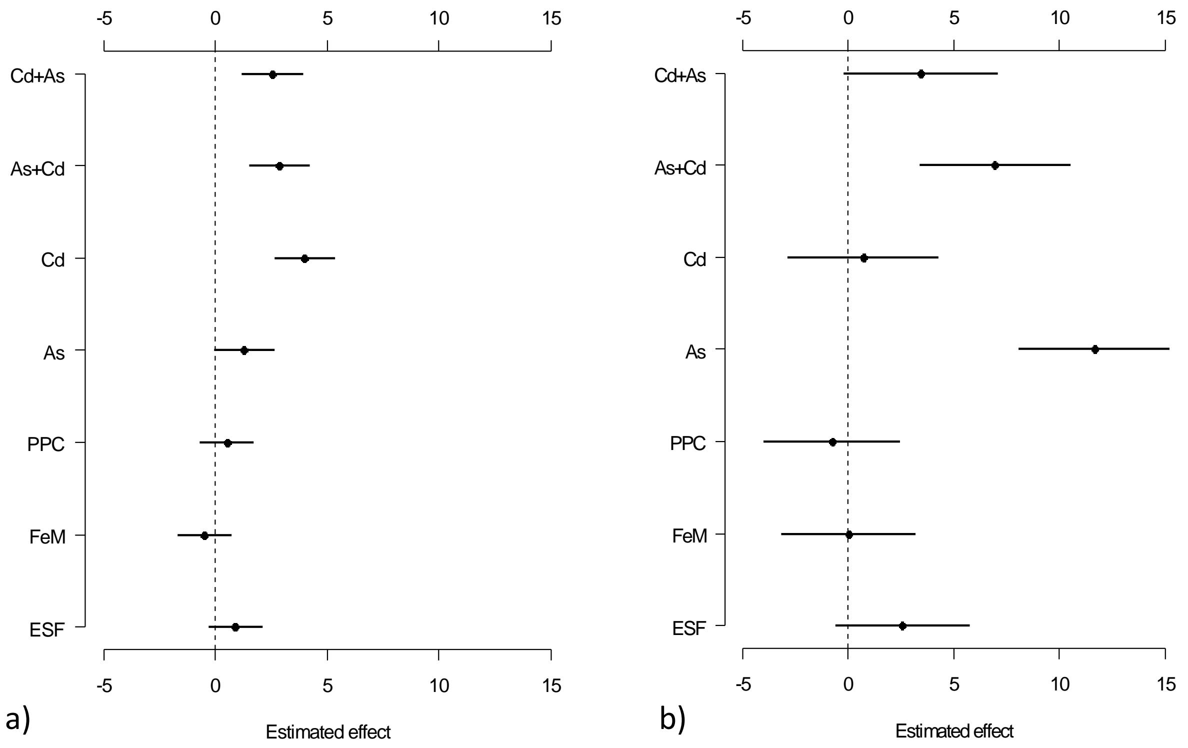
801 **Figure 7**



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