

## Parasitism perturbs the mucosal microbiome of Atlantic Salmon

Llewellyn, M. S.; Leadbeater, S.; Garcia, C. ; Sylvain, F-E.; Custodio, M. ; Ang, K. P.; Powell, F. ; Carvalho, Gary; Creer, Simon; Elliot, J.; Derome, N.

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1 Title: **Parasitism perturbs the mucosal microbiome of Atlantic Salmon**

2

3 Authors: **Llewellyn, M.S.\*<sup>1</sup>, Leadbeater, S.\*<sup>2</sup> Garcia, C.<sup>3</sup>, Sylvain F-E<sup>3</sup>, Custodio, M.<sup>4</sup>**  
4 **Ang, K. P. <sup>5</sup> Powell, F.,<sup>5</sup> Carvalho, G. R.,<sup>6</sup> Creer, S.<sup>6</sup> Elliot, J.<sup>5</sup> Derome, N.<sup>3</sup>**

5

6 1 School of Life Sciences, University of Glasgow, Glagsow UK

7 2 St Andrew's Marine Station, Department of Fisheries and Oceans, New Brunswick, Canada

8 3 Universite Laval, Quebec, Canada

9 4 Universidade Federale do Rondonia, Porto Vehlo, Brazil

10 5 Cooke Aquaculture, Canada

11 6 Marine and Fisheries Genetics Laboratory, University of Wales, Bangor

12 \*Contributed equally to this study

13 Correspondence to [martin.llewellyn@glasgow.ac.uk](mailto:martin.llewellyn@glasgow.ac.uk)

14

15 Abstract

16 Interactions between parasite, host and host-associated microbiota are increasingly  
17 understood as important determinants of disease progression and morbidity. Salmon lice,  
18 including the parasitic copepod *Lepeophtheirus salmonis* and related species, are perhaps the  
19 most important problem facing Atlantic Salmon aquaculture after feed sustainability. Salmon  
20 lice parasitize the surface of the fish, feeding off mucus, scales and underlying tissue.  
21 Secondary bacterial infections are a major source of associated morbidity. In this study we  
22 tracked the diversity and composition of *Salmo salar* skin surface microbiota throughout a  
23 complete *L. salmonis* infection cycle among 800 post-smolts as compared to healthy controls.  
24 Among infected fish we observed a significant reduction in microbial richness (Chao1,  
25  $P=0.0136$ ), raised diversity (Shannon,  $P<7.86e-06$ ) as well as highly significant  
26 destabilisation of microbial community composition (Pairwise Unifrac, beta-diversity,  
27  $P<1.86e-05$ ;  $P = 0.0132$ ) by comparison to controls. While undetectable on an individual  
28 level, network analysis of microbial taxa on infected fish revealed the association of multiple  
29 pathogenic genera (*Vibrio*, *Flavobacterium*, *Tenacibaculum*, *Pseudomonas*) with high louse  
30 burdens. We discuss our findings in the context of ecological theory and colonisation  
31 resistance, in addition to the role microbiota in driving primary and secondary pathology in  
32 the host.

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35

## 36 **Introduction**

37 New data from epidermal (e.g. [1]) and intestinal (e.g. [2-4]) parasitic disease systems  
38 suggest major roles for host-associated microbiota in promoting effective host immunity (e.g.  
39 [1]) or driving host pathology [2]. More widely, commensal microbiota – especially in the  
40 gut - are known to exert ‘colonization resistance’ on potential opportunistic pathogens,  
41 inhibiting over-growth and pathogenesis (e.g. [5]). In aquatic systems major pathogens and  
42 putative opportunists are frequently identified as asymptomatic infections. As such, the host-  
43 associated microbiome can act as the source of many disease agents which emerge as  
44 important pathogens under the right conditions [6].

45 Salmon lice are copepod ectoparasites of salmon. Several species of the two main genera,  
46 *Lepeophtheirus* and *Caligus*, are distributed globally and infect both Pacific and Atlantic  
47 salmonid species [7]. Costs and losses attributed to sea louse infection, estimated at €300M  
48 million annually, are the single greatest pathogen burden on the global salmonid aquaculture  
49 industry [8]. In the North Atlantic, a single species predominates (*Lepeophtheirus salmonis*),  
50 causing year-round infestations of Atlantic Salmon (*Salmo salar*) housed in marine cages,  
51 with concomitant ramifications for fish health as well as aquaculture economics and  
52 sustainability.

53 *L. salmonis* are the cause of substantial morbidity in their salmonid hosts. Pathology arises  
54 primarily through louse feeding behaviour whereby their rasping maxillae scrape mucus,  
55 scales, and underlying tissue into their digestive tract [9]. Osmoregulatory dysfunction, fluid  
56 and blood loss result. There is evidence that *L. salmonis* secrete several proteases to assist  
57 with feeding [10]. A further significant source of host pathology is secondary bacterial  
58 infections (e.g. *Aeromonas salmonicida* and *Piscirickettsia salmonis*, among others) that  
59 often accompany salmon lice infection [9,11,12]. In addition to breaking down physical  
60 barriers, it is thought that *L. salmonis* secrete immunosuppressive chemicals (e.g.  
61 Prostaglandin E, Trypsin), that down-regulate T-cell and other functions required to  
62 effectively cope with bacterial pathogens [13,14]. Experimental *S. salar* co-infections  
63 between a Chilean copepod species *Caligus rogercresseyi* and the bacteria *P. salmonis* show  
64 that survival rates in the co-infected fish (0% after 53 days) are substantially lower than in  
65 fish infected with *P. salmonis* alone (c.57% over the same period) [11]. Whilst there is an  
66 increasing understanding of salmonid immunity to copepod pathogens, as well as to  
67 secondary agents, nothing is known about the role commensal microbes may play in such  
68 infections.

69

70 In this study we set out to explore the evolution of the host epidermal microbial community  
71 during *L. salmonis* infection of marine-phase *S. salar* raised in aquaculture conditions. We  
72 aimed to assess the extent of association between features of the epidermal microbiome and  
73 different intensities of parasite burden. To achieve this we employed 16S rRNA amplicon  
74 deep sequencing on the epidermal mucosa of a subset of 1200 *S. salar* post smolts (800 tests,  
75 400 controls) experimentally infected with *L. salmonis*. Substantial perturbation of microbial  
76 community structure and composition was observed in infected fish, consistent with a  
77 reduction in the ‘colonization resistance’ of the system. Network analysis suggested a  
78 correlation with increasing louse load and multiple potential pathogens. Together, our data  
79 highlight the role of parasite-perturbed host associated microbiota as drivers of disease, as  
80 well as new potential targets for therapeutic interventions.

81

## 82 **Results**

83 **Experimental infection outcomes** Exposure of post-smolts to 40 *L. salmonis* copepodids /  
84 fish resulted in final louse counts ranging between two and 67 adult lice per individual (See  
85 frequency distribution in Figure S1). Significant differences in louse load (ANOVA,  
86  $P=0.0035$ ) were noted between tanks (Figure S2). Weight gain differences were noted  
87 between some infected and control tanks at  $T_3$  (Figure 1). A mixed-model incorporating tank  
88 as a random effect showed a clear effect of lice on fish weight overall (Figure 1,  $P = 0.00679$ )  
89 . Only mucus samples from Test tank3 & Test tank 4 were only 16S rRNA sequenced at the  
90 final time point ( $T_3$ ), a decision taken prior to and weight / growth calculations. For the four  
91 test tanks, where individual fish were recaptured on multiple samplings, individual growth  
92 rates (mass change (g)  $\text{day}^{-1}$ ) were calculated (mean:  $1.118 \text{ g day}^{-1}$ , range:  $-1.57$  to  $3.55$ ). No  
93 correlation was observed between individual growth rate and louse load (Linear regression,  
94  $P>0.05$ ,  $R^2 = 0.01667$ ). Among the 50 salmon families included in our study (all survivors),  
95 no impact of family was noted on louse density (ANOVA,  $P=0.425$ ). For the infected fish  
96 for which we could determine individual growth rate ( $N=36$ ), no effect of family on growth  
97 rate was detected.

98

## 99 **Microbial alpha and beta diversity destabilisation in response to *L. salmonis* infection**

100 After error filtering, alignment and chimera removal, a total dataset of 4,512,783 reads was  
101 generated across all samples which clustered into 1754 97% OTUs (for sample numbers, see  
102 Supplementary Information). This dataset was then rarefied to 13,700 reads per sample and  
103 low abundance OTUs filtered out (<100 total). Rarefaction curves confirmed saturation at  
104 this depth across the dataset (Figure S3). Again treating tank as a random effect, alpha

105 diversity (Shannon) and richness (Chao1) were compared across test and control tanks and  
106 sampling points. A significant decline in Chao1 richness (Figure 2, ,  $P=0.0136$ ) was noted  
107 between test and control tanks at  $T_2$  but a significant increase in Shannon diversity at  $T_3$   
108 (Figure 2,  $P<7.86e-06$ ). (Models:  $\text{Chao1} \sim \text{Time} * \text{Treatment} + (1 | \text{Tank})$ ;  $\text{Shannon} \sim \text{Time} * \text{Treatment} + (1 | \text{Tank})$ ). Very strikingly, we noted strong evidence for beta-diversity  
109 destabilisation of host mucosal microbiota in fish infected by pre-adult ( $T_2$ ) and adult lice  
110 ( $T_3$ ) (Figure 3,  $T_2 P<1.86e-05$   $T_3, P = 0.0132$ ; Model:  $\text{Beta\_Div} \sim \text{Time} * \text{Treatment} + (1 | \text{Tank})$ ). No significance was obtained for treatment (infected or not) at earlier time points.  
111 Destabilisation can be clearly observed in the principal coordinates analysis based on Unifrac  
112 distances displayed in Figure 4. As is observable from Figure 3, destabilisation involves an  
113 increase in the mean beta-diversity and its variance with time. As such, beta-diversity in both  
114 test tanks experienced a ‘shot-gun’ spread of increasing dissimilarity over the course of  
115 infection, compared to the two control tanks. As well as the important role of time in defining  
116 microbiome composition, other features of interest in Figure 4 include clear clustering of all  
117 water samples ( $T_{0-3}$ ) with all mucus samples at  $T_{0\&1}$  (Figure 4, Panel B). By contrast, biofilm  
118 samples were distributed more widely across different time points (Figure 4, Panel E).  
119 Samples taken from pooled *L. salmonis* intestines were highly divergent with respect to their  
120 microbial composition (Pairwise Unifrac, Figure 4, Panel F), although fairly similar among  
121 tanks. Multivariate permutational analysis of beta diversity undertaken in *vegan* at each time  
122 point for test and control samples were significant at every time point ( $T_0$ - $T_3$ ,  
123 PERMANOVA,  $P<0.001$ ), indicative of standing compositional differences between test and  
124 control tanks prior to the addition of copepodids. However,  $R^2$  estimates did increase between  
125 test and control tanks over the course of infection, suggesting an increasingly important role  
126 of *L. salmonis* infection in explaining the variance between treatments as infection progressed  
127 (PERMANOVA,  $R^2$ ,  $T_0 : 0.2608$ ;  $T_1:0.2726$ ;  $T_2:0.3351$ ;  $T_3:0.3492$ ,  $p<0.001$  in all cases).  
128  
129

130  
131 **Dominant microbial taxa, taxon associations and networks.** At the genus level,  
132 *Tenacibaculum* was perhaps the most abundant taxon across all samples in the experiment,  
133 including mucus and water in both tests and controls (Figure 5). *Tenacibaculum* was present  
134 but relatively less abundant in louse samples compared to other genera. Additional genera  
135 present at high abundances globally included *Vibrio*, *Pseudomonas* and *Lewinella*. *Vibrio*  
136 was particularly abundant among *L. salmonis* intestine samples, as was the genus *Arcobacter*  
137 and NS10\_marine\_group, a member of family Cryomorphaceae. To more robustly assess  
138 changes in taxon abundance in test and control tanks, we applied a Kruskal-Wallis test [15].  
139 In view of standing variation present at  $T_0$  between infected and control fish, direct

140 comparisons between treatments at T<sub>3</sub> would be meaningless. Therefore, we compared taxon  
141 abundance in control and infected tanks respectively between T<sub>0</sub> and T<sub>3</sub> and noted differences  
142 between these two comparisons (Figure 6). Genera significantly (P<0.001 after Bonferroni  
143 correction) more abundant at T<sub>3</sub> in infected fish but not controls included Rhizobiales and  
144 NS10\_marine\_group (family Cryomorphaceae). Only *Arthrobacter* were more abundant at T<sub>3</sub>  
145 in controls than in infected fish. Less abundant taxa in controls between T<sub>0</sub> and T<sub>3</sub> but not  
146 infected fish were individual OTUs within family Saprospiraceae, order Alteromonadales and  
147 order Gammaproteobacteria. The relative abundance of individual genera containing known  
148 salmonid pathogen species: *Tenacibaculum*, *Vibrio*, *Flavobacterium*, *Pseudomonas* was not  
149 higher among *L. salmonis* - infected fish at T<sub>3</sub> as compared to the control T<sub>0</sub>-T<sub>3</sub> comparison  
150 (Figure 6). We also explored any correlation with individual OTUs and louse load in the  
151 larger cohort of infected fish. No significant negative associations were uncovered (bacterial  
152 taxa associated with low louse loads). However, two OTUs – one belonging to  
153 *Verrucomicrobia*, the other *Lewinella* were consistently associated with increasing louse load  
154 (P<0.001 after Bonferroni correction) in all three tests applied. Consistent with Figure 4,  
155 *Arcobacter*, presumably of louse origin, was also positively associated with louse load at T<sub>3</sub>.  
156 Network analysis, including louse load as a continuous variable, partitioned the 50 most  
157 abundant OTUs in infected fish into two correlated groups (Figure 7), one large guild  
158 comprising mainly commensals, the other containing a number of putative pathogenic genera  
159 (*Pseudomonas*, *Tenanicibaculum*, *Flavobacterium*, among others). Importantly, significant  
160 associations were apparent between the commensal guild and lower louse load and the  
161 pathogenic guild and higher louse abundances on individual fish (Figure 7). Thus, while  
162 individual associations between given microbial taxa and increasing louse abundance were  
163 limited – second order, multi-taxa associations were clearly at play.

164

165 **Discussion.** Commensal microbiota may play a fundamental role in mediating host-parasite  
166 interactions (e.g. [1-3]). The aim of this study was to explore the impact of *L. salmonis*  
167 infection on the microbiota associated with Atlantic Salmon skin mucus in the context of  
168 salmon pathology, louse life-cycle stage (T<sub>0-3</sub>), and susceptibility to intense louse infections  
169 as well as secondary bacterial infections. We were successfully able to demonstrate the  
170 destabilizing influence that parasitism exerts on salmon skin microbiota. We did not  
171 demonstrate a link between louse infection and *individual* secondary pathogens. However,  
172 network analysis did reveal pathogenic and non-pathogenic guilds present within the  
173 communities of infect fish that correlated with high-intensity and low intensity infections  
174 respectively. We can thus conclude that perturbation of the mucosal microbiome may

175 promote pathology via proliferation of endogenous pathogenic genera and/or via decreased  
176 colonization resistance to exogenous opportunists.

177 Numerous experimental studies have charted the detrimental impact of louse infection on  
178 marine phase Atlantic salmon in terms of basic morbidity and stress (e.g. [16]) as well as  
179 detailed immunological and transcriptional responses [17,18]. Our data generally corroborate  
180 these studies in terms of reduced fish performance in three out of four of our infected tanks.  
181 However, the limited time of exposure of the post-smolts to adult lice resulted in mass  
182 changes that were borderline with respect to controls. Mortality associated with louse load  
183 was not observed. Nonetheless, we did achieve our primary aim in obtaining intense *L.*  
184 *salmonis* loads in *S. salar* that developed through to adult stage (mean parasites per fish:  
185 23.53), providing the opportunity to track microbial diversity over the time course of  
186 infection.

187 The composition of the *S. salar* associated intestinal microbiome is increasingly well  
188 understood in both wild [19] and aquaculture [20] settings. Furthermore, the relative  
189 contributions of environment and host to shaping euryhaline teleost gut microbial diversity  
190 have also been estimated [21]. Data concerning the epidermal mucosal microbiome in  
191 salmonids are less common, especially in the marine phase. Boutin et al., 2013 have  
192 extensively characterized freshwater salmonid mucosal microbiota in brook char (*Salvelinus*  
193 *fontinalis*) in the context of emergent opportunistic infections and stress [22,23]. Dominant  
194 genera in our study (e.g. *Tenacibaculum*, *Lewinella*, *Vibrio*) were highly divergent with  
195 respect to those uncovered by Boutin et al., with the possible exception of *Pseudomonas*  
196 species [22]. Human skin microbiota are known to be among the most temporally unstable  
197 assemblages in the human body, as well as showing high levels of inter-individual variation  
198 [24]. The high degree of sharing apparent between environmental (principally water) and  
199 salmon skin microbiota stands in stark to sharing between environmental samples and *S.*  
200 *salar* gut microbiota [19]. It is also apparent that time (rather than infection status) is the  
201 major driver behind many differences one sees between microbial assemblages in this study  
202 (Figures 4&5). However, fluctuations in environmental microbiota did not seem to be the  
203 root cause of such differences. Instead, most water samples were associated with salmon  
204 mucus samples at T<sub>0</sub>-T<sub>1</sub> only, while salmon mucus a samples T<sub>2</sub>&T<sub>3</sub> were divergent and  
205 distinct from those in the water. It is not clear whether skin microbiota might eventually  
206 converge on a stable state with respect to time, or whether, like in other vertebrate systems,  
207 skin communities are continually subject to high levels of stochastic temporal change (e.g.  
208 [24]).

209 Sampling point (time) was not the only driver of microbiome community dynamics. Infection  
210 with *L. salmonis* did play an increasingly important role in defining microbial community  
211 identity as infection progressed, as revealed by multivariate analyses. In addition to  
212 community identity, we were able to demonstrate that community richness and beta-diversity  
213 were both impacted. ‘Destabilization’ of host-associated microbiota in comparison to healthy  
214 controls is a consistent feature of diseased states in both non-communicable (e.g. Crohns  
215 disease [25]) and communicable disease (e.g. Giardia [2]). The direction that these so-called  
216 ‘dysbioses’ take is a matter for debate. Simple reductions in microbial diversity and/or  
217 richness can be associated with conditions such as Crohns [26]. Directional shifts in  
218 community identity can also be detected in *Plasmodium*-infected mice [27]. Moreover,  
219 microbial co-occurrence networks shift in bowel cancer and changes in microbiome  
220 functional metabolic signatures can be detected in periodontitis [28,29]. The impact of such  
221 microbial dysbiosis on the host is less clear, and may indeed be either a primary,  
222 deterministic feature that allow opportunistic disease to occur or a secondary, neutral feature  
223 of primary pathogenesis with little more than diagnostic significance. Given the importance  
224 of secondary infections in the *L. salmonis* system, the destabilization of surface microbiota  
225 may, however, have a direct impact on host health – perhaps primarily via the declining  
226 ‘colonization resistance’ exerted by skin commensals that may result. Invasion ecologist  
227 Charles E. Elton first hypothesized that diverse communities might resist invasion more  
228 effectively than stable ones [30]. Various modifications of this argument linking aspects of  
229 microbial diversity to invasibility (i.e. colonization resistance) can be uncovered throughout  
230 the literature (reviewed in [31]). Fluctuating alpha and beta-diversity in infected fish did not  
231 significantly impact the abundance of putative pathogens in our study at individual level. For  
232 example OTUs of genus *Tenacibaculum* (to which *Tenacibaculum maritimum*, the etiological  
233 agent of salmon ulcerative tenacibaculosis belongs [32]) were abundant in almost all fish  
234 sampled, irrespective of whether or not they were infected with *L. salmonis*. Individual  
235 OTUs that were significantly associated with louse load among infected fish (one belonging  
236 to phylum Verrucomicrobia, the other classified as *Lewinella*) were not attributable to any  
237 known pathogen. OTUs found associated with sea lice intestines showed some interesting  
238 features. The capacity of *L. salmonis* to propagate disease agents has been the subject of  
239 some discussion in the literature (e.g. [33]). *Vibrio*, a genus comprising several major fish  
240 pathogens [6], amongst other commensal taxa, was highly abundant in louse samples,  
241 although also present among fish and environmental samples in test and control tanks. One  
242 bacterial OTU (NS\_10: Cryomorphaceae) was very clearly associated with louse infection  
243 and was amplified exclusively from lice intestines and test tanks T<sub>2</sub> and T<sub>3</sub>. Whilst the

244 importance of this the bacterium is not clear, the data suggests an ability to proliferate in the  
245 louse and transfer effectively from one host to another and a role as an indirectly transmitted  
246 pathogen cannot be ruled out.

247 Whilst associations between louse load and individual bacterial taxa do not suggest a clear  
248 link between parasite burden and the abundance specific secondary disease agents, network  
249 analyses were less equivocal. In line with previous work on microbial assemblages from  
250 salmonid skin mucus, co-occurring guilds of bacteria (respectively putative commensals or  
251 pathogens) persist whose relative abundance can be modulated by stress [22] – in our case  
252 corresponding to parasite load. Establishing the role of such community dynamics in driving  
253 opportunistic disease or transmissible disease susceptibility is a crucial goal of future  
254 research. As such, maintaining stability in skin surface microbial assemblages via pre- pro- or  
255 syn-biotics may provide an effective means of mitigating disease in parasitized fish. Co-  
256 infection experiments are vital in this context, involving paired macro- and micro- pathogens  
257 to simulate the real world scenarios (e.g. [11]). Thus our study underlines the importance of  
258 taking a holistic approach that incorporates changing host, parasite and microbiome to  
259 appreciate their relative roles in modifying disease outcome.

## 260 **Materials and Methods:**

261 **Experimental procedures:** Salmon post-smolt (mean mass at experiment outset 149g +/-  
262 13.1g SE) from 50 salmon families were internally Passive Integrated Transponder (PIT)  
263 tagged and distributed randomly across six 1000L tanks in a flow through system at the  
264 Fisheries and Oceans Canada marine facility St. Andrews Biological Station (St. Andrews,  
265 New Brunswick (NB), Canada). All fish handling and procedures were approved by DFO  
266 Maritimes & Gulf / CFIA Regional Animal Care Committee (File Number 14-13) and carried  
267 out under the direct supervision of a trained Department of Fisheries and Oceans Canada  
268 operative in strict compliance with regulations set out by the Canadian Council for Animal  
269 Care (<http://www.ccac.ca/>). Water conditions were maintained at 11-14°C with a salinity of  
270 30-33g L<sup>-1</sup>. Each tank housed a maximum of 200 fish at a density under 40kg m<sup>-3</sup> and water  
271 quality parameters (temperature and oxygen) were monitored daily. Fish were fed with  
272 commercial salmon feed (2.5 mm) at 1-2% body weight per day and oxygen was added to  
273 maintain a saturation level between 90 and 105% (8-10mg/l). Following an acclimation  
274 period of three weeks, four of the six tanks salmon were challenged with infective *L.*  
275 *salmonis* copepodids at a concentration of 40 copepodids per fish (8 copepodids L<sup>-1</sup>) for 1  
276 hour. Copepodids were hatched from egg strings collected from gravid female lice gathered  
277 at a commercial salmon farm by technical staff from the Huntsman Marine Sciences Centre  
278 (HMSC), St. Andrews, NB, Canada. Water flow to the experimental exposure tanks was

279 stopped just prior to addition of lice and fish were observed closely during the infection  
280 event. Jumping, flashing and behaviours such as rapid swimming were observed which is  
281 consistent with lice infection. After 1 hour, water flow was resumed and fish were not  
282 handled until the required sampling time point.

283

284 At 48 hours prior to infection ( $T_0$ ), 6 days ( $T_1$ ), 22 days ( $T_2$ ) and 35 ( $T_3$ ) after infection,  
285 bacterial community sampling was undertaken. Mucus samples comprised skin swabs along  
286 one full lateral surface of the fish (including the gill operculum). Samples from two control  
287 (Tank C1&C2 - uninfected) and two test (Tank Test\_1&Test\_2 - infected) tanks were taken  
288 at sample point  $T_{0-3}$ . In addition, samples were taken from two further test tanks at  $T_3$  (Tank  
289 Test\_3&Test\_4, identical conditions to Test\_1&Test\_2) to provide further insight on the  
290 impact of adult lice. A single inflowing water bacterial community sample was taken per time  
291 point (10 litres filtered through a 0.2  $\mu\text{m}$  filter). Biofilm samples were taken along the sides  
292 of each tank per time point also. During sampling, all fish in each tank were sedated using  
293 Aquacalm at 0.9mg/l and 25 fish from each tank per time point were sampled randomly using  
294 individual sterilised soft-mesh nets to avoid cross-contamination and to avoid dislodging lice.  
295 Length (cm) and mass (g) were also recorded. Skin pH data for 10 fish were collected, while  
296 a blood sample for serum cortisol determination was collected for 5 fish per tank at time  
297 points  $T_{1-3}$ . At day 35 ( $T_3$ ) all fish were euthanized with Tri-methanosuphonate (TMS) at  
298 100-150 mg/l in individual nets to account for mobile lice loss and lice count data, weight  
299 length and sex were recorded. At  $T_3$ , 10 adult lice per tank were collected and treated with  
300 0.1% hypochlorite solution for 30 minutes to remove adherent microbes, washed with  
301 microbe free water, pooled and frozen for gut microbial analysis.

302

### 303 **16S rDNA amplicon sequence analysis**

304 Mucus, environmental (biofilm, water) and louse samples were collected in sterile micro-  
305 centrifuge tubes and immediately stored in liquid nitrogen ( $-196^\circ\text{C}$ ) until DNA extraction at  
306 the Institut de Biologie Intégrative et des Systèmes, at the Université Laval (Québec, QC).  
307 DNA was extracted from all samples using the Qiagen DNeasy blood and tissue kit according  
308 the manufacturers instructions. Amplification of the 16S rRNA V4 region was achieved with  
309 primers 519\_f 5'-CAGCMGCCGCGGTAA-3' and 785\_r 5'-TACNVGGGTATCTAATCC-  
310 3' using Takara *Taq* Polymerase (CloneTech, USA), and a final concentration of 1 pmol of  
311 each primer [19]. Reaction conditions were  $95^\circ\text{C}$  for five minutes, followed by  $30^\circ\text{C}$  cycles  
312 and of  $95^\circ\text{C}$  for 30 seconds,  $55^\circ\text{C}$  for 30 seconds and  $72^\circ\text{C}$  for 30 seconds, followed by a  
313 final elongation step of  $72^\circ\text{C}$  for 10 minutes. Each amplification was run in triplicate

314 (technical replicates) and pooled to minimise PCR bias, purified using an AxyPrep™ Mag  
315 PCR Clean-Up Kit (Corning, USA). Sequence libraries were dual indexed using Illumina  
316 Nextera multiplex barcodes and sequenced in a single run on an Illumina MiSeq platform. V4  
317 was chosen in the light of its widespread use to profile vertebrate-associated microbiota as  
318 well as its suitability for Illumina paired end sequence read lengths at the time of sequencing  
319 [34].

320 Amplicon data were processed as described previously ([19]). Briefly, SICKLE [35] was  
321 used for error screening ( $>Q30$ ) and assembly of each paired end read into a single  
322 overlapping 290bp fragment from the 16S rRNA V4 hypervariable region was achieved in  
323 PANDASeq [36]. Sequences were aligned against the *E. coli* 16S rRNA gene and trimmed in  
324 Mothur [37] prior to operational taxonomic unit clustering in UPARSE at 97% identity [38].  
325 Putatively chimeric OTUs were filtered out in reference to the genomes online database  
326 (GOLD v.5) in UCHIME [39]. Subsequently, the following steps were undertaken in QIIME  
327 [15]: after exclusion of chimeric OTUs, samples containing  $<13,700$  reads were discarded  
328 and all samples were rarefied to an even depth of 13,700 reads. 13,700 represented the  
329 optimal minimum depth at which saturation was achieved while still including the maximum  
330 number of sample. OTUs with fewer than 100 reads or that only occurred in a single sample  
331 were filtered out as a step to improve accuracy and diversity estimates [40].

332

### 333 **Statistical and diversity analyses**

334 Fish mass and growth rate (where the same individuals were resampled at different time  
335 points – mean mass gain (g) day<sup>-1</sup>) were recorded throughout the experiment. Differences in  
336 mass between time points and between test (infected) and controls (uninfected) were plotted  
337 and assessed for significance using mixed models incorporating different tanks as a random  
338 effect in R using lme4 (*lmer(Weight ~ Time\_point\*Test + (1/Tank\_Number))*) and tested for  
339 significance using a likelihood ratio test in the same package (*anova(null, model)*) [41]. For  
340 the microbial samples themselves, Shannon diversity and Chao1 richness estimators were  
341 calculated for each rarefied sample in QIIME [15]. Mixed models were also applied to assess  
342 the distribution of variation in these parameters per treatment (fixed), tank (random) and  
343 sample point (fixed) using lme4 and lmerTest [41]. To evaluate differences in community  
344 composition (beta-diversity), unweighted Unifrac distances were calculated and plotted [42].  
345 Differences in beta-diversity between treatments and tanks were also assessed using mixed  
346 models in lme4 [41]. Beta-diversity comparisons between all samples were also subjected to  
347 principal coordinates analysis, also in QIIME [15]. Differences in microbiome composition  
348 between test (Test tank 1 & Test Tank 2) and control (Control Tank 1 & Control 2) tanks at

349 each time point (T<sub>0-3</sub>) were tested using a permutation-based multivariate analyses of  
350 variance (PERMANOVA) in ADONIS in the Vegan package in R [43]. OTU abundances,  
351 genus and order-level taxonomic classifications were calculated and plotted. Differential  
352 abundance of majority OTUs (i.e. comprising 95% of all samples) were compared between  
353 times T<sub>0</sub> and T<sub>3</sub> in control and infected fish treatments respectively and tested for significance  
354 using a non-parametric Kruskal-Wallis test in QIIME. Among infected fish from four tanks at  
355 T<sub>3</sub>, correlations were explored between microbial diversity and abundance and sea louse load  
356 as well as individual fish growth rate (mass (g) day<sup>-1</sup>) via several Bonferroni-corrected  
357 correlation tests in QIIME including: Pearson, Kendal and Spearman rank tests. Only  
358 consistently occurring OTUs across these measures were reported. Finally, network analysis  
359 was achieved in Cytoscape v.3.2.1 based on correlations between the relative abundance of  
360 the top 50 OTUs on the test fish (T<sub>0</sub>-T<sub>3</sub>) in relation to lice load. Spearman correlations and  
361 node weightings were calculated in the R packages *multtest*, *Hmisc*, *parallel* and *iterators*.  
362 Correlations were considered significant when the Spearman correlation value was > 0.6 and  
363 the correlation p-value (corrected with Bonferroni) was < 0.05.

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479

#### 480 **Authors Contributions**

481

482 Authors: ML, SL, & GC carried out the research. ML, MC, & FS undertook analyses. KA,  
483 FP, & JE contributed resources and reagents. ML, ND, FS, SC, GC, SC & ND wrote the  
484 paper.

485

486 **Additional Information**

487

488 I declare that the authors have no competing interests as defined by Nature Publishing Group,  
489 or other interests that might be perceived to influence the results and/or discussion reported in  
490 this paper.

491

492 **Figure 1 – Impact of *Lepeophtheirus salmonis* infection on salmon growth during the**  
493 **experiment.** Mean values for fish mass with error bars showing +/- standard error are shown  
494 per tank and time point in test and control tanks. An analysis of variance indicates a  
495 borderline insignificant impact of infection on fish mass across all six tanks ( $P=0.082$ ), and  
496 significant when only the four tanks (Test (Tt)1, Test(Tt) 2,C1,C2) from which longitudinal  
497 microbiome sampling had occurred ( $P=0.0007$ ).

498

499 **Figure 2 – Alpha diversity (Shannon) and richness (Chao1) variation in *Salmo salar***  
500 **skin mucosal microbiota in response to infection with the sea louse *Lepeophtheirus***  
501 ***salmonis*.** Box plots show diversity and richness profiles at each sampling point T<sub>0</sub>-T<sub>3</sub>. A  
502 significant decline in Chao1 richness (Figure 2) was noted between test and control tanks at  
503 T<sub>2</sub> ( $P=0.0136$ ). Shannon diversity increased at T<sub>3</sub> ( $P<7.86e-06$ ). \* denotes significance level.

504

505 **Figure 3 – Pair-wise beta diversity measurements show destabilisation of *Salmo salar***  
506 **skin mucus bacterial assemblages in response to infection with the sea louse**  
507 ***Lepeophtheirus salmonis*.** Box plots indicate variation in inter-sample pairwise Unifrac  
508 distance per tank and sampling point T<sub>1</sub>-T<sub>4</sub>. Significant increases in inter-sample variation  
509 was noted at the Times 2&3 between control and infected tanks (T<sub>2</sub>  $P<1.86e-05$  T<sub>3</sub>,  $P =$   
510  $0.0132$ )

511

512 **Figure 4 – Relationship between microbiota sampled from *Salmo salar*, *Lepeophtheirus***  
513 ***salmonis* and environmental samples (water, biofilm) over the course of experimental**  
514 **infection.** A composite multidimensional scaling (MDS) plot of sample clustering is based  
515 on a single principal coordinates analysis (PCoA) of pairwise un-weighted Unifrac distances  
516 between all samples. The left hand plot figure depicts samples coloured by time point  
517 (T<sub>0</sub>=red, T<sub>1</sub>=blue, T<sub>2</sub>=orange, T<sub>3</sub>=green). The right hand plot depicts the same plot coloured  
518 by sample type (skin mucous=blue, water samples=green, tank biofilm=red, lice=orange)

519

520 **Figure 5 – Taxonomic classifications and abundances of OTUs recovered among *Salmo***  
521 ***salar*, *Lepeophtheirus salmonis* and water samples.** The bubble shows mean abundance of  
522 core OTU taxonomic assignments (y axis, present in >85% of samples, represented by >100  
523 sequences) in each sample group respectively (test (infected) vs control (uninfected) at each  
524 time point) on the x-axis. Variance associated with mean abundances are included in  
525 Supplementary data.

526

527 **Figure 6 – Comparisons of mean abundance of bacterial taxa between infected and**  
528 **uninfected fish.** Plots show log abundance of different taxa (y axis) compared between T<sub>0</sub>  
529 and T<sub>3</sub> of all control (A) and test (B) tanks, respectively (x axis). Error bars are +/- standard  
530 error. Based on a Kruskal-Wallis test, data point (closed circles) colours in T<sub>3</sub> indicate where  
531 an OTU was significantly less abundant than at T<sub>0</sub> (blue), more abundant (red) or not  
532 significantly different (black). Abundance differences between taxa in control (top) and test  
533 (bottom) treatments for T<sub>0</sub> (left) - T<sub>3</sub> (right) comparisons (that are still significant after  
534 Bonferroni correction) are marked up by green dashed boxes. Putative secondary pathogens  
535 are listed in black (and indicated by the black dashed circles). Listed in red are taxa that were  
536 more abundant at T<sub>3</sub> of control or infected fish respectively. Listed in blue were taxa that are  
537 less abundant given the same criteria.

538

539 **Figure 7 - Network of bacterial taxa based on co-abundance of the 50 most abundant**  
540 **bacterial genera on all infected fish between samplings T0 and T3.** The abundance of the  
541 sea lice on each fish has been used as a factor. Each node represents a taxon or louse  
542 abundance. An edge between two samples indicates a Spearman correlation index > 0.7  
543 between the two samples and a correlation p-value corrected with Bonferroni < 0.05. The  
544 size of each node is proportional to the number of edges to which it is connected. The two  
545 main clusters are labeled green (putative commensal) and red (putative pathogens). High lice  
546 abundance correlations refer to taxa which are positively correlated with lice abundance  
547 (Spearman correlation > 0.6), whereas low lice abundance correlations refer to taxa  
548 negatively correlated with lice abundance (Spearman correlation < -0.6).

549