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1	Intensive aquaculture selects for increased virulence and interference competition in bacteria
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23	

# 25 Abstract

26 Although increased disease severity driven by intensive farming practices is problematic in food 27 production, the role of evolutionary change in disease is not well understood in these environments. 28 Experiments on parasite evolution are traditionally conducted using laboratory models, often 29 unrelated to economically important systems. We compared how the virulence, growth and 30 competitive ability of a globally important fish pathogen, Flavobacterium columnare, change under 31 intensive aquaculture. We characterized bacterial isolates from disease outbreaks at fish farms during 32 2003-2010, and compared F. columnare populations in inlet water and outlet water of a fish farm 33 during the 2010 outbreak. Our data suggest that the farming environment may select for bacterial 34 strains that have high virulence at both long and short time scales, and it seems that these strains 35 have also evolved increased ability for interference competition. Our results are consistent with the 36 suggestion that selection pressures at fish farms can cause rapid changes in pathogen populations, 37 which are likely to have long-lasting evolutionary effects on pathogen virulence. A better 38 understanding of these evolutionary effects will be vital in prevention and control of disease 39 outbreaks to secure food production.

40

#### 42 Background

43 Human interventions in food production create environments where the ecology, epidemiology and 44 evolution of disease differ from nature. Increased host density and decreased host diversity can 45 reduce the costs of pathogen transmission, and, according to the predictions of virulence evolution 46 theory, highly virulent strains may emergence as a consequence [1,2]. Indeed, intensive farming 47 environments are considered evolutionary hotspots, where the enhanced transmission and frequency 48 of infections could promote virulence evolution in pathogen populations [3-7]. This can be especially 49 important for environmentally transmitted opportunistic pathogens that often have the ability to 50 survive and replicate outside hosts. These opportunistic pathogens do not suffer from the 51 transmission-virulence trade-off similarly to the obligate pathogens, that are dependent on host 52 survival during transmission, which may significantly alter pathogen virulence [8].

53

54 It is common that hosts in nature are co-infected by several pathogen strains. The within-host 55 competitive interactions have been traditionally linked to virulence via faster growth of more virulent 56 parasite strains which is expected to influence dynamics of disease epidemics [1,2,9-14]. However, 57 bacterial interactions towards each other are mainly negative [15]. Competition over limited 58 resources can promote selective interference competition mechanisms targeted to exclude 59 competitors through the release of toxins [16,17], and lead to decreased virulence during bacterial 60 infection [18,19]. It is thus likely that the interactions between co-existing pathogen strains influence 61 the development and persistence of disease epidemics, and the population dynamics of pathogens.

62

63 Although these abovementioned theories and laboratory studies have provided insight on pathogen 64 evolution, very little is still known about how anthropogenic changes, like intensive farming, 65 influence virulence evolution [20-22]. Such empirical evidence is crucial for testing the theoretical 66 predictions, but also for understanding disease ecology and evolution. More than anything, we need 67 data outside of the laboratory from the economically relevant systems [21]. Information on the 68 diseases threatening crop, livestock and aquaculture production is essential for securing world food 69 production. Here, we study how selection in intensive aquaculture influences the virulence and the 70 competitive ability, at both long and short time scales, in the globally significant fish pathogen 71 Flavobacterium columnare (Bacteroidetes) [23,24]. First, we explore temporal changes in virulence 72 and competitive ability using F. columnare strains isolated from disease outbreaks in 2003-2010. 73 Second, we study locally occurring selection by comparing bacterial isolates originating from inlet 74 water and outlet water of a fish farm during one outbreak season in summer 2010. We observe that 75 intensive aquaculture seems to select for increased F. columnare virulence, as well as ability for interference competition, at both short and long timescales. This indicates that the more virulent strains have higher fitness under intensive farming conditions, and demonstrates that the intensive farming environments can be used as model systems to understand disease dynamics and evolution of virulence.

80

#### 81 Methods

82 Experimental set-up

83 In this study we used two different sets of F. columnare strains to study evolution of bacterial 84 virulence and competitive ability on both at temporal and spatial scale. In the first set, we used 85 strains isolated during 2003 - 2010. In the second set we compared the characteristics of bacteria 86 isolated in 2010 from the inlet and the outlet water of a fish farm. In both study settings we studied 87 the bacterial growth rate, the bacterial virulence in zebra fish, and the ability of the bacteria to inhibit 88 the growth of other strains, and analysed if these traits changed in time and place. In addition, the 89 data from 2003-2010 was used to analyse the bacterial competition in finite resources in liquid 90 culture, and the growth of the inlet water and outlet water strains were compared in two different 91 resource concentrations.

92

### 93 Bacterial strains, their isolation and genetic characterization

94

The bacteria were isolated originally from three different fish farms in Central and Northern Finland during columnaris disease epidemics and from environment in 2003-2010 (Table 1) using standard culture methods on Shieh medium [25], [26] or AO-agar [27]. Isolates for the dataset were randomly chosen from a large collection of uncharacterized bacterial strains without any a priori information on their competitive ability or virulence, except for strains H2, B067, B185, B245, B405 and B407 that have been used (separately) in our previous studies [28-30].

101

102 The bacterial strains from inlet water upstream of a fish farm [29] and from downstream and outlet 103 water of the same farm were collected in summer 2010 (Table 2). Both locations (inlet water and outlet water) were sampled on 8<sup>th</sup> and 21<sup>st</sup> June, on 5<sup>th</sup>, 12<sup>th</sup>, 19<sup>th</sup> and 26<sup>th</sup> July, and on 2<sup>nd</sup>, 16<sup>th</sup> and 104 30<sup>th</sup> August in 2010 (Supplementary Table 1). Nine bacterial isolates were randomly selected from 105 106 both locations for further analyses. After isolation, the bacteria were maintained frozen in -80°C with 107 10% glycerol and 10% fetal calf serum. For the analyses, the bacterial strains were grown in Shieh 108 medium at room temperature (RT, approximately 24°C) under constant shaking (110 rpm) 24-48h 109 and enriched overnight.

- 111 The strains collected in 2003-2010 and the inlet water strains were genetically characterized in an
- 112 earlier study using Multilocus Sequence Analysis (MLSA) [31] and Automated Ribosomal
- 113 Intergenic Spacer Analysis (ARISA) [29]. The MLSA method produces genetic clustering
- 114 comparable to the ARISA method [31] and the two methods can be used interchangeably. The outlet
- 115 water strains and one inlet water strain (B355), were genotyped in this study using ARISA, as
- described earlier [29,32] (see Supplementary Material for more details). The possible presence of
- 117 plasmids in the strains was studied from 3.8 ml of overnight grown turbid cultures using a QIAprep®
- 118 Spin Miniprep kit (Qiagen) following manufacturer's instructions. *Flavobacterium* sp. strain B330
- harbouring a natural plasmid was used as a positive control. DNA was run in 0.8 % agarose gel and
- 120 visualized under UV light to detect the presence/absence of plasmids.
- 121

# 122 Interference competition assays

123 The inhibitory activity of F. columnare strains was studied both in time (17 strains isolated in 2003-124 2010) and space (nine inlet water strains vs. nine outlet water strains). Within each set of strains, 125 inhibition was tested reciprocally using a double layer method and the assays were replicated three 126 (strains from 2003-2010) or four (inlet vs. outlet water strains from 2010) times. The optical density 127 (OD, at 570 nm) of the bacterial cultures was measured with spectrophotometer and adjusted 128 between 0.250 and 0.290. Three hundred µl of fresh overnight-grown "recipient" bacterial culture 129 was mixed with 3 ml of soft Shieh agar (0.7 %) tempered to 47 °C and poured on Shieh agar plates. 130 Aliquot of the bacterial culture was centrifuged at 17 000 x G for 3 minutes in RT. Five microliters 131 of the supernatant of the "donor" cultures were spotted on the surface of the top agar. Following an 132 incubation of 48h at RT, the plates were checked to see whether the "donor" strain had caused a 133 growth inhibition of the underlying "recipient" bacterial lawn. The inhibition was ranked as 0 = no134 inhibition, 1 = inhibition.

135

The interaction between *F. columnare* strains B067 and B185 was further characterized with filtered supernatant (0.2  $\mu$ m PES filter, VWR) and by cross streaking on agar plates in 10 replicates. In the first experiment, turbid overnight grown liquid culture and filtered supernatant of strain B185 were cross streaked with B067 by using 1  $\mu$ l loop. In the second experiment, 300  $\mu$ l of turbid liquid culture of B067 was mixed with Shieh soft agar and plated, and 10  $\mu$ l of B185 culture and filtered supernatant was applied on the soft agar. Growth inhibition was monitored after 48 h incubation.

- 142
- 143

#### 144 Bacterial growth measurements

145 A temperature-controlled spectrophotometer (Bioscreen C®, Growth curves Ltd, Helsinki, Finland) 146 was used to monitor the growth of the bacterial strains. Before the growth measurements of the both 147 study sets, the optical densities of the fresh overnight-grown bacterial cultures were adjusted to 148 0.10-0.20 in A570 to minimize the differences in the initial turbidity between strains. Forty  $\mu$ l of 149 each bacterial strain was inoculated onto 400 µl of sterile Shieh culture media on a BioScreen 150 Honeycomb plate (100-well-plate, Oy Growth Curves Ab Ltd) in five replicates per strain, both in 151 normal and diluted medium. The growth data was measured at 25 °C for 96 h at 5 min intervals 152 (absorbance at 420–580 nm, wide band option).

153

154 The growth parameters were calculated from the raw data by utilizing matlab script written by TK in which the maximal growth rate is found from log<sub>2</sub>-transformed data by fitting linear regressions on 155 156 25 time-point sliding windows. The highest linear (log transformation linearizes the exponential 157 growth) slope found in sliding windows equals the maximal growth. The yield is found as a maximal 158 average optical density over 25 time point's sliding window in the raw data. The area under curve 159 (AUC) sums the OD data over the entire measurement period to indicate the cumulative amount of 160 biomass attained during the time.

161

#### 162 Bacterial competition in liquid culture

163 We also studied competition in liquid culture (in finite resources) using the strains isolated in 2003-164 2010. Cultures and measurements were done similarly as above with the single strains (see above), 165 but with 1:1 mixture of bacterial strain pairs (compared to individual bacterial strain bacteria diluted 166 1:1 with dH<sub>2</sub>0) and OD at 600 nm was measured every five minutes for 95 hours. Area under curve 167 (AUC), a measure that describes the cumulating amount of biomass a given strain and strain-strain 168 combinations can produce within a given time, gave roughly the same results as the yield. From the 169 obtained AUC we calculated interaction indexes for the bacterial strains as

 $interaction index = \frac{growth (strain 1 alone) + growth (strain 2 alone)}{growth (strain 1 alone) + growth (strain 2 alone)}$ 

growth(strain 1 and 2 together)

170

172

173 For the data analysis, the interaction index was arcsin-transformed (see data analysis below).

174

175 Virulence in zebra fish To measure the virulence of the studied bacterial strains, unsexed, adult, disease-free zebra fish were obtained from Core Facilities (COFA) and research services of Tampere (Tampere University, Finland). The zebra fish response to *F. columnare* infection is qualitatively similar to the common host of the pathogen in aquaculture, rainbow trout [33]. The optical density of the overnight-grown bacterial culture (570 nm) was measured and the corresponding bacterial density in colony forming units (cfu) was calculated according to our previously fitted standard curve. The infection method and bacterial dose used were optimized in preliminary experiments.

183

184 The bacterial exposure with nine inlet water strains and nine outlet water strains was done using 14 185 replicate fish per strain. In addition, 14 control fish received sterile growth medium and served as a 186 negative control group. In total, 266 individual zebra fish were used in the experiment. Five ml of 187 sterile Shieh culture medium mixed with bacterial culture (pure culture medium in the negative 188 control) was pipetted directly into each aquarium to reach the infective dose of  $1 \times 10^4$  colony 189 forming units (cfu) ml<sup>-1</sup> in the water throughout the experiment, as a continuous exposure. The fish 190 were then monitored for 11 d for disease symptoms and morbidity. For the first 3 days, during the 191 most acute phase of the disease, the fish were monitored every hour and when the progression of the 192 epidemic ceased the monitoring points were decreased accordingly, including at least two checks per 193 day.

194

The virulence of strains isolated in 2003-2010 was tested similarly but with small modifications. Infection with each bacterial strain (11 strains of the dataset) was done to 10 replicate fish. In addition, 10 fish in the negative control group were exposed to sterile culture medium, thus the total number of individual fish in this experiment was 120. In this experiment the bacteria were mixed in 550  $\mu$ l of Shieh medium and pipetted into aquaria to reach a dose of 2.5 x 10<sup>5</sup> cfu ml<sup>-1</sup>. The fish were monitored every hour for 40 hours.

201

During the experiments the fish were held in individual 750 ml plastic aquaria with 500 ml of ground water (average t=24.7°C). Morbid fish that had lost their natural swimming buoyancy and did not respond to external stimuli were considered dead and removed from the experiment. All the remaining healthy fish at the end of the experiment were euthanized by cutting the spinal cord under terminal aenesthesia with MS-222 (Sigma). All fish were weighted and a bacterial culture sample was taken from the gills on Shieh agar supplemented with tobramycin [26] to ensure the cause of death to be columnaris disease.

The experiments were conducted according to the Finnish Act on the Use of Animals for Experimental Purposes, under permission ESAVI-2010-05569/Ym-23 granted for L-RS by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

214

#### 215 Data-analyses

216 The inhibition data of inlet water and outlet water strains was analysed using generalized linear 217 mixed models implemented in function glm, in R. Inhibition data was modeled with quasipoisson 218 distribution, explaining pooled replicates (within each reciprocal combination, considered as separate 219 combination) with 4 levels fixed factor representing all combinations of receiver and donor being an 220 inlet or outlet strains (without multiple comparisons). The inhibition data of strains isolated in 2003-221 2010 was analyzed with similar method from averaged data, but using year of isolation of donor and 222 recipient strains as continuous covariates (both rank transformed). The replicates were pooled due to 223 problems in mixed model fitting of random effects of strain combination that prevented accurate 224 estimates of 2003-2010 data. The results obtained from mixed models, in both datasets, give 225 equivalent biological interpretation of the data that is presented here.

226

In competition in liquid culture there are no producer or receiver strains that could be distinguished, and we could only test the effects of average isolation year and difference of the isolation years on the interaction index, in addition to the strain combination identity. The growth traits of all bacterial isolates, and the competition indices for time series 2003-2010, were analysed with mixed models in SPSS.

232

233 Virulence of bacterial strains in both of the datasets (time series and the inlet vs outlet water strains) 234 was first analysed by Kaplan Meier survival analysis (log rank Mantel cox). The fish alive at the end 235 of experiments were treated as censored data. In addition to the log rank analysis, we also used 236 average longevity as a measure of virulence with strains isolated in 2003-2010. However, in the 237 dataset of the inlet and outlet water strains only 10% of the fish infected with the inlet water strains 238 (and 40 % infected with the outlet water strains) entered a moribund state during the experiment, 239 precluding the use of the fish longevity as a surrogate of virulence. Therefore, for the inlet and outlet 240 water strains, we used the log rank Mantel cox survival analysis, and also arcsin-square transformed 241 mortality percentage as a measure of virulence. The mortality percentages of fish were tested with t-242 tests allowing for the unequal variances [34].

- 244 **Results**
- 245

#### 246 Temporal evolution in *F. columnare* strains isolated in 2003-2010

247 Inhibitory toxin production, competitive ability and virulence were significantly associated with the 248 time of isolation in strains from different fish farms and environmental locations (2003-2010). The isolation year had a significant effect on bacterial virulence (log rank Mantel cox,  $\chi^2$ = 55.338, df=3, 249 250 p<0.001). The more recently isolated bacteria were significantly more virulent (average longevity of 251 the infected fish) than the ones isolated earlier (b=-1.716, p=0.014) (Figure 1a). In line with the 252 virulence, the more recently isolated strains were more likely to inhibit the earlier isolates (b=0.004, 253 p=0.0181). The sensitivity of the bacteria to the interference by the other strains was independent of 254 the isolation time (year (rank-transformed) of isolation of the inhibited isolate b=0.002, p=0.2975) 255 (see Supplementary Table 2a for original data). The year of isolation did not have an effect on the 256 bacterial growth parameters (maximum growth rate, yield or area under curve AUC) whereas the 257 strain identity did (Supplementary Table 3).

258

To elaborate the benefits of interference in resource competition, we studied the intensity of competition between the isolates in co-culture, where the competition on finite resources is expected to lead to lower production of biomass if the competing strains use the same resource. The competition was more intense (indicated by lower interaction index) between strains that on average had been isolated more recently, than between the strains that were isolated earlier (Table 3, Figure 2). The difference in the isolation times of the competing strains did not significantly affect the interaction index (Table 3).

266

The cross-streaking experiments of the strains B185 and B067 indicated that toxin production in *F*. *columnare* could be contact-dependent. When cross-streaked on agar-plate, in nine out of ten replicates clear inhibitory zones (similar to Dienes lines) [35,36] were observed (Supplementary Figure 1), but only when supernatant containing bacterial cells was used. Sterile filtered supernatant did not cause any inhibition in any of the experiments.

272

#### 273 Local, short timescale evolution

The farming environment was observed to have a significant impact on the population structure of the bacterial populations occurring in the inlet and outlet water. The bacterial population from the outlet water of the fish farm was genetically more homogenous than the population from the inlet

- 277 water upstream of the farm (Table 2). Some of the genotypes in the inlet water were not detected in
- 278 outlet water, indicating that the fish farming environment may select for specific genotypes.
- 279

Place of isolation (inlet or outlet water of a fish farm) had a significant effect on bacterial virulence (log rank Mantel cox,  $\chi^2$ = 33.471, df=2, p<0.001). Mean percentage of mortality in fish infected with the outlet water isolates was 32.5%, and 10.3% with the inlet water strains (t=3.155, p=0.006, on arcsin square transformed proportion of the dead fish) (Figure 1b). No background mortality in the fish exposed to sterile growth medium (negative control group) was observed.

285

286 When the outlet water isolates acted as donors in an inhibition test against the inlet water isolates, the 287 risk of inhibition (0.225) seemed to be higher (z=-2.154, p=0.033) than when the outlet water strains 288 were let to inhibit other outlet water strains (0.167). Other types of inhibition pairs produced 289 intermediate inhibition risks and did not statistically differ from the other pairs (Table 4 and 290 Supplementary Table 2b). Since level of significance (p=0.033) of the only significant pairwise 291 comparison is not dramatically different from 0.05, there is a possibility that significance is 292 overstated without corrections for multiple testing, leading to acceptance error (type I error). 293 However, several papers suggest that multiple corrections could very easily lead to rejection errors 294 (type II), especially with small datasets, as here [37]. Hence, as we have not adopted corrections the 295 obtained result should be considered tentative.

296

297 To examine the effects resource concentrations on bacterial growth we studied the growth of the inlet 298 and the outlet water bacterial strains in two resource concentrations: in the standard growth medium 299 and in the diluted (0.5x) medium. In yield and AUC the outlet water strains (yield: 0.853, se: 0.052; 300 AUC: 647.6, se: 40.98) excelled over the inlet water strains (yield: 0.668, se: 0.052; AUC: 486.6, se: 301 40.98), but the growth rates of the two groups were comparable (inlet water: 0.216, se: 0.012; outlet 302 water: 0.199, se: 0.012) (Table 5, Supplementary Figure 2). Interestingly, though, the growth of the 303 outlet water strains was more sensitive to the differences in resource concentrations (low resource: 304 0.176, se: 0.013; high resource: 0.222, se: 0.013, Table 5) than the growth of the inlet water strains 305 (low resource: 0.208, se: 0.013; high resource: 0.225, se: 0.013).

306

Fifteen bacterial strains (including strains from both datasets) were screened for presence ofplasmids, but none were observed (Supplementary Figure 3).

- 309
- 310 Discussion

311 Since the mid-1990's, F. columnare outbreak frequency, severity of symptoms and disease-related 312 mortality have significantly increased in aquaculture, and the evolution of virulence has been 313 suggested as an explanation [23]. Our study finds that the bacterial strains isolated most recently, 314 during the period of more difficult disease outbreaks, are more virulent and have a higher 315 competitive ability than the strains isolated earlier. In addition, our findings about the F. columnare 316 populations from the inlet and outlet water of a fish farm are congruent with the suggestion of 317 selection of more virulent strains in aquaculture. The strains isolated from outlet water were more 318 virulent and able to produce higher maximum population sizes than the inlet water strains, indicating 319 that the bacteria with the best ability to exploit fish populations benefit the most in the intensive 320 aquaculture conditions.

321

322 Opportunistic lifestyle and ability to persist outside host opens less stringent trajectories for virulence 323 evolution, in contrast to the obligate pathogens restricted by the transmission-virulence trade-off [8], 324 but factors that increase or limit virulence evolution in opportunistic pathogens are still poorly 325 understood. F. columnare can be considered an opportunist, as it is known to survive long periods 326 outside the host and transmit efficiently from dead hosts [38-40]. A weak trade-off between 327 virulence and other fitness traits, like transmission, provided by the outside-host survival could thus 328 promote the evolution of high virulence in F. columnare. Moreover, in aquaculture, new susceptible 329 fish populations are introduced at the farm annually, creating conditions similar to serial passage 330 which further decreases the costs of virulence [9].

331

332 In addition to the factors related to host abundance, the bacterial communities in aquaculture are 333 shaped by increased concentrations of nutrients and chemical and antibiotic treatments within the 334 rearing units. Eutrophication of the aquatic environment increases parasitic and bacterial diseases via 335 direct and indirect effects in the food web [41,42]. While the role of eutrophication on F. columnare 336 epidemics is still unclear, the increased nutrient concentrations may support the outside-host growth 337 of this pathogen, giving the more virulent strains a greater advantage (Supplementary Figure 2), see 338 also [43]. On the other hand, the use of chemotherapy can relax the competitive interactions between 339 bacteria by eliminating sensitive species. This may lead to more frequent or virulent outbreaks of 340 opportunistic diseases if the use of antibiotics increases the intensity of the within-species 341 competition [44] or selects for faster transmission rate [45]. The increasing amount of antibiotics 342 used in food production [46] may thus affect microbial communities beyond the traditionally 343 expected environmental effects [47-49]. Therefore, to secure the global food production it is vital to 344 understand the factors that select for virulent pathogen strains.

346 On top of intensive farming practices *per se*, also other ecological and evolutionary factors underpin 347 the evolution of more virulent and competitive bacteria at long timescales. A general increasing trend 348 in disease species diversity and outbreak frequency has been observed during last decades, but the 349 reasons for this are still largely unknown [50]. One contributing factor is the warming climate, that 350 causes changes in disease ecology, outbreak dynamics and seasonality [51-53]. Due to the global 351 warming, the longer outbreak period for columnaris disease [23] increases the bacterium-host and 352 bacterium-bacterium interactions, allowing greater opportunities for evolution of both virulence and 353 competitive ability.

354

Strain-strain interactions can have a significant role in bacterial disease dynamics via competition in 355 356 both within-host and outside-host environments [54-58], and the surrounding microbial community 357 has been shown to have significant effects on the evolution of interference [59,60]. However, how 358 interference competition is associated with intensive farming is not properly understood. Our data 359 suggest that the most recently isolated bacteria seem to have the highest capacity for interference 360 competition and, on average, the competition between the most recently isolated strains is the most 361 intense. Although the strains isolated from the inlet water were able to inhibit each other and the 362 outlet water strains, significant differences in inhibition was observed only when the outlet water 363 isolates acted as inhibitors. The strains isolated from outlet water seemed to be able to inhibit the 364 growth of the inlet water strains but tolerated well the toxins produced by other outlet water strains, 365 most likely due to more homogeneous population structure resulting in less competitive interactions 366 [61]. While these results are in accordance with the general expectations of the evolution of 367 interference competition in mixed populations [54,57], whether these interactions are relevant during 368 disease outbreaks is unknown, and type I error in the interpretation of the results is possible. 369 Nevertheless, different bacterial population structure was observed in these two locations even 370 during the same sampling dates that are directly comparable (Table 2). These results suggest that the 371 competition pressures differ within and outside the farming environment, and that the interference, as well as toxin tolerance, could be beneficial for the virulent strains in the farming environment where 372 373 invasions by multiple strains are frequent.

374

Although this study does not aim to characterize the mechanism of growth inhibition, bacteriocins
have been reported previously in *F. columnare* [62]. Our data, however, indicate that the toxin

377 production in *F. columnare* may require a direct contact between bacterial cells (see Supplementary

Figure 1), but more studies are needed to identify the cell-cell interactions in this species in detail.

13

380 Previous experimental studies have often demonstrated a direct trade-off between toxin production 381 and both growth rate and virulence. Virulence of co-infection with a toxin-producing and a toxin-382 sensitive bacterial strain leads to a decrease in the total virulence of infection [18,19,63]. In contrast 383 to traditional assumptions, our data show no evidence of costs in toxin production for growth 384 (growth rate, population size) or virulence in F. columnare. Although the competitive ability and 385 virulence increased in time, the bacteria isolated in 2003-2010 did not differ in their growth features. 386 Similarly, the growth rate of the virulent bacteria isolated from the outlet water did not exceed the 387 growth rate of the less competitive and low-virulence inlet water strains. However, the outlet water 388 population reached higher population sizes regardless of the nutrient conditions. It seems that in this study system the same factors selecting for increased virulence might simultaneously also select for 389 390 increased competitive ability. It remains unknown whether virulence and competitive ability are 391 genetically linked and if their benefits are traded off with other life-history traits.

392

393 To conclude, in accordance with the theoretical predictions [1,7-9,21], our data are consistent with 394 the hypothesis that the intensive farming conditions (high host densities, increased transmission 395 opportunities, co-infections, possibility for serial passage, availability of nutrients, use of 396 chemotherapy) can select for pathogen strains with the ability to produce large population sizes, high 397 virulence that have high competitive ability under short time scales. This indicates a genetic 398 difference in populations of high- and low-virulence bacterial strains resulting in selection for strains 399 with an increased ability to exploit the fish host as a nutrient source. To reveal the possibility of 400 horizontal gene transfer by conjugation as a mechanism for increased virulence, we assayed the 401 presence of plasmids in the F. columnare bacterial strains, but similarly to previous studies [28] none 402 were found. Therefore, other possible genetic mechanisms causing the changes in virulence 403 (transduction, chromosomal transformation, mutations) [64] remain to be solved, and will require 404 whole genome sequencing of several strains. Also the recent achievements in genetic manipulation 405 techniques [65,66] and genome sequencing [67] are likely to provide detailed insight into the 406 mechanisms behind F. columnare pathogenicity.

407

408 Our results indicate selection for pathogen virulence and competitive ability in both long and short 409 time scales. The bacterial strains isolated in 2003 were significantly less virulent than the strains 410 isolated later, which correlates with the observed increase in the severity of columnaris outbreaks 411 during the last decade [23]. Interestingly, a similar pattern was observed already during one outbreak 412 season, as the strains isolated from the outlet water of a fish farm were more virulent than strains

413 isolated from the inlet water. These results indicate that the selection pressures at fish farms can 414 cause changes in pathogen populations, which may have long-lasting effects on pathogen virulence. 415 The global changes in nutrients and climate can further select pathogen traits at a wider temporal 416 scale. Indeed, we cannot rule out the possibility that the changes in bacterial characteristics observed 417 in this paper are unrelated to aquaculture. Nevertheless, aquaculture has a major role in securing the 418 world protein production, but disease epidemics severely affect the profitability of the industry [68-419 70]. Understanding the disease ecology and evolution in the man-made environments is important in 420 securing sustainable livestock and aquaculture production. In addition to the benefits in the applied 421 field, the studies on pathogens in the intensive farming systems can provide much needed empirical 422 field data on the evolution of virulence.

423

#### 424 Data accessibility

425 All data used in this paper are publicly available in supplementary file and in Dryad 426 (doi:10.5061/dryad.nk76k).

427

## 428 Competing interests

429 Authors do not have competing interests.

430

### 431 Authors' contributions

L-RS, TK, JB and JM designed the study. L-RS, TK, EL, HK and RP collected and analysed the
data. All authors participated in drafting and critically revised the manuscript. All authors approved
the final version of the manuscript.

435

436

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600

# 635 TABLES

- 636 Table 1. Flavobacterium columnare strains isolated in 2003-2010 and used in this study. Genetic
- 637 group is based on MLSA analysis which corresponds with the ARISA genotyping used with strains
- 638 in Table 2 [31].

Isolation source	Bacterial strain	Year of isolation	Fish species/water sample	Genetic group
A, fish farm Central Finland	B431	2003	Grayling Thymallu thymallus	А
	B067	2007	Trout Salmo trutta	А
	B185	2008	Rearing tank water	G
B, fish farm				
Central Finland	H2	2003	Rainbow trout Oncorhynchus mykiss	Н
	B429	2003	Pikeperch Zander lucioperca	Н
	B430	2003	Pikeperch Z. lucioperca	Е
	B425	2007	Rainbow trout O. mykiss	
	B245	2009	Rearing tank water	С
	B259	2009	Rearing tank water	С
	B402	2010	Whitefish Coregonus lavaretus	С
	B366	2010	Outlet water of a fish farm	С
C, Nature (Lake)	B405	2010	Lake Jyväsjärvi	С
D, Nature (River)	B407	2010	River upstream the fish farm B	G
E, fish farm Northern Finland	B428	2006	Atlantic salmon Salmo salar	
	B426	2006	Atlantic salmon S. salar	С
	B420	2009	Atlantic salmon S. salar	G
	B421	2009	Atlantic salmon S. salar	С

- 642 nature, from river upstream of a fish farm (inlet water) in Central Finland (farm B in Table 1) and
- from the outlet water or from the river immediately downstream of the outlet water tube of the same
- farm of the farm in summer 2010. Genetic grouping is based on Automated Ribosomal Intergenic
- 645 Spacer Analysis (ARISA).

Isolate	Inlet	Outlet	Genetic	Time of isolation	Reference	
	water	water	group	(2010)		
B395	Xa		G	21 <sup>st</sup> June	[29]	-
B396	Xa		А	٠٠	"	
B397	$X^{b}$		С	٠٠	"	
B398	$X^b$		А	٠٠	"	
B400	$X^{b}$		А	19 <sup>th</sup> July	"	
B404	X <sup>a</sup>		С	2 <sup>nd</sup> August	"	
B355	Х		А	٠٠	This study	
B406	$X^{b}$		С	16 <sup>th</sup> August	[29]	
B407	$X^{b^*}$		G	٠٠	"	
B339		Xc	С	5 <sup>th</sup> July	This study	
B340		X <sup>c</sup>	С	۰۵	۲۵	
B350		X <sup>a</sup>	E	2 <sup>nd</sup> August	٠٠	
B351		X <sup>a</sup>	E	٠٠	٠٠	
B366		X <sup>c</sup>	С	٠٠	٠٠	
B370		$X^{b}$	E	16 <sup>th</sup> August	٠٠	
B374		$X^{b}$	E	٠٠	٠٠	
B375		$X^{b}$	E	٠٠	٠٠	
B379		Xc	E	30 <sup>th</sup> August	٠٠	

<sup>a</sup>river, biofilm

<sup>b</sup>river, free water

\* 400m upstream of the water intake

<sup>c</sup> outlet water of the farm

Table 3. Effects of the average year of isolation and the difference of the isolation years on growth
inhibition (i.e. interference, studied on agar plates) and competition (interaction studied in liquid
medium) in pairs of *Flavobacterium columnare* isolates, collected during disease epidemics in 20032010.

### Inhibition

Parameter	Estimate	Std. Error	t	Sig.
Average year of isolation	0.0034	0.002	2.078	0.039
Difference in years of isolation	-0.0001	0.002	0.764	0.446

#### **Interaction index**

Parameter	Estimate	Std. Error	df	t	Sig.
Average year of isolation	-0.0255	0.009	104.707	-2.759	0.007
Difference in years of isolation	0.0024	0.007	103.641	0.355	0.724
(Strain combination 0.0215 s.e:0.0036, Wald Z:5.911, p<0.001, Residual: 0.008, s.e.:0.001, Wald					
Z:6.959, p<0.001)					

- **Table 4.** Pairwise comparisons of risk of inhibition (interference competition) by inlet water and
- outlet water strains in different combinations of donors and receivers (each reciprocal combination
- 668 considered as separate combination). I = inlet water strains isolated from upstream of a fish farm, O
- 669 = strains isolated from outlet water or downstream of the farm.

	Donor O, Reciever I	Donor I, Reciever O	Donor O, Reciever O
Donor I, Reciever I	z=1.084, p=0.2790	z=0.098, p=0.922	z=-1.086, p=0.278
Donor O, Reciever I		z=-0.986 p=0.325	z=-2.154, p=0.033
Donor I, Reciever O			z=1.183, p=0.238

- **Table 5.** Results of the mixed model analysis exploring the growth differences between the inlet
- 674 water and the outlet water isolates (Location) of *F. columnare* measured in high and low resource
- 675 concentrations. The model also contains a random effect of the strain identity to control for the non-
- 676 independency of observations arising from repeated growth measurements.

	Maximal growth rate (change in OD h -1)		
	F	df1,df2	р
Location	0.968	1,16.054	0.34
Resource concentration	27.113	1,163.047	< 0.001
Location x Resource concentration	5.826	1,163.047	0.017
Strain identity	Wald Z:	2.477	0.013

	Yield		
	F	df1,df2	р
Location	6.372	1,15.989	0.023
Resource concentration	173.173	1,163.002	< 0.001
Location x Resource concentration	0.004	1,163.002	0.947
Strain identity	Wald Z:	2.729	0.006

	AUC		
	F	df1,df2	р
Location	7.724	1,15.989	0.013
Resource concentration	136.565	1,163.002	< 0.001
Location x Resource concentration	1.302	1,163.002	0.255
Strain identity	Wald Z:	2.722	0.006

#### 680 FIGURE LEGENDS

681

- 683 *rerio*) A) The mean longevity (±S.E., open squares, left axis) and survival percentage (dark circles,
- right axis) of the infected zebra fish (n=10 in each bacterial exposure) after exposure to bacterial
- 685 strains isolated in 2003-2010. **B**) Survival percentage of zebra fish (n=14 in each bacterial exposure)
- after exposure to bacterial strains isolated from inlet water (B355-B407) and outlet water (B399-
- 687 B397) of a fish farm (dark circles for individual strains, open circles for mean survival±S.E.).
- 688

Figure 2. Changes in (arcsin-transformed) interaction index of the competing pairs of
 *Flavobacterium columnare* strains on limited resources in relation to the mean isolation time of the

691 pair of isolates. Lower index indicates higher competition. Circle indicates the mean of the two

- 692 replicates of the tested *F. columnare* strain pairs.
- 693



- **FIGURE 2**



709	
710	Supplementary material
711	
712	Intensive aquaculture selects for increased virulence and interference competition in bacteria
713	
714	Lotta-Riina Sundberg*, Tarmo Ketola, Elina Laanto, Hanna Kinnula, Jaana K.H. Bamford, Reetta
715	Penttinen, and Johanna Mappes
716	

#### 717 Supplementary methods

718

#### 719 *Genetic characterization of bacteria*

720 The strains isolated in 2003-2010 were genetically characterized in a previous study using the 721 Multilocus Sequence Analysis developed for F. columnare [1]. The inlet water strains were 722 characterized in an earlier study [2] with the Automated Ribosomal Intergenic Spacer Analysis 723 (ARISA), which corresponds with the MLSA typing [1]. The outlet water F. columnare strains and 724 one inlet water strain (B355) were genotyped in this study, essentially as described earlier [2,3]. 725 Shortly, DNA from overnight cultures was isolated with Fermentas Life Sciences GeneJET Genomic 726 DNA Purification Kit (#K0722) according to manufacturer's instructions. Ribosomal intergenic 727 spacer region was amplified by PCR using primers rD1f (5'-GGCTGGATCACCTCCTT-3', with 6FAM label, [4] and 23Sr (5'-GGGTTBCCCCATTCRG-3', [5]. The PCR products were denatured 728 729 with formamide mixed with GeneScan<sup>TM</sup> 1200 LIZ Size Standard (1:20 mixture), separated with an 730 ABI Prism 3130xl Genetic Analyser, and visualized with GeneMapper v.5.0 software (all Applied

731 Biosystems, Carlsbad, California, USA).

**Supplementary Table 1.** *Flavobacterium columnare* occurrence (indicated by grey bar) at Fish farm

B on sampling dates in 2010. The asterisk indicates that an obtained bacterial isolate was used in the

736 experiments (details in Table 2 in main text).

Date	Inlet water	Outlet water	Water temperature°C
February 23 <sup>th</sup>			0.5
April 26 <sup>th</sup>			
May24 <sup>th</sup>			
June 8 <sup>th</sup>			14
June 21 <sup>st</sup>	*		15.5
July 5 <sup>th</sup>		*	20.5
July 12 <sup>th</sup>			
July 19 <sup>th</sup>	*		24.8
July 26 <sup>th</sup>			22.2
August 2 <sup>nd</sup>	*	*	22
August 16 <sup>th</sup>	*	*	20.8
August 30 <sup>th</sup>		*	15.8
October 11 <sup>th</sup>			8
November 15 <sup>th</sup>			1.7

- 740
- 741

Supplementary Table 2. The inhibition profiles of *Flavobacterium columnare* strains isolated a) in 2003-2010 and b) from inlet water (B355-B407) and outlet water (B339-B379) of a fish farm during summer 2010. The number in the cell represents the frequency of inhibition. Experiments were done in three (strains 20013-2010) or four (inlet, outlet waters strains) independent replicates. Supernatant of each overnight grown bacteria ('donors', top row) were spotted on each bacteria mixed in soft agar ('recipients', columns below) in a double layer plate assay. The presence of inhibition was recorded after 48h.

- 749 a)
- 750

			2003 A	2003 B	2003 B	2003 B	2006 E	2006 E	2007 B	2007 A	2009 E	2009 E	2009 A	2009 B	2009 B	2010 C	2010 D	2010 B	2010 B
Year	Farm	Code	B431	H2	B429	B430	B428	B426	B425	B067	B420	B421	B185	B245	B259	B405	B407	B402	B366
2003	Α	B431		0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	2
2003	в	H2	0		0	3	0	0	1	0	0	0	0	1	0	2	0	1	2
2003	в	B429	0	0		2	0	0	0	0	0	0	0	0	0	2	0	1	0
2003	В	B430	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0
2006	E	B428	0	0	0	0		0	0	0	0	0	2	0	0	0	1	0	0
2006	E	B426	0	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0
2007	В	B425	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0
2007	Α	B067	0	0	0	2	0	1	1		0	0	1	0	0	2	0	0	1
2009	E	B420	0	0	0	1	0	0	2	0		0	0	1	0	1	0	0	3
2009	E	B421	0	0	0	1	0	0	0	0	1		1	0	0	1	0	0	0
2009	Α	B185	0	0	1	1	1	0	1	0	0	0		1	1	1	0	1	2
2009	В	B245	0	0	0	0	0	0	0	0	1	0	1		0	0	0	0	0
2009	в	B259	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0
2010	С	B405	0	0	0	0	0	0	0	0	1	0	0	0	0		0	0	0
2010	D	B407	1	0	1	3	1	1	3	0	0	0	0	2	3	1		2	2
2010	в	B402	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0		1
2010	В	B366	0	0	0	0	0	0	0	0	2	0	1	0	0	0	1	0	

753

b)

		B355	B395	B396	B397	B398	B400	B404	B406	B407	B339	B340	B350	B351	B366	B370	B374	B375	B379
Inlet	B355		0	0	2	1	0	0	1	0	1	2	0	1	0	1	2	0	1
Inlet	B395	4		3	2	2	3	3	4	4	2	4	0	3	3	3	4	1	4
Inlet	B396	1	0	1	1	1	0	1	2	2	1	2	0	2	1	2	1	1	1
Inlet	B397	1	2	0		0	0	0	1	2	0	1	0	1	0	0	2	0	1
Inlet	B398	2	1	0	1		1	1	2	1	0	2	0	1	3	2	2	1	1
Inlet	B400	2	1	0	1	0		1	1	1	0	2	0	2	2	1	1	1	1
Inlet	B404	0	1	0	0	0	0		3	1	0	0	0	1	0	1	1	0	1
Inlet	B406	3	1	1	0	1	0	0		1	1	0	0	1	0	1	3	0	1
Inlet	B407	1	2	0	1	0	0	1	2		0	0	0	0	0	1	1	0	1
Outlet	B339	1	1	0	0	0	0	0	0	1		2	0	1	0	2	1	0	1
Outlet	B340	0	1	0	0	0	0	0	1	0	0		0	1	0	1	1	0	0
Outlet	B350	1	1	0	1	1	0	1	2	0	1	2		1	0	1	3	0	1
Outlet	B351	1	0	0	3	0	0	2	2	0	1	3	0		2	1	3	0	0
Outlet	B366	2	4	2	1	2	1	1	2	2	2	2	0	1		3	2	0	1
Outlet	B370	0	1	1	1	0	0	2	3	0	1	3	0	0	0		0	0	0
Outlet	B374	0	0	0	2	0	0	2	3	0	0	1	0	0	0	0		0	0
Outlet	B375	1	1	0	3	1	0	1	3	0	0	1	0	0	1	1	1		0
Outlet	B379	0	1	0	2	1	0	1	2	0	0	2	0	0	1	2	1	0	

Supplementary Table 3. Effects of the year of isolation and the strain identity on growth of
 *Flavobacterium columnare* strains isolated in 2003-2010.

Maximal growth rate				
(change in OD h -1)	Estimate	Std. Error		Sig.
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.504	0.012
Yield	Estimate	Std. Error		Sig.
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.502	0.012
Area under curve	Estimate	Std. Error		Sig.
Year of isolation	-12.93	22.99	F:-0.562	0.584
Strain ID	62088.88	24388.15	Wald Z: 2.546	0.011

- the intersection of agar plate culture of *Flavobacterium columnare* strains. A) Control B185 + B185,
- 765 B) B185 (horizontal) and B067 (vertical).











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