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Title: Intensive aquaculture selects for increased virulence and interference competition in bacteria

Year: 2016

Version:

Please cite the original version:

Sundberg, L.-R., Ketola, T., Laanto, E., Kinnula, H., Bamford, J., Penttinen, R., & Mappes, J. (2016). Intensive aquaculture selects for increased virulence and interference competition in bacteria. *Proceedings of the Royal Society B: Biological Sciences*, 283(1826), Article 20153069. <https://doi.org/10.1098/rspb.2015.3069>

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

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20 **KEY WORDS: aquaculture, evolution, fish farming, *Flavobacterium columnare*, pathogen,**
21 **virulence**

22

23

24

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

41

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

76 interference competition, at both short and long timescales. This indicates that the more virulent
77 strains have higher fitness under intensive farming conditions, and demonstrates that the intensive
78 farming environments can be used as model systems to understand disease dynamics and evolution
79 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

95 The bacteria were isolated originally from three different fish farms in Central and Northern Finland
96 during columnaris disease epidemics and from environment in 2003-2010 (Table 1) using standard
97 culture methods on Shieh medium [25], [26] or AO-agar [27]. Isolates for the dataset were randomly
98 chosen from a large collection of uncharacterized bacterial strains without any a priori information
99 on their competitive ability or virulence, except for strains H2, B067, B185, B245, B405 and B407
100 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
104 outlet water) were sampled on 8th and 21st June, on 5th, 12th, 19th and 26th July, and on 2nd, 16th and
105 30th August in 2010 (Supplementary Table 1). Nine bacterial isolates were randomly selected from
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.

110

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
116 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
119 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 = no
134 inhibition, 1 = inhibition.

135

136 The interaction between *F. columnare* strains B067 and B185 was further characterized with filtered
137 supernatant (0.2 µm PES filter, VWR) and by cross streaking on agar plates in 10 replicates. In the
138 first experiment, turbid overnight grown liquid culture and filtered supernatant of strain B185 were
139 cross streaked with B067 by using 1 µl loop. In the second experiment, 300 µl of turbid liquid
140 culture of B067 was mixed with Shieh soft agar and plated, and 10 µl of B185 culture and filtered
141 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 µl 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
 155 which the maximal growth rate is found from log₂-transformed data by fitting linear regressions on
 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 dH₂O) 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

170

$$171 \quad \text{interaction index} = \frac{\text{growth}(\text{strain 1 and 2 together})}{\text{growth}(\text{strain 1 alone}) + \text{growth}(\text{strain 2 alone})}$$

172

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

174

175 *Virulence in zebra fish*

176 To measure the virulence of the studied bacterial strains, unsexed, adult, disease-free zebra fish were
177 obtained from Core Facilities (COFA) and research services of Tampere (Tampere University,
178 Finland). The zebra fish response to *F. columnare* infection is qualitatively similar to the common
179 host of the pathogen in aquaculture, rainbow trout [33]. The optical density of the overnight-grown
180 bacterial culture (570 nm) was measured and the corresponding bacterial density in colony forming
181 units (cfu) was calculated according to our previously fitted standard curve. The infection method
182 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 1×10^4 colony
189 forming units (cfu) ml^{-1} 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

195 The virulence of strains isolated in 2003-2010 was tested similarly but with small modifications.
196 Infection with each bacterial strain (11 strains of the dataset) was done to 10 replicate fish. In
197 addition, 10 fish in the negative control group were exposed to sterile culture medium, thus the total
198 number of individual fish in this experiment was 120. In this experiment the bacteria were mixed in
199 550 μl of Shieh medium and pipetted into aquaria to reach a dose of 2.5×10^5 cfu ml^{-1} . The fish were
200 monitored every hour for 40 hours.

201

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

209

210 The experiments were conducted according to the Finnish Act on the Use of Animals for
211 Experimental Purposes, under permission ESAVI-2010-05569/Ym-23 granted for L-RS by the
212 National Animal Experiment Board at the Regional State Administrative Agency for Southern
213 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

227 In competition in liquid culture there are no producer or receiver strains that could be distinguished,
228 and we could only test the effects of average isolation year and difference of the isolation years on
229 the interaction index, in addition to the strain combination identity. The growth traits of all bacterial
230 isolates, and the competition indices for time series 2003-2010, were analysed with mixed models in
231 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].

243

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
249 isolation year had a significant effect on bacterial virulence (log rank Mantel cox, $\chi^2= 55.338$, $df=3$,
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

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

266

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

272

273 Local, short timescale evolution

274 The farming environment was observed to have a significant impact on the population structure of
275 the bacterial populations occurring in the inlet and outlet water. The bacterial population from the
276 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

280 Place of isolation (inlet or outlet water of a fish farm) had a significant effect on bacterial virulence
281 (log rank Mantel cox, $\chi^2=33.471$, $df=2$, $p<0.001$). Mean percentage of mortality in fish infected with
282 the outlet water isolates was 32.5%, and 10.3% with the inlet water strains ($t=3.155$, $p=0.006$, on
283 arcsin square transformed proportion of the dead fish) (Figure 1b). No background mortality in the
284 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

307 Fifteen bacterial strains (including strains from both datasets) were screened for presence of
308 plasmids, 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.

345

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

355 Strain-strain interactions can have a significant role in bacterial disease dynamics via competition in
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
372 well as toxin tolerance, could be beneficial for the virulent strains in the farming environment where
373 invasions by multiple strains are frequent.

374

375 Although this study does not aim to characterize the mechanism of growth inhibition, bacteriocins
376 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
378 Figure 1), but more studies are needed to identify the cell-cell interactions in this species in detail.

379

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
389 study system the same factors selecting for increased virulence might simultaneously also select for
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**

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

435

436

437 **Acknowledgements**

438 We would like to thank Dr Heidi Kunttu and Dr. Päivi Rintamäki for donating the bacterial isolates
439 used in this study, Dr. Kunttu for help during the experiments, Dr Nina Pekkala, Mr Petri Papponen
440 and MSc Katja Neuvonen for technical assistance in laboratory, and Dr Emily Burfield-Steel and Dr
441 Andrés López-Sepulcre for helpful comments on the manuscript. This work was supported by the
442 Finnish Centre of Excellence Program of the Academy of Finland, CoE in Biological Interactions
443 2012-2017 (#252411, J.M. and J.B.), by Academy of Finland grants #272995 (L.-R.S.) and #278751
444 (T.K.), and by Maj and Tor Nessling Foundation (J.B.).

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- 634

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 <i>Thymallu thymallus</i>	A
	B067	2007	Trout <i>Salmo trutta</i>	A
	B185	2008	Rearing tank water	G
B, fish farm Central Finland	H2	2003	Rainbow trout <i>Oncorhynchus mykiss</i>	H
	B429	2003	Pikeperch <i>Zander lucioperca</i>	H
	B430	2003	Pikeperch <i>Z. lucioperca</i>	E
	B425	2007	Rainbow trout <i>O. mykiss</i>	
	B245	2009	Rearing tank water	C
	B259	2009	Rearing tank water	C
	B402	2010	Whitefish <i>Coregonus lavaretus</i>	C
	B366	2010	Outlet water of a fish farm	C
C, Nature (Lake)	B405	2010	Lake Jyväsjärvi	C
D, Nature (River)	B407	2010	River upstream the fish farm B	G
E, fish farm Northern Finland	B428	2006	Atlantic salmon <i>Salmo salar</i>	
	B426	2006	Atlantic salmon <i>S. salar</i>	C
	B420	2009	Atlantic salmon <i>S. salar</i>	G
	B421	2009	Atlantic salmon <i>S. salar</i>	C

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640

641 **Table 2. *Flavobacterium columnare* strains used in this study.** The strains were isolated from
 642 nature, from river upstream of a fish farm (inlet water) in Central Finland (farm B in Table 1) and
 643 from the outlet water or from the river immediately downstream of the outlet water tube of the same
 644 farm of the farm in summer 2010. Genetic grouping is based on Automated Ribosomal Intergenic
 645 Spacer Analysis (ARISA).

Isolate	Inlet water	Outlet water	Genetic group	Time of isolation (2010)	Reference
B395	X ^a		G	21 st June	[29]
B396	X ^a		A	“	“
B397	X ^b		C	“	“
B398	X ^b		A	“	“
B400	X ^b		A	19 th July	“
B404	X ^a		C	2 nd August	“
B355	X		A	“	This study
B406	X ^b		C	16 th August	[29]
B407	X ^{b*}		G	“	“
B339		X ^c	C	5 th July	This study
B340		X ^c	C	“	“
B350		X ^a	E	2 nd August	“
B351		X ^a	E	“	“
B366		X ^c	C	“	“
B370		X ^b	E	16 th August	“
B374		X ^b	E	“	“
B375		X ^b	E	“	“
B379		X ^c	E	30 th August	“

^a river, biofilm

^b river, free water

* 400m upstream of the water intake

^c outlet water of the farm

646

647 **Table 3.** Effects of the average year of isolation and the difference of the isolation years on growth
 648 inhibition (i.e. interference, studied on agar plates) and competition (interaction studied in liquid
 649 medium) in pairs of *Flavobacterium columnare* isolates, collected during disease epidemics in 2003-
 650 2010.

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Inhibition

<i>Parameter</i>	<i>Estimate</i>	<i>Std. Error</i>		<i>t</i>	<i>Sig.</i>
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

<i>Parameter</i>	<i>Estimate</i>	<i>Std. Error</i>	<i>df</i>	<i>t</i>	<i>Sig.</i>
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)

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666 **Table 4.** Pairwise comparisons of risk of inhibition (interference competition) by inlet water and
 667 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.

670

	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			$z= 1.183, p=0.238$

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672

673 **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.
 677

Maximal growth rate (change in OD h⁻¹)			
	F	df1,df2	p
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	p
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	p
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

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679

680 **FIGURE LEGENDS**

681

682 **Figure 1.** Virulence of *Flavobacterium columnare* in experimentally infected zebra fish (*Danio*
683 *rerio*) **A)** The mean longevity (\pm S.E., open squares, left axis) and survival percentage (dark circles,
684 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)
686 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 \pm S.E.).

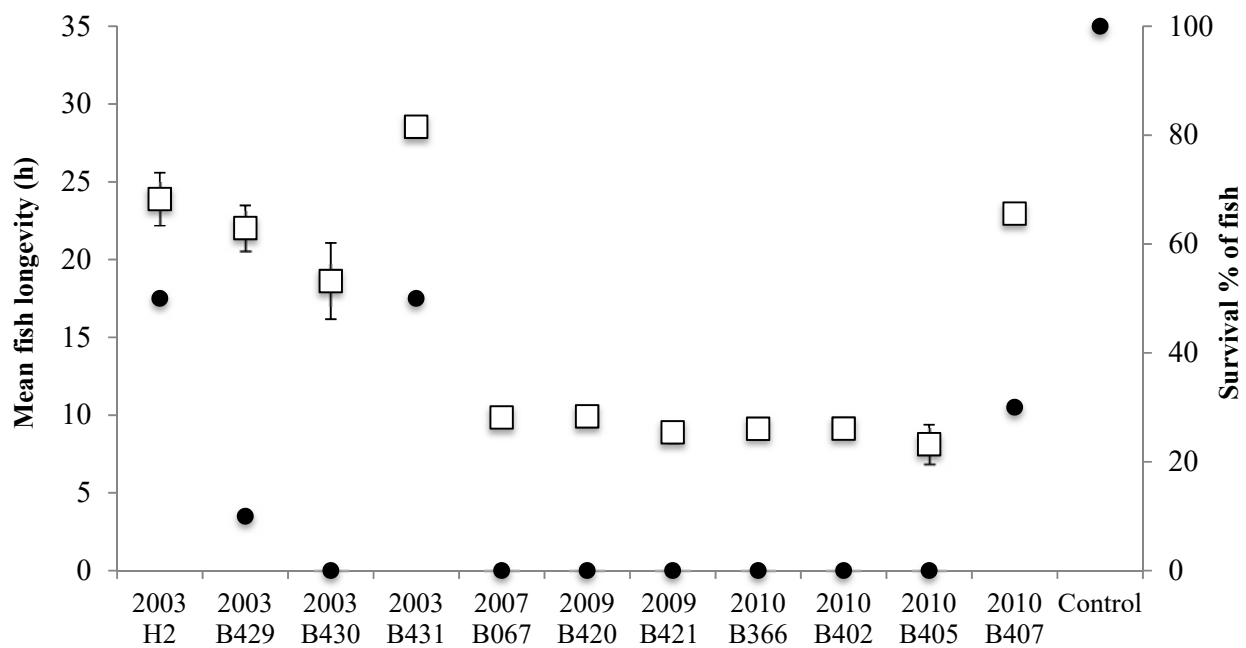
688

689 **Figure 2.** Changes in (arcsin-transformed) interaction index of the competing pairs of
690 *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

694 **FIGURE 1**

695 **A)**

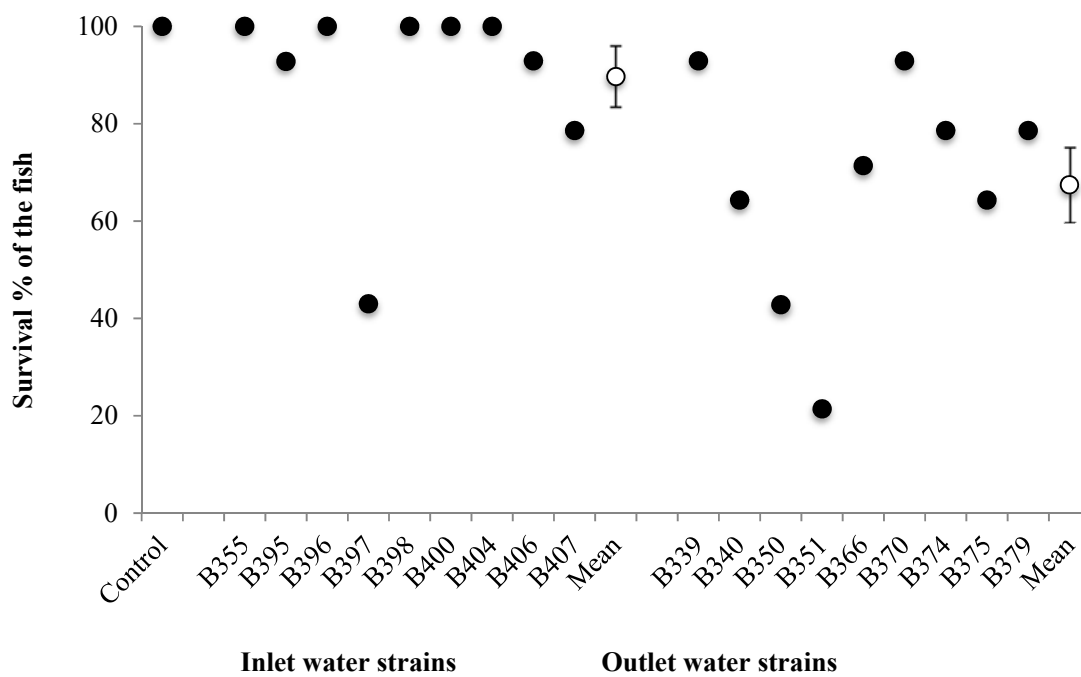


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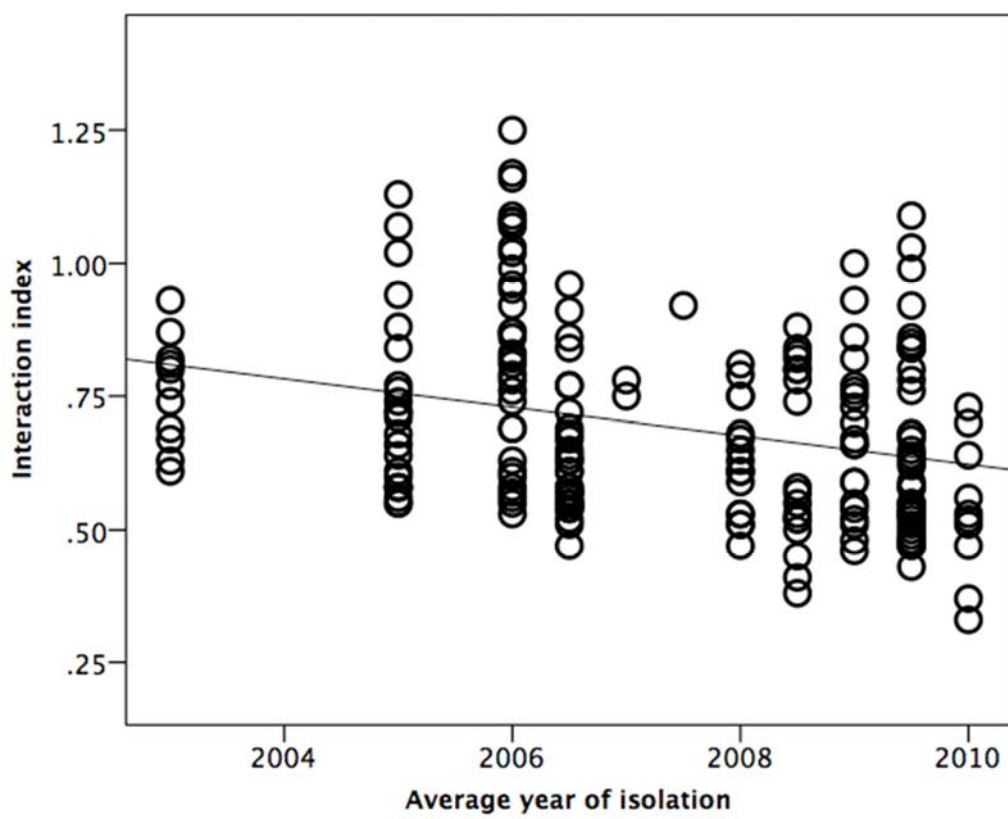
699 **B)**



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701

702 **FIGURE 2**
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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
728 6FAM label, [4] and 23Sr (5'-GGGTTBCCCCATTCRG-3', [5]. The PCR products were denatured
729 with formamide mixed with GeneScan™ 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).

732

733

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

735 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).

737

Date	Inlet water	Outlet water	Water temperature°C
February 23 th			0.5
April 26 th			
May 24 th			
June 8 th			14
June 21 st	*		15.5
July 5 th		*	20.5
July 12 th			
July 19 th	*		24.8
July 26 th			22.2
August 2 nd	*	*	22
August 16 th	*	*	20.8
August 30 th		*	15.8
October 11 th			8
November 15 th			1.7

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742 **Supplementary Table 2.** The inhibition profiles of *Flavobacterium columnare* strains isolated **a)** in
 743 2003-2010 and **b)** from inlet water (B355-B407) and outlet water (B339-B379) of a fish farm during
 744 summer 2010. The number in the cell represents the frequency of inhibition. Experiments were done
 745 in three (strains 20013-2010) or four (inlet, outlet waters strains) independent replicates. Supernatant
 746 of each overnight grown bacteria ('donors', top row) were spotted on each bacteria mixed in soft
 747 agar ('recipients', columns below) in a double layer plate assay. The presence of inhibition was
 748 recorded after 48h.

749 a)

750

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

751

752

753 b)

754

	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
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	1	2	0	1	0	2	1	0	1	0
Outlet	B340	0	1	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0
Outlet	B350	1	1	0	1	1	0	1	2	1	2	0	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	0	1	3	2	0	1	0
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	1	1	1	0	0
Outlet	B379	0	1	0	2	1	0	1	2	0	2	0	0	1	2	1	0	0

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

Maximal growth rate

(change in OD h ⁻¹)	<i>Estimate</i>	<i>Std. Error</i>		<i>Sig.</i>
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.504	0.012

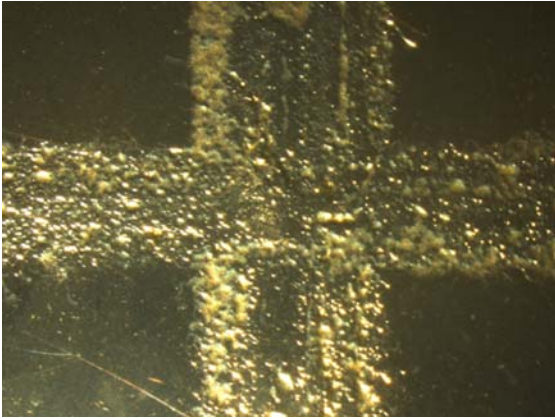
Yield	<i>Estimate</i>	<i>Std. Error</i>		<i>Sig.</i>
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.502	0.012

Area under curve	<i>Estimate</i>	<i>Std. Error</i>		<i>Sig.</i>
Year of isolation	-12.93	22.99	F:-0.562	0.584
Strain ID	62088.88	24388.15	Wald Z: 2.546	0.011

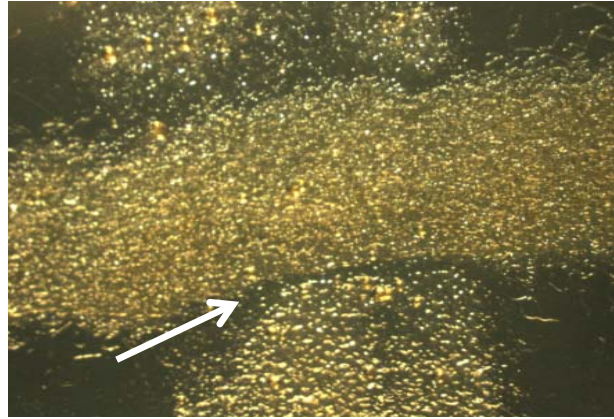
762

763 **Supplementary Figure 1.** Growth inhibition similar to Dienes lines (indicated by white arrow) in
764 the intersection of agar plate culture of *Flavobacterium columnare* strains. A) Control B185 + B185,
765 B) B185 (horizontal) and B067 (vertical).

A



B



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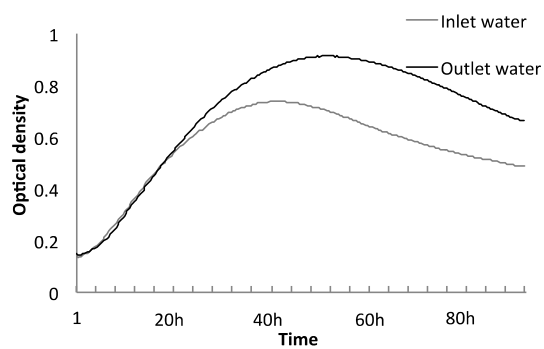
770

771 **Supplementary Figure 2.** Growth (measured as optical density over time) of the inlet water and the
772 outlet water *Flavobacterium columnare* strains in a) standard culture medium and b) 0.5x diluted
773 medium.

774

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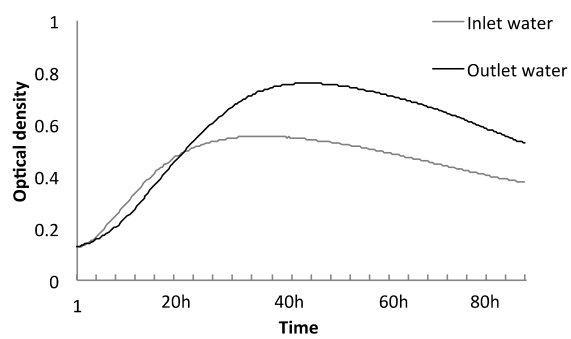
776 a)



777

778

b)



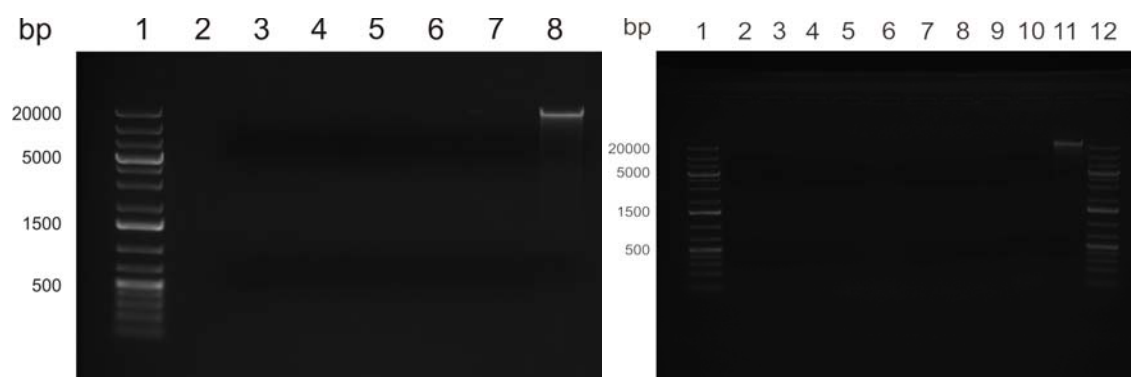
779 **Supplementary Figure 3.** Plasmid screening for *F. columnare* isolates. Lane 1: GeneRuler™ 1kb
780 Plus DNA Ladder (Fermentas). A) Lanes 2 to 7: B067, B185, B245, B405, B407, B420. Lane 8:
781 *Flavobacterium* sp. B330 (positive control). B) Lanes 1 and 12: GeneRuler™ 1kb Plus DNA Ladder
782 (Fermentas). Lanes 2 to 10: B366, B404, B375, B349, B406, B339, B400, B407, B395. Lane 11:
783 *B330 Flavobacterium* sp. B330 (positive control).

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785

786 **A****B**

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