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Effect of resource availability on evolution of virulence and competition in an environmentally transmitted pathogen

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Abstract

Understanding ecological and epidemiological factors driving pathogen evolution in contemporary time scales is a major challenge in modern health management. Pathogens that replicate outside the hosts are subject to selection imposed by ambient environmental conditions. Increased nutrient levels could increase pathogen virulence by pre-adapting for efficient use of resources upon contact to a nutrient rich host or by favouring transmission of fast-growing virulent strains. We measured changes in virulence and competition in Flavobacterium columnare, a bacterial pathogen of freshwater fish, under high and low nutrient levels. To test competition between strains in genotype mixtures, we developed a quantitative real-time PCR assay. We found that a virulent strain maintained its virulence and outcompeted less virulent strains independent of the nutrient level and resource renewal rate while a less virulent strain further lost virulence in chemostats under low nutrient level and over long-term serial culture under high nutrient level. Our results suggest that increased outside-host nutrient levels might maintain virulence in less virulent strains and increase their contribution to epidemics in aquaculture. The results highlight a need to further explore the role of resource in the outside-host environment in maintaining

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strain diversity and driving evolution of virulence among environmentally growing pathogens.

Key words: bacterium, Flavobacterium columnare, fish disease, interference competition, qPCR, resource competition

Background

Evolution can have a significant impact on the properties and responses of organisms in an ecological time scale over a few tens or hundreds of generations (Geerts, et al. 2015, Stuart, et al. 2014, Yoshida, et al. 2003). These effects can be especially pronounced in organisms with short generation times, such as microbes (Hiltunen, et al. 2014). Evolutionary changes resulting in more serious disease and epidemics are presenting major challenges for health management (Griette, et al. 2015), and emphasize the importance of understanding the ecological and epidemiological factors underlying the evolutionary changes for disease control (Bull and Lauring 2014, Cressler, et al. 2016, Kennedy, et al. 2016, Lively, et al. 2014).

Hosts and the nutrition they offer are among the fundamental selective environments encountered by pathogens. However, in pathogens capable of environmental reproduction, the outside-host environment might be equally important by favouring phenotypic properties that increase fitness in that particular environment (Brown, et al. 2012) and selecting for genotypes conferring these properties (New, et al. 2014). Thus, outside-host conditions can be important for the ecological and evolutionary trajectories of environmentally replicating opportunistic pathogens.

Microbial competitive ability in a particular resource environment is mainly governed by resource utilization traits such as growth rate (Litchman, et al. 2015). For environmentally growing bacteria, fast growth is essential in fighting against competing microbes but will also increase the probability of being the first to infect a host upon contact. High resource availability may therefore increase the share of fast-growing strains in host infections.

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The resource environment may also direct selection on resource utilization traits (Litchman, et al. 2015) and promote diversification in resource use (Cooper and Lenski 2000, Jasmin and Kassen 2007, Szappanos, et al. 2016). Resource utilization traits can be traded off against each other, such that adaptation to one resource environment may decrease the competitive ability of a microbe on alternative resources (Cooper and Lenski 2000, Litchman, et al. 2015). Converserly, growth in matching outside-host resources might shorten the lag phase in growth and speed up the ability to exploit host resources (Brown, et al. 2012, Ketola, et al. 2016, Velicer and Lenski 1999). There is also evidence that virulence genes are affected by the nutrient environment, such that growth in rich resources in the outside-host environment could prime environmentally growing opportunistic pathogens for higher virulence towards the host (Brown, et al. 2012, Ketola, et al. 2016, Rohmer, et al. 2011).

Intensive farming is suggested to favour increased pathogen virulence for example via increased contact rates, fast turnover of hosts and strain competition (Kennedy, et al. 2016, Mennerat, et al. 2010, Pulkkinen, et al. 2010). However, in addition to epidemiological factors, intensive farming environments differ from the natural environment with regard to ambient conditions such as the availability of resource for environmentally growing microbes. Nutrient enrichment is increasingly being connected with an increase in the incidence of infectious and parasitic diseases (Aalto, et al. 2015). Recent work has also shown the potential of higher resource availability in the outside-host environment to induce increased virulence in environmentally growing opportunistic pathogens (Kinnula, et al. 2017b, Penttinen, et al. 2016, Wedekind, et al. 2010).

Increased virulence at fish farms over the course of the last 40 years has been suggested for Flavobacterium columnare, an environmentally growing bacterial pathogen of freshwater fish (Kunttu, et al. 2012, Suomalainen, et al. 2006a), potentially due to selection for certain genotypes of the bacterium (Pulkkinen, et al. 2010, Sundberg, et al. 2016). Apart from diseased fish, F. columnare is frequently isolated from lake water and biofilms (Kunttu, et al. 2012), and the bacterium can be efficiently transmitted to a new host also from dead fish (Kunttu, et al. 2009b). Notably, higher ambient nutrient concentration leads to increased virulence of F. columnare in fish challenge experiments (Kinnula, et al. 2017b, Penttinen, et al. 2016). In aquaculture, high fish densities increase water nutrient levels due to fish excretion, faeces and

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uneaten fish feed (Lalonde, et al. 2015). A positive association between growth rate and virulence (Pulkkinen, et al. 2010) could indicate the potential for selection towards higher virulence under high nutrient availability for this environmentally growing pathogen.

Here we examined the hypothesis that high nutrient levels in the outside-host environment in fish farms select for more virulent strains of F. columnare by i) increasing the overall virulence of the strains towards fish hosts and/or ii) favouring virulent strains over less virulent strains in competition. To study the first question, we initially cultured a virulent and less virulent strain under a low and high nutrient level in monocultures. After having evolved under a certain nutrient level, we tested if the overall virulence in the population had changed in comparison to ancestral strains (fish challenge tests in vivo). In order to study the second question about the effect of nutrient level on competition between a virulent and less virulent strain, we initiated co-cultures with a 1:1 ratio of each strain and followed genotype frequencies using a real-time quantitative PCR (qPCR) assay developed for this purpose. We also tested for growth inhibition between the strains, because interference (direct) competition is common for F. columnare growing on surfaces (Ashrafi, et al. 2017, Sundberg, et al. 2016).

We performed two separate experiments, each with the same virulent but a different less virulent bacterial strain. The two experiments differed in the rate of resource renewal. In the first experiment, bacteria were inoculated into batch cultures and only the small volume of culture removed upon sampling was replaced with fresh medium (low rate resource renewal). In the second experiment, high rate resource renewal was executed at high nutrient level with daily serial transfer of a small aliquot of bacterial culture to fresh growth medium in test tubes and at low nutrient level by constant flow-through of the medium in chemostats (Velicer and Lenski 1999). In the high rate renewal experiment, the co-cultures initiated from ancestral strains were terminated after three weeks when the virulent strain had outcompeted the less virulent strain. While monocultures were maintained as they were, another set of co-cultures was initiated using bacteria evolved at the respective nutrient level in monocultures in order to study if potential adaptation to a given nutrient level changes the outcome of competition. For the high rate resource renewal experiment we also tested if biofilm formation by the strains explains the outcome of competition.

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We expected that cultivation at a high nutrient level would pre-adapt the bacteria to rapidly exploit the nutrient rich host and thereby increase their virulence in fish challenge tests. Concerning competition, we expected that the virulent strain would grow faster (Pulkkinen, et al. 2010) and outcompete the less virulent strain under a high nutrient level. However, under a low nutrient level, the slow-growing less virulent strain was expected to win because reproduction of the virulent strain should be restricted by the high nutrient requirement of a high growth rate (Hibbing, et al. 2010).

Methods

Bacterial strains and culture conditions

The virulent strain B402 was isolated from a diseased fish at a fish farm. It was compared pairwise in two separate experiments with a less virulent strain (B407 or B398) isolated from river water (Kunttu, et al. 2012). The virulence of these strains had been tested previously in fish challenge experiments (Kunttu, et al. 2012). The strains were genotyped based on automated ribosomal intergenic spacer analysis (ARISA) (Kunttu, et al. 2012), and more recently, multilocus sequence analysis (MLSA) (Ashrafi, et al. 2015).

All strains were preserved as stock cultures containing 10% foetal calf serum and 10% glycerol at −80°C. The stocks were revived by cultivation in 5 mL of modified Shieh medium (hereafter, Shieh medium) (Song, et al. 1988) at room temperature and constant shaking (120 RPM) for 24 h and subsequent renewal by 1:10 dilution into fresh medium and cultivation for another 24 h.

Bacterial cultures were grown either under a low or high nutrient level. For each nutrient level, the Shieh medium was adjusted to the desired concentration with sterile deionized water. Water was used instead of saline buffer, because of low tolerance of F. columnare to salt water (Suomalainen, et al. 2006a). Bacterial densities used in inoculation were determined by measuring the optical density of the revived culture at 595 nm and comparing to a previously determined relationship between optical density and colony forming unit (CFU mL^{-1}) counts. During the experiment, the

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bacterial growth in the cultures was quantified as CFU mL^{-1} by serial dilution and cultivation on Shieh agar plates.

Batch experiment (low rate resource renewal)

In batch cultures, one-tenth of culture volume was replaced with fresh medium upon sampling (see supplementary Fig. S1A). Bacteria (the virulent strain B402 and the less virulent strain B407) were grown in 5% (low nutrient level) and 50% (high nutrient level) Shieh medium. The total culture volume was 30 mL in 50 mL plastic test tubes, with three replicates per strain or co-culture. The estimated starting number for bacteria was 1.0×10^4 CFU mL^{-1} in the monocultures and 0.5×10^4 CFU mL^{-1} for each of the two strains (1:1 ratio) in the co-cultures. The batch cultures were kept at 25 °C in a shaker incubator under constant agitation (120 RPM).

Samples were collected from all cultures on days 0–5, 7, 9, 11, 16, 21 and 35 (Table S1). On each sampling day, 3.1 mL subsamples were collected from each tube and replaced with fresh medium. For both mono- and co-cultures, 100 µL was used for plate cultivation. For co-cultures, in order to determine the proportions of two bacterial strains in the sample, 3 mL was used for DNA extraction for qPCR. On day 21, samples collected from the bacterial populations evolved in monoculture were tested for virulence in a fish challenge experiment in vivo and compared to ancestral isolates.

High rate resource renewal experiment

High rate resource renewal was executed with continuous supply of a low nutrient level (2%) of Shieh medium in chemostats (a flow-through microcosm) or with daily serial transfer at a high nutrient level (20%) of Shieh medium in test tubes (Velicer and Lenski 1999) (Fig. S1B). The concentration of Shieh medium at the low nutrient level could not be kept at 5% used in the batch experiment because of causing blockage of chemostat tubes, and was decreased to 2%. Consequently, the concentration of the high nutrient level was decreased from 50% to 20%, in order to maintain the same (i.e. 10×) relative difference between the low and high nutrient levels. The strains used were B402 (virulent) and B398 (less virulent). The chemostat volume was approximately 455 mL. The chemostats were assembled aseptically and magnetic stirrer bars were placed at the bottom of the chemostats. A constant flow of fresh 2% Shieh medium was supplied from sterile 5 L flasks to the bottom of the chemostat

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with a Gilson peristaltic pump at dilution rate 0.21–0.24 day^{-1}. During the experiment, the medium flask was replaced at ca. 20 day intervals. The overflow from the top of the chemostat was collected via tubing to a separate waste bottle and discarded. For the high nutrient level, the total culture volume was 30 mL in 50 mL plastic test tubes. In order to maintain the bacterial population at the exponential growth phase, a 50 µL subsample was transferred to a new tube containing 30 mL of fresh medium each day. The culture tubes were kept under constant agitation (120 RPM) in the same room as the chemostats, with temperature adjusted at 22.6 ± 0.1°C. Each tube culture was replicated three times and each chemostat twice.

The monocultures were maintained continuously for 53 days (Table S1). Samples collected from the evolved populations on days 24 and 53 were stored at −80°C as described above and later tested for virulence in a fish challenge experiment in vivo in comparison to ancestral isolates. Co-cultures were started with ancestral strains simultaneously with the monocultures. However, the co-cultures were discarded on day 20 after the virulent strains had become dominant during the first two weeks at both nutrient levels in all replicates and no further change had been observed in the following week (see Fig. 2B). A second set of co-cultures was initiated with samples taken from monocultures on day 25 and, for one chemostat (due to a fail in first inoculation) on day 31 (Table S1). The purpose of the second set of co-cultures was to study if the competitive outcome would change after the strains had already been adapting to a certain nutrient level. The second set of co-cultures was maintained for 28 days.

The estimated starting number for bacteria was 1.0×10^4 CFU mL^{-1} in the test tube monocultures and 0.5×10^4 CFU mL^{-1} for each of the two strains (1:1 ratio) in the co-cultures. Chemostat monocultures were inoculated with 1.0×10^6 CFU mL^{-1} of bacteria and the first co-cultures with 0.5×10^6 CFU mL^{-1} of each of the two strains. The second co-cultures were inoculated with bacteria collected from the monocultures and diluted to the desired bacterial concentration using data from the most recent CFU-measurements. The concentration of bacteria in one of the monocultures used for inoculation was so low that one co-culture had to be started with a lower-than-desired concentration, with a total concentration of 5.0×10^4 CFU mL^{-1} (2.5×10^4 CFU mL^{-1} of each strain). Another co-culture started at the same time was lost and was re-started 6 days later with a total concentration of 1.0×10^6

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CFU mL^{-1} (0.5×10^6 CFU mL^{-1} of each of the two strains). The number of days since inoculation was used to compare growth or the proportion of competing strains in co-cultures (Table S1).

For tube cultures, the plate counting was performed for the culture remaining after the daily transfer of 50 µL to fresh medium and for chemostats for samples taken with a sterile needle and syringe through two ports near the top and bottom of the chemostat (total sample volume approximately 40 mL) mixed in equal proportions. For co-cultures, the remaining culture in the tube and the chemostat sample were used for DNA extraction and subsequent qPCR.

Biofilm formation in the high rate resource renewal experiment

In order to evaluate if a difference in the relative amount of biofilm formation between the virulent and less virulent strain in the high rate resource renewal experiment affected their growth and competition, we quantified the relative amount of biofilm formed during 48 h incubation at the low or high nutrient level. Bacterial cultures (strains B402 and B398) were inoculated into 200 µL of 2% (1.0×10^6 CFU mL^{-1}) or 20 % (1.0×10^4 CFU mL^{-1}) Shieh medium in eight replicate wells per strain × nutrient level in 96 well spectrophotometer plates. After 48 h, the liquid phase was discarded from the wells, the wells were rinsed with distilled water, and the biofilm bound to the walls of the wells was dyed with 200 µL of 1% crystal violet. After 10 min, the plate was overturned to discard the liquid phase of the wells, and the wells were rinsed with distilled water. Overturning and rinsing was repeated three times and after the final rinse, the wells were visually confirmed to be dry. Subsequently, 96% ethanol was added to dissolve the dye. After 24 h, a spectrophotometer was used to measure the optical density of wells at 570 nm wavelength.

Assessment of genotype frequencies in co-cultures

To evaluate the frequency of the virulent and less virulent strain in co-culture we developed a quantitative PCR (qPCR) assay including primer design and testing of primer specificity and efficiency. We used sequences of the F. columnare gene trpB (encoding tryptophan synthase subunit B) (Ashrafi, et al. 2015) to design primers

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specific to genotype C and universal primers that amplify all five genotypes found in Finland (genotypes A, C, E, G and H). The proportions of two bacterial strains in co-culture were then determined using ΔCq (Briand, et al. 2008). In brief, standard curves were prepared using samples containing a known proportion of virulent strain (B402: genotype C) DNA mixed with less virulent strain (B407: genotype G: or B398: genotype A) DNA and by plotting the proportion of the virulent strain against the ΔCq value of the sample ($\Delta Cq = Cq_{specific\ primers} - Cq_{universal\ primers}$). The qPCR-reactions with specific and universal primers were always performed in the same well plate, and the ΔCq values were calculated using the mean of three or sometimes two technical replicates (outliers were removed from the data, sometimes leaving only two of the three replicates for analysis). The unknown samples were analysed similarly together with a positive control sample containing DNA from the strains at a 1:1 ratio. The positive control was used to normalize the ΔCq values of the samples before determining the proportion of the virulent strain in a sample using the constructed standard curve. For further details, including DNA extraction and qPCR assay validation see Supplementary Information (Tables S2 and S3, and Figs S2–S4).

Interference competition

To evaluate the potential for interference competition via secreted products in explaining the outcome of competition in co-cultures, we examined the growth inhibition between the pairs of strains used in the experiments with the double layer method (Sundberg, et al. 2016). Briefly, an aliquot of bacterial culture was centrifuged (17 000 × g, 3 min) and 5 μL of the supernatant was pipetted on top of a Shieh agar plate with a soft agar (0.7%) overlay containing 300 μL of the other bacterial strain. Three replicates were prepared for each strain. Plates were checked after 48 h to detect if the supernatant had inhibited the growth of the strain beneath.

Fish challenge experiments

Prior to fish challenge experiments, ancestral bacterial strains were revived from frozen stock cultures and all bacterial samples from different nutrient levels were precultured in 100% Shieh as described above (basic culture conditions). Thus the outcome of the challenge test was likely not affected by the nutrient levels added to the fish containers.

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Challenge experiments were performed with rainbow trout fingerlings obtained from a fish farm using ground water, ensuring that the fish had no previous exposure to F. columnare. The fish were maintained in the laboratory in aerated well water in glass aquaria at a 12:12 L:D cycle at 17°C and fed with commercial pellets. The water temperature was gradually increased to 25°C (+1–1.5 °C per day) over the course of ten days prior to the bacterial challenge. The fish were transferred individually into transparent plastic containers with 0.5 L of aerated well water at 25°C. The containers were randomly assigned to different treatments (10 replicates per treatment) and the cultures, adjusted to final concentration of 1.5×10^3 CFU ml^{-1} , were added to the containers in 0.1 mL of 100% Shieh according to the continuous challenge method (Kinnula, et al. 2015). The fish were monitored at one hour intervals for signs of bacterial infection and morbidity. Due to the rapid development of disease symptoms, the diseased fish were mostly unresponsive to external stimuli and were killed by cutting the spinal cord with scissors leading to instant death. A bacterial sample was collected from the gills with a sterile loop and spread on F. columnare selective agar plates containing modified Shieh medium and tobramycin (Decostere, et al. 1997). Fish weight was recorded. The fish surviving until the end of experiments were euthanized with an overdose of MS-222, sampled for bacteria on gills and weighed.

The disease caused by F. columnare, i.e. columnaris disease, is a threat especially to young salmonids, with a rapid disease progress in freshwater fish farms. The challenge experiments were performed with rainbow trout fingerlings (Oncorhynchus mykiss) (age 0+) with a mean weight of 2.10±0.69 g when testing bacteria from the batch experiment and 0.39±0.12 g when testing bacteria from the high rate resource renewal experiment. For this size range, previous studies have confirmed a qualitatively similar progress of infections (Kinnula, et al. 2015, Laanto, et al. 2014, Sundberg, et al. 2014). The experiments were finished after 45 h (batch experiment) or 49 h (high rate resource renewal experiment). The fish challenge experiments were conducted according to the Finnish Act on the Use of Animals for Experimental Purposes under the license number ESAVI/10184/04.10.07/2014 granted to Jouni Taskinen by the National Animal Experiment Board of the Regional State Administrative Agency of Southern Finland.

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Statistical analyses

Competition and growth

The data was analysed separately for the batch experiment and the high rate resource renewal experiment. For the high rate resource renewal experiment, the data was divided into two parts according to the duration of the two sets of co-cultures (days 0–24 and 25–53). The data was analysed in R version 3.4.3 using Bayesian mixed models using Markov chain Monte Carlo estimation with the package MCMCglmm (Hadfield 2010). To study the outcome of competition in co-cultures using qPCR-data, the proportion of the virulent strain in co-culture was analysed by using nutrient level (low vs. high) as a fixed factor and day since inoculation as a continuous factor. The non-independence of multiple observations from the same replicate was accounted for by including replicate identity as a random factor. Bacterial growth (CFU ml^{-1}) was compared between monocultures and co-cultures using nutrient level (low vs. high) and strain or strain combination (virulent, less virulent, co-culture) as fixed factors. The day since inoculation was included as a continuous covariate and replicate identity as a random factor. The models for studying competition based on qPCR data were fitted with the Gaussian distribution and the models on the effect of nutrient level on growth were fitted with the Poisson distribution to account for overdispersion of the data. As model selection with deviance information criterion (DIC) criteria might not be reliable for non-Gaussian data, the models were fitted including all main effects and 2-way interactions. For part 1 (days 0–24) in the high rate resource renewal experiment, 3-way interactions were also included. Inverse-Wishart priors with a low degree of information were used for both fixed and random effects (Hadfield 2010). Diagnostic tools from the Coda package (Plummer, et al. 2006) were used to determine the number of the iterations (600 000), length of thinning (130) and burn-in period (100 000) for the models. The Gelman-Rubin test and visual inspection of traces on three chains were used to check model convergence. The potential scale reduction factor was close to 1 among chains in all analyses. Differences between factors are considered significant if the 95% credible intervals (CI) for the posterior means do not overlap with zero.

In order to evaluate if the outcome of competition is affected by differences in growth between the strains in different nutrient levels in the beginning of the experiments

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(days 0-1), initial population growth was calculated for each monoculture as $\ln(N_{day1}/N_{day0})/1$ day (Lenski, et al. 1991). N_{day0} was the initial inoculation density and N_{day1} the population density on day 1 as determined by plate counting. These values were compared between the virulent and less virulent strain at each nutrient level separately for the batch experiment and the high rate resource renewal experiment using ANOVA.

Biofilm formation

For evaluation of the effect of biofilm formation on the outcome of growth and competition in the high rate resource renewal experiment, the optical density values obtained from the separate biofilm experiments for the virulent and less virulent strain under low and high nutrient levels were compared using ANOVA with log-transformed data.

Fish challenge tests

For analysing data from in vivo fish challenge experiments, generalized linear models with binomial distribution were used to determine the effect of bacterial strain identity and treatment (ancestral isolate, evolved under low or high nutrient level) on the morbidity of fish. The analyses were performed separately for the batch experiment and for the high rate resource renewal experiment. Strain and treatment were included as fixed factors. Fish weight was included as a continuous covariate to control for the effect of surface area and respiration rate on the infection risk of a fish. Model selection was based on the Akaike information criteria (AIC), performed with the stepAIC function in the package MASS (Venables and Ripley 2002) in R, starting from the full model including all 3-way interactions. In the challenge experiment for bacteria evolved in the high rate resource renewal experiment, samples collected from the cultures on day 24 and day 52 were considered independent of each other and included as fixed treatment factors (ancestor, high nutrient day 24, high nutrient day 52, low nutrient day 24, low nutrient day 52).

Results

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Fish challenge experiments

Batch culture experiment

Culturing bacteria in batches with low rate resource renewal at low or high nutrient levels did not change the virulence of the bacteria towards rainbow trout fingerlings in the challenge experiment when compared to the ancestral isolates. The best model explaining the outcome of the challenge experiment according to the AIC included only the effect of strain (Table 1). Namely, the virulent strain B402 caused higher morbidity in the rainbow trout than the less virulent strain B407 in all treatments (Figs 1A, S5A).

High rate resource renewal experiment

Populations evolved under high rate resource renewal at low or high nutrient levels for different lengths of time (24 days or 52 days) were compared to the ancestral isolates in their ability to cause morbidity in fish. The best model according to AIC included an interaction between the strain (virulent, less virulent) and the treatment (ancestral, low nutrient at day 24 or 52, or high nutrient at day 24 or 52). The morbidity caused by the virulent strain B402 was higher than that caused by the less virulent strain B398. The nutrient level did not affect the morbidity caused by the virulent strain B402. However, for the strain B398, which was originally less virulent, morbidity in fish decreased after culturing as compared to that caused by the ancestral strain. This was especially evident after culturing at the low nutrient level but also occurred after culturing at the high nutrient level for a longer time period (Table 2, Figs 1B, S5B).

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Competition and growth

Batch experiment

The proportion of the virulent strain determined with the qPCR assay did not differ between high and low nutrient level (MCMCglmm model: the 95% credible intervals for the posterior means overlapped zero for the low nutrient level and interaction between low nutrient level and day; Table S4, Fig. 2A). The proportion of the virulent strain increased during the experiment as indicated by the positive deviance of day from zero (posterior mean for day: 0.003: 95% CI: 0.001–0.005).

The bacterial concentrations of the virulent strain in monoculture, the less virulent strain in monoculture and the competition treatment did not differ from each other, they did not change during the experiment and the nutrient level did not affect the concentrations (Table S4, Fig. 3A,B). This is shown by the posterior distribution credible intervals which overlapped with zero for all variables considered in the MCMCglmm model.

The initial population growth during the first 24 h showed different patterns among the strains in the monocultures in the low and high nutrient levels (ANOVA: strain $F_{1,8}$ = 59.139, $p < 0.001$: nutrient $F_{1,8}$ = 89.048, $p < 0.001$, nutrient × strain $F_{1,8}$ = 28.010, $p < 0.001$). Initial population growth was higher in the monocultures of the virulent compared to the less virulent strain in the high nutrient level, while no difference between the strains was observed in the low nutrient level (for pairwise comparisons, see Table S7). Both strains had higher initial growth when grown at high compared to low nutrient level (Table S7).

High rate resource renewal experiment

When the co-cultures were started from ancestral strains, the proportion of the virulent strain did not differ between high and low nutrient level (MCMCglmm model: 95% CI for the posterior means overlapped zero for the low nutrient level and the interaction

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between low nutrient level and day; Table S5, Fig. 2B). The proportion of the virulent strain increased from the initial 50% to approximately 100% during the experiment as indicated by the positive deviance of day from zero (MCMCglmm model: 0.020: 95%CI: 0.010–0.030).

The concentration of the less virulent strain in monocultures at the low nutrient level was lower than the concentration of the virulent strain during the first three days (Fig 3C: 1. co-culture). This is indicated by the negative posterior mean −5.479 and 95% CI (from −9.029 to −1.662) not overlapping zero for the interaction between the less virulent strain and low nutrient level. The concentration of the co-culture did not differ from the concentration of the virulent strain monoculture at the low nutrient level (co-culture × low nutrient interaction: posterior mean 0.722: 95% CI: −3.067–4.257; Table S5). However, after 6 days, the monocultures of the less virulent strain at low nutrient level reached almost the same concentration as the virulent strain monocultures and the co-culture (Fig. 3C), as suggested by a significant positive deviance from zero for the posterior mean for the 3-way interaction between less virulent strain, low nutrient level and day (0.353: 95%CI: −0.007–0.670: Table S5, Fig. 3C). At the high nutrient level, the growth of the less virulent strain monocultures, the virulent strain monocultures and the co-cultures did not differ from each other (95%CI of less virulent strain B398 and co-culture overlapped zero; Table S5, Fig. 3D: 1. co-culture).

The initial population growth during the first 24 h differed between the strains in the low and high nutrient levels in the monocultures (ANOVA: strain $F_{1,6} = 88.258$, $p < 0.001$: nutrient level $F_{1,6} = 279.167$, $p < 0.001$: nutrient level × strain $F_{1,6} = 87.872$, $p < 0.001$), but in the opposite direction as compared to the batch experiment. The virulent and the less virulent strain had similar initial growth at the high nutrient level while at the low nutrient level the initial growth was higher for the virulent strain compared to the less virulent strain (for pairwise comparisons, see Table S7). Initial population growth was higher for both strains at the high compared to low nutrient level (Table S7).

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In the second part of the experiment testing whether adaptation to a particular nutrient level in monocultures changed the competition outcome (Fig. 2C), the proportion of the virulent strain in the competition treatment increased during the experiment (MCMCglmm model: posterior mean for day 0.029, 95% CI: 0.021– 0.037, Table S6, Fig. 2C). However, the two chemostat (low nutrient level) co-cultures behaved very differently, thus nutrient level was not included in the statistical analysis. The inoculations taken from the monocultures at day 24 did not result in equal proportion of the less virulent and virulent strains in co-cultures, but the proportion of the virulent strain was lower, ca. 30 % for the high resource treatment and the other one of the chemostats and only a few percent for the other chemostat. In spite of lower proportion at the start, and remaining at very low level for 7–10 days in the high nutrient level co-cultures and the other chemostat, the virulent strain eventually outcompeted the less virulent strain at both nutrient levels (Fig. 2C).

The concentrations of the virulent and less virulent strain monocultures in the second part of the high rate resource renewal experiment (Fig. 3C,D: 2. co-culture) were higher than those of the co-cultures (MCMCglmm model co-culture posterior mean: −3.645: 95% CI: from −5.020 to −2.228: Table S6). This can be explained by continuous maintenance of the monocultures since the beginning of the first part while co-cultures were started with a lower concentration. However, the concentration of the co-culture increased during the experiment (co-culture × day: 0.170: 95% CI: 0.098–0.239; Fig. 3C–D). The bacterial concentrations were lower at the low nutrient level (posterior mean: −1.640: 95% CI: from −3.013 to −0.153) and they decreased during the experiment compared to the high nutrient level (low nutrient level × day posterior mean: −0.239: 95% CI: from −0.293 to −0.179: Table S6: Fig. 3C–D). There was no difference in growth between the less virulent and the virulent strain (95% CI for the posterior mean of the less virulent strain B398 overlapped zero: Table S6).

Biofilm formation

In the experiment evaluating relative differences in the amount of biofilm formation between the virulent and less virulent strain in the high rate resource renewal experiment, the less virulent strain B398 formed more biofilm than the virulent strain B402 (Fig. 4:

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ANOVA: $F_{1,23} = 6.65$, $p = 0.02$). There was no interaction between the strain and resource level ($F_{1,23} = 2.33$, $p = 0.14$).

Interference competition

In the experiment evaluating the potential for interference competition via products secreted by the strains, the virulent strain B402 inhibited the growth of the less virulent strain B407 used in the batch experiment in all three replicates, but no inhibition was observed vice versa (Table 3). There was no inhibition between the virulent strain B402 and the less virulent strain B398 used in the high rate resource renewal experiment (Table 3).

Discussion

Both the within-host and the outside-host environment affect the ecology and evolution of environmentally growing opportunistic pathogens (Brown, et al. 2012). The two environments may select for different properties, for example, in resource use although growth in one environment may also pre-adapt for utilisation of resources in the other environment (Ketola, et al. 2016, New, et al. 2014). Environmental resources may thus affect pathogen virulence by producing changes in host utilisation rates. Here we tested the hypothesis that higher nutrient availability in the outside host environment increases the virulence of the environmentally growing opportunistic fish pathogen, Flavobacterium columnare. We expected that evolving in a high nutrient environment would increase virulence due to selection for rapid resource use. As virulent strains of F .columnare have higher growth rates than less virulent strains (Pulkkinen, et al. 2010), we also expected that high nutrient conditions would favour virulent strains in competition.

Increased resource availability in the outside-host environment could increase virulence in F. columnare by increasing bacterial population size, and thus the dose encountered by the host (Kinnula, et al. 2017b, Kinnula, et al. 2015), or by facilitating host invasion

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via virulence factor activation (Kinnula, et al. 2017b, Penttinen, et al. 2016), such as increased expression of tissue-degrading enzymes (Penttinen, et al. 2016). In the current experiments, however, the immediate nutrient environment did not affect virulence, as we pre-cultured all bacteria in 100% Shieh medium and adjusted the dose prior to the fish challenge. Therefore any changes in virulence were expected to be due to adaptation to the nutrient environment where the strain was evolving. In contrast to our expectations, we did not detect an increase in virulence after evolving in the high nutrient level in either the virulent or the less virulent strain compared to ancestral strains. Instead, the virulent strain maintained its virulence at the same high level independent of the nutrient level and resource renewal rate. These results suggest that increased resources in the outside-host environment do not affect virulence in highly virulent strains. However, the less virulent strain decreased in virulence during cultivation at constant low-level nutrient supply in chemostats or in long-term serial cultivation in tubes in the high rate resource renewal experiment. Loss of virulence has been commonly observed in pathogenic micro-organisms as an outcome of serial transfer in vitro (Ford, et al. 2002, Gonzalez-Carrillo, et al. 2016, Moody, et al. 1990, Songe, et al. 2014), pointing to trade-offs between long-term survival in the outside-host and within-host environment (Ferenci 2016) or relaxed selection for virulence in the outside-host environment (Mikonranta, et al. 2012). An increased supply of resources might therefore maintain virulence at a higher level in less virulent strains in the outside-host environment.

In F. columnare, virulence is associated with rhizoid colony morphology, with a loss of virulence upon change into either of two other morphs (rough or soft) (Kunttu, et al. 2011, Kunttu, et al. 2009a). Maintenance of F. columnare for 5 months in stationary starvation conditions led to diversification in colony morphology and virulence such that the rhizoid morph became more virulent and rough morph less virulent than the ancestral strain (Sundberg, et al. 2014). The strains used in the current experiment originally exhibited rhizoid colonies upon cultivation on agar plates and no change was observed during cultivation in batches. Bacteria from chemostats, however, formed colonies that were intermediate between rhizoid and soft morphs (Fig. S6). The appearance of cells giving rise to soft colonies might thus have decreased the overall virulence of the chemostat population. Resources in the outside-host environment might contribute to the virulence of F. columnare via diversification of cell types forming different colony morphologies, which might be related to trade-offs in the acquisition of nutrients in outside-host and within-host environments. The

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mechanisms behind the different colony morphologies and their virulence in F. columnare are unclear, but virulence is associated with gliding motility of the cells which in turn is affected by nutrient availability (Laanto, et al. 2014).

In direct competition over resources, the competitor with the fastest turnover rate of resources into biomass is expected to win. In F. columnare, individually assessed growth rates have been found to have a good agreement with the outcome of competition in liquid culture (Ashrafi, et al. 2017). However, in the current experiments, the outcome of competition in co-culture could be explained only in two cases by differences in the initial growth rates of the strains measured in monocultures at the respective nutrient levels. Instead, we found that the virulent strain was superior in competition both at the high and low nutrient level regardless of the resource renewal rate. The virulent strain outcompeted the less virulent strains in co-cultures in a few days even after adaptation to the prevailing nutrient level and after starting from a very low proportion (see Fig. 2C). Similar genotype-based dominance has been detected previously (Kinnula, et al. 2017a), and more studies are needed to clarify whether the traits harboured by the dominant strains are common to all virulent genotypes of F. columnare.

Apart from direct competition over resources, microbes compete with each other with various indirect mechanisms, including chemical interference (Cornforth and Foster 2013, Hibbing, et al. 2010). For F. columnare, interference competition is common for colonies growing on surfaces (Ashrafi, et al. 2017, Kinnula, et al. 2017a, Sundberg, et al. 2016), but it is not clear whether it plays a role in planktonic growth for this bacterium (Ashrafi, et al. 2017). Here we confirmed growth inhibition of the less virulent strain B407 by the virulent strain B402, which might explain the competition outcome in the batch experiment. The strains used in the high rate resource renewal experiment did not inhibit each other's growth.

In addition to resource level, resource renewal rate can affect different traits in growth (Finkel 2006, Velicer and Lenski 1999). Even though we expected that bacteria grown under a high nutrient level would be selected for fast growth (Frank 2010, Litchman, et al. 2015, Velicer and Lenski 1999), it is possible that conditions in the batches with low

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rate renewal of resources selected similar traits under both low and high nutrient level. Without continuous renewal of nutrients, the bacteria might have been adapting to fast growth only until reaching carrying capacity long before the next resource renewal. This saturation phase might then select for the survival of bacterial cells that are capable of remaining viable in starvation conditions (Arias, et al. 2012) or capable of saprophytic usage of their conspecific cells. F. columnare has been shown to survive and remain infective for several months when maintained in water without added nutrients (Arias, et al. 2012, Kunttu, et al. 2009b, Sundberg, et al. 2016), and this feature has been attributed to a saprophytic capacity in the bacterium, supported by experiments showing reproduction in fish carcasses (Kunttu, et al. 2009b), [however, see (Arias, et al. 2012) for an opposing view]. It is also possible that nutrients were depleted in the tube cultures with high rate resource renewal despite the high dilution aimed at maintaining the cultures at exponential growth stage prior to daily serial transfer, and these conditions might therefore also have selected for survival rather than for high growth rate (Velicer and Lenski 1999).

As opposed to batch cultures, the conditions in flow-through chemostats, favouring cells that can grow and multiply at the lowest nutrient level, were expected to favour the less virulent strain (Gresham and Hong 2015, Litchman, et al. 2015, Velicer and Lenski 1999). In the high rate resource renewal experiment, the less virulent strain had a propensity to form biofilm on chemostat walls, and its higher relative biofilm forming capacity compared to the virulent strain was confirmed in a separate experiment on 96-well plates at both nutrient levels. As no inhibition was detected between the strains used in this experiment, the competition outcome in chemostats was possibly driven by the less virulent strain diverting part of the population growth into the biofilm instead of liquid culture. At the high nutrient level, however, the daily transfer might have prevented formation of biofilm by the less virulent strain, as no differences in concentration between strains were seen. However, adhesion capacity has not been found to be connected with virulence in F. columnare (Kunttu, et al. 2009a, Suomalainen, et al. 2006b). In fish farming conditions, the tendency to form biofilm could give at least a short-term competitive advantage or a possibility to survive also for less virulent strains in situations where the entire water mass of a fish tank is renewed.

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In addition to fish farms, F. columnare is commonly isolated from lake water and biofilms at shore (Kunttu, et al. 2012). The selection for survival and infectivity might greatly differ between these two environments. In the lake water the resource level is much lower than the lowest resource level used in this study (2% Shieh medium). In a fish farming environment, excess nutrients are released to the water from fish excretion, faeces and uneaten feed in varying concentrations depending for example on the fish species, biomass, water exchange rate, feeding rate and temperature (Lalonde, et al. 2015). Thus the resource quality available in the outside-host environment closely matches the resources provided by the host and could pre-adapt the bacteria for faster host exploitation. Our results suggest that the outside-host nutrient environment has a lower impact on strains that are highly virulent, but rich resources in the outside-host environment might be relevant for the maintenance of virulence in less virulent strains and increase the contribution of less virulent strains in columnaris epidemics in aquaculture. As inter-strain interactions between co-infecting strains might determine the outcome of infection (Kinnula, et al. 2017a), further studies are needed on the role of outside-host resources in maintaining strain diversity and driving the evolution of virulence among environmentally growing pathogens.

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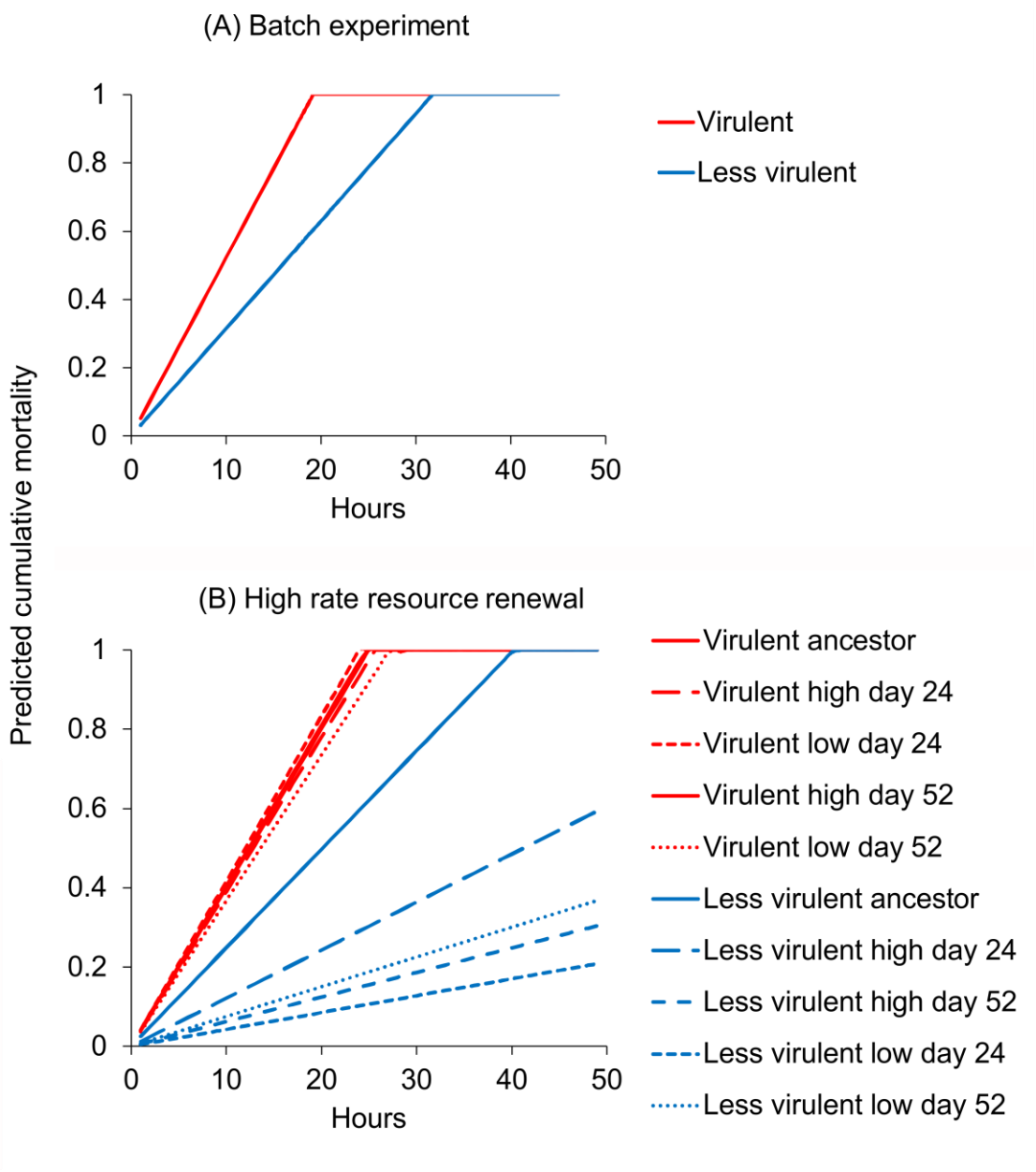

Fig. 1 The predicted cumulative mortality of rainbow trout (Oncorhynchus mykiss) fingerlings in continuous challenge experiments with Flavobacterium columnare. A) In fish challenge with bacteria cultivated in monocultures in the batch experiment, the risk depended only on the strain identity of the bacterium, but not on whether it was an ancestral isolate or had been cultured under a low or high nutrient level. B) In fish challenge with bacteria from the monocultures from the experiment with high rate resource renewal, culturing in different nutrient environments did not change the virulence in the virulent strain (high = high nutrient level; low = low nutrient level). The less virulent strain decreased in virulence after cultivation at the low nutrient level and after the longer cultivation period (52 days) at the high nutrient level.

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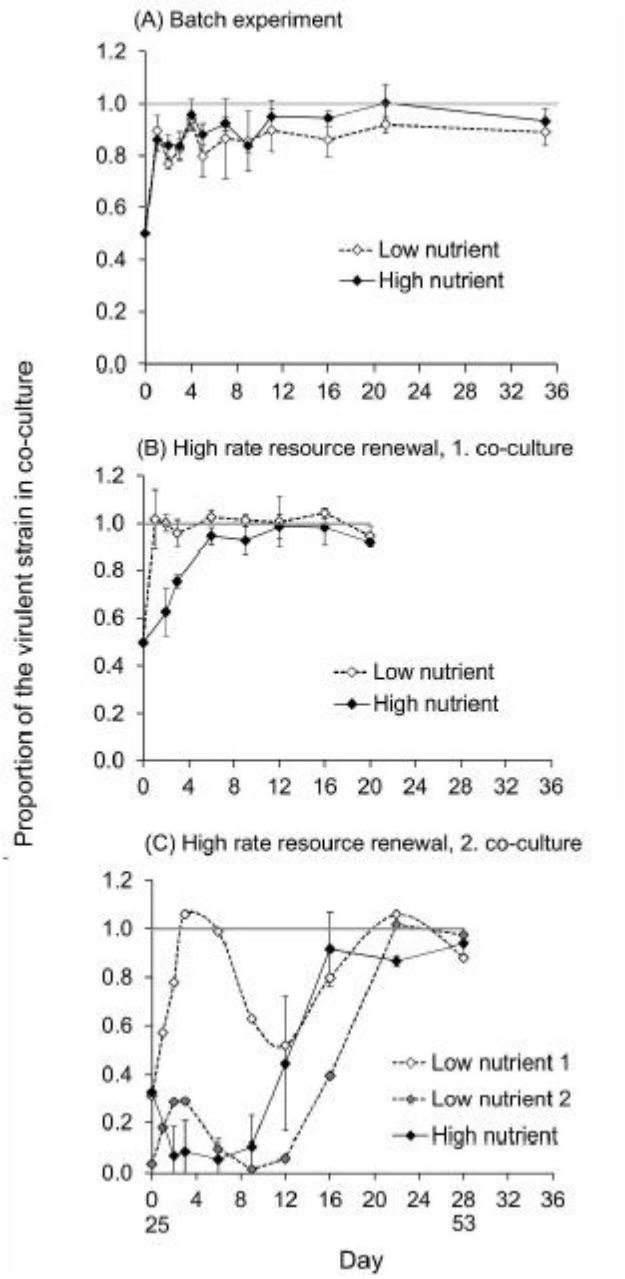

Fig. 2 The proportion of the virulent strain in co-cultures (mean ± SE): A) in the batch experiment: B) in the experiment with high rate resource renewal started from ancestral isolates (1. co-culture): and C) in the experiment with high rate resource renewal after starting the co-cultures with bacteria from the monocultures after adaptation to a particular nutrient level (2. co-culture). In panel C, the proportion of the virulent strain is shown separately for both low nutrient level replicates due to large differences between replicates. Note that due to technical reasons (see Supporting information), the calculated proportion of the virulent strain may slightly exceed 1. The two values below the x-axis in C) denote the corresponding days for monocultures in comparison to Fig. 3C–D.

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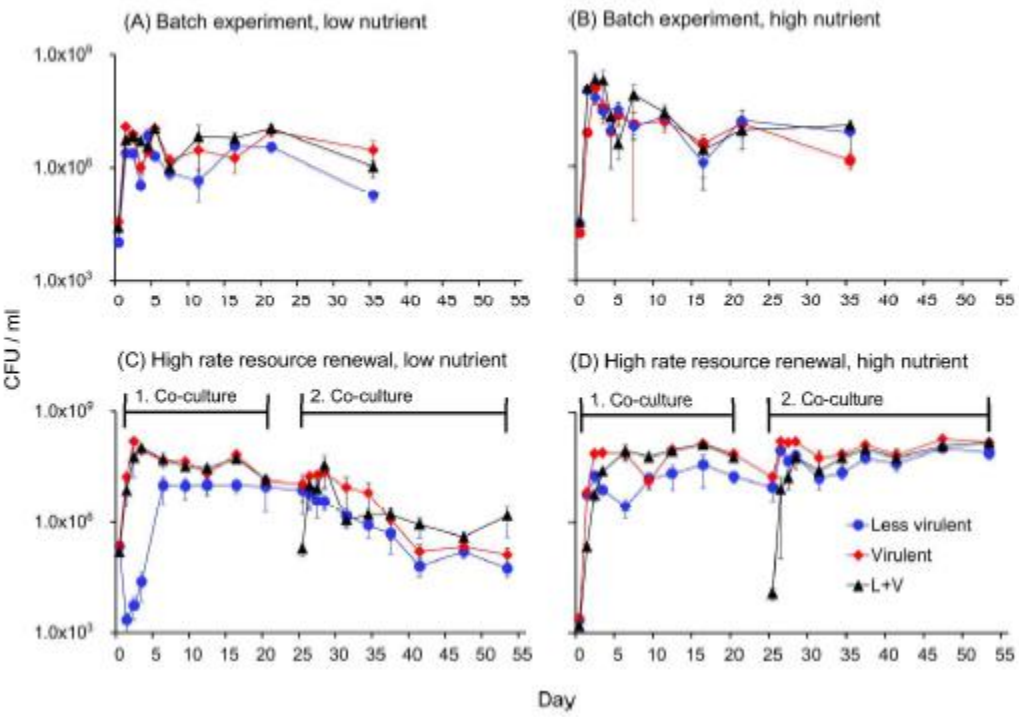

Fig. 3 The bacterial concentrations (CFU ml^{-1}) in monocultures of the less virulent strain (Less virulent), monocultures of the virulent strain (Virulent) and co-cultures (L+V). A) Batch experiment at nutrient levels 5% (low nutrient) and B) 50% Shieh medium (high nutrient). C) The experiment with high rate resource renewal at 2% Shieh medium (low nutrient) and D) 20% Shieh medium (high nutrient). In the beginning of the high rate resource renewal experiment the co-cultures were started from ancestral isolates and discarded at day 20 (1. co-culture). New co-cultures were started (2. co-culture) with bacteria from the monocultures after adaptation to a certain nutrient level. Monocultures were maintained continuously for the whole duration of the high rate resource renewal experiment.

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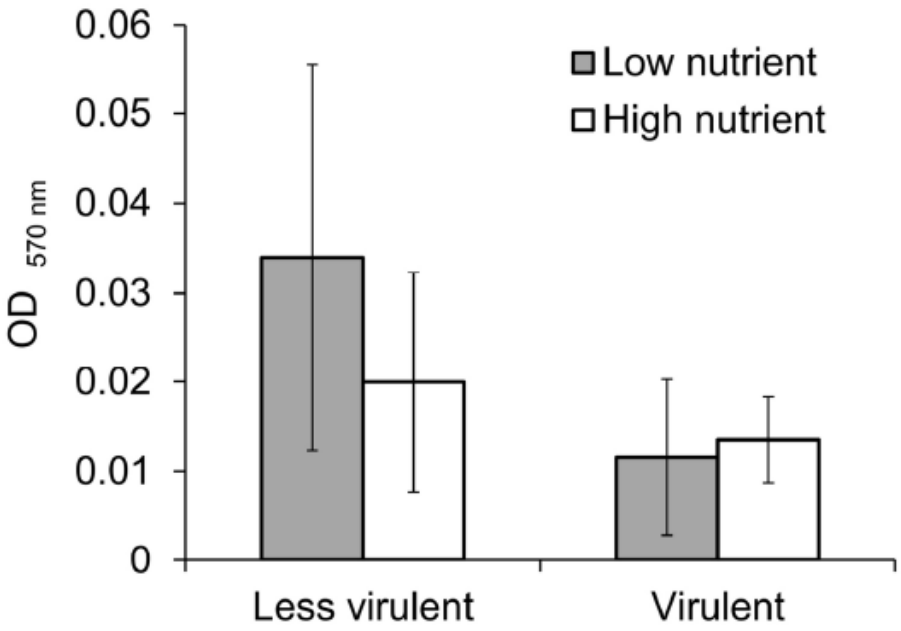

Fig. 4 Relative biofilm formation (mean ± SD) of the virulent strain B402 and the less virulent strain B398 used in the high rate resource renewal experiment. Relative biofilm formation was quantified after 48 h incubation at high (20% Shieh medium) or low (2% Shieh medium) nutrient level as optical density measured with a spectrophotometer at 570 nm after crystal violet staining.

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Table 1 Model selection and the summary of the best fitting model on the morbidity risk of the rainbow trout in the fish challenge experiment testing the strains used in the batch experiment. Model selection is based on the Akaike information criterion (AIC). The best fit model estimating the morbidity risk of the host (rainbow trout) within time is marked with bold. The p-value indicates the significance of the term removed from the higher model based on the log-likelihood test (LRT).

Model [a]	**AIC**	**DF**	**LRT**	**p**
S+T+W+S:T+S:W+T:W+S:T:W	302.65	128		
S+T+W+S:T+S:W+T:W	298.91	130	0.255	0.88
S+T+W+S:T+T:W	297.08	131	0.178	0.67
S+T+W+S:W+T:W	295.28	132	0.374	0.83
S+T+W+S:T+S:W	294.93	132	0.026	0.99
S+T+W+S:T	293.10	133	0.170	0.68
S+T+W+S:W	291.30	134	0.832	0.83
S+W+S:W	288.45	136	1.149	0.56
S+T+W	289.43	135	0.130	0.72
S+W	286.65	137	1.218	0.54
W	293.28	138	0.943	0.33
S	**285.63**	**138**	**8.626**	**0.003**

Summary of the best fitting model

Source	**Estimate**	**SE**	**z value**	**p**
(Intercept)[b]	-2.9	0.13	-23.607	< 0.001
Strain(Less virulent)	-0.53	0.18	-2.988	0.003

[a] S = strain (virulent B402, less virulent B407); T = treatment (ancestor, low nutrient level, high nutrient level); W = fish weight; + = main effect; colon = interaction.
[b] The intercept includes the effect of the virulent strain.

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Table 2 Model selection, summary of the best fitting model and the significance of the effect of the variables included in the best fitting model on the morbidity risk of the rainbow trout in the fish challenge experiment testing the strains used in the high rate resource renewal experiment. Model selection is based on the Akaike information criterion (AIC). The best fit model estimating the morbidity risk of the host (rainbow trout) within time is marked with bold. The p-value indicates the significance of the term removed from the higher model based on the log-likelihood test (LRT).

Model[a]	**AIC**	**DF**	**LRT**	**p**
S+T+W+S:T+S:W+T:W+S:T:W	424.52	200		
S+T+W+S:T+S:W+T:W	419.59	204	3.064	0.547
S+T+W+S:T+S:W	412.58	208	0.991	0.911
S+T+W+S:T	411.49	209	0.913	0.339
S+T+S:T	**410.97**	**210**	**8.947**	**0.062**

Summary of the best fitting model

Source	**Estimate**	**SE**	**z value**	**p**
(Intercept)[b]	−3.17	0.32	−9.805	**< 0.001**
Strain(Less virulent)	−0.50	0.47	−1.079	0.2808
High day 24	−0.04	0.37	−0.104	0.9175
High day 52	−0.01	0.37	−0.030	0.9761
Low day 24	0.03	0.40	0.076	0.9396
Low day 52	−0.10	0.40	−0.254	0.7996
Less virulent: high day 24	−0.69	0.57	−1.223	0.2212
Less virulent: high day 52	−1.39	0.32	−2.267	**0.0234**
Less virulent: low day 24	−1.82	0.72	−2.515	**0.0119**
Less virulent: low day 52	−1.11	0.64	−1.732	0.0833

Significance of variables included in the best fitting model

Source	**DF**	**Deviance**	**Residual devi**	**p**

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				anc e
Null			182.32	
Bacterial strain	1	78.62	103.71	**<0.001**
Treatment	4	3.81	99.90	0.432
Strain:treatment	4	8.95	90.95	0.062

[a] S, strain (virulent B402, less virulent B407); T = treatment (ancestor, low nutrient level day 24 sample, low nutrient level day 52 sample, high nutrient level day 24 sample, high nutrient level day 52 sample): W = fish weight; + = main effect; colon = interaction. [b] The intercept includes the effect of the ancestral virulent strain.

Table 3 The results of the interference competition between the strains used in the experiments. The number indicates the times when the strain on top layer inhibited the growth of the strain in bottom layer in three replicate tests.

Batch experiment			**High resource renewal experiment**		
	Top layer			Top layer	
Bottom layer	B402	B407	Bottom layer	B402	B398
B402		0	B402		0
B407	3		B398	0	

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