ANTAGONISTIC EFFECT OF INDIGENOUS SKIN BACTERIA OF BROOK CHARR (*Salvelinus fontinalis*) AGAINST *Flavobacterium columnare* AND *F. Psychrophilum*

Running Title: Probiotic effect of Brook charr skin bacteria

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SUMMARY

Industrial fish production exposes fish to many potential stressful conditions, which in turn may induce infections by opportunistic pathogens. Probiotics appear to be a promising way to prevent opportunistic infections in aquaculture. In this study, we tested the inhibitory potential of mucus endogenous bacterial community of brook charr *(Salvelinus fontinalis)* against two major pathogens *Flavobacterium columnare* and *Flavobacterium psychrophilum*. Nine bacterial strains were isolated from brook charr skin mucus and tested for potential antagonistic activity. Results from both agar diffusion assay and broth co-culture assay showed the presence of antagonism. We identified seven bacterial strains, collected from unstressed fish, which exerted strong antagonism against *F.psychrophilum* and/or *F.columnare*. Those strains were mixed and used to treat columnaris disease in an *in vivo* experiment in which four distinct fish families were tested. This treatment resulted in an substantial decrease of mortality (54% to 86%) across fish families. Those results clearly indicate that directly deriving candidate from the host microbiota is very suitable to develop probiotics. It thus allow to meet criteria for an efficient and durable management of opportunistic diseases in aquaculture: ability to adhere and colonize the host mucus, and an antagonistic effect against pathogen which is harmless for the host and safe for its environment.

INTRODUCTION

Over the last decade, the aquaculture industry has greatly intensified its productivity and is now a major economic and social activity in many countries (FAO 2007). Massive production in fish farms may expose fish to stressful conditions (handling, high density, transportation, poor water quality), which in turn induce infection by opportunistic pathogens (Snieszko 1974; Barton and Iwama 1991; Wakabayashi 1991). Opportunistic pathogens are naturally present in the water and harmless most of the time (Cahill 1990; Crump *et al.* 2001). However, infection is triggered when the host encounters stressful conditions. As a consequence, opportunistic infectious diseases constitute a major problem for the aquaculture industry. For example, some strains of *F. columnaris* are able to induce 100% mortality in 3 days salmon fingerlings (Michel *et al.* 1999; Pulkkinen *et al.* 2010).

Infections are usually prevented and controlled by intrusive veterinary medicines and chemical substances like synthetic antimicrobial agents (trimethoprim, sulphadiazine, oxolinic acid, oxytetracycline hydrochloride and amoxycillin trihydrate) (Rangdale *et al.* 1997). However, the beneficial effects of antimicrobial agents are counterbalanced by the selection of resistant pathogens (Siegman-Igra *et al.* 1987; Decostere *et al.* 1997; Decostere *et al.* 1998; Nematollahi *et al.* 2003) which gain resistance by horizontal transfers of plasmids containing resistance genes (Lewin 1992; Sørum 2006; Hesami *et al.* 2010). Therefore, alternative strategies to prevent opportunistic infections in aquaculture are strongly needed. The development of probiotics appears to be the most promising way to reach this goal (Verschuere *et al.* 2000; Merrifield *et al.* 2010).

The definition of probiotic agents evolved over time. The term "probiotic" is generally used to denote bacteria that promote the health of other organisms. In the aquaculture context, a probiotic agent has been defined as a bacteria (viable or not) which improves host health when added to the food or in the surrounding environment (Moriarty 1998). The current strategy to develop probiotic for a given host species is to test a probiotic agent already proven to be efficient in another host species (Villamil *et al.* 2003; Mohamed and Ahmed Refat 2011). However, when transferred into a different environment, the probiotic agent will likely lose its probiotic properties, and possibly become harmful for the host (Courvalin 2006). Therefore, to ensure the harmlessness of a probiotic in a given host species, it is recommended to isolate it from the host's endogenous bacterial community (Balcazar *et al.* 2006). Probiotics improve host health either by inducing the host immune response or by exerting direct inhibitory effects on pathogens. Four action mechanisms of probiotics have been defined: i) competitive exclusion (Garriques and Arevalo 1995; Moriarty 1997; Gomez-Gil *et al.* 2000; Balcazar 2003; Balcázar *et al.* 2004; Vine *et al.* 2004), ii) nutritional contribution (Sakata 1990; Ringø *et al.* 1995), iii) influences on the environment (Dalmin *et al.* 2001), iv) improvement of immune response and antimicrobial effect (Kamei *et al.* 1988; Sakai *et al.* 1995; Rengpipat *et al.* 2000). Among these four inhibitory mechanisms, the competitive exclusion is the most promising to look for probiotic candidates. Indeed, it is more difficult for a given pathogen to adapt to the different factors involved in competitive exclusion mechanisms than to a single specific antimicrobial substance (Smith 1993; Moriarty 1998). The usual experimental procedure to screen for candidate probiotics is to perform *in vitro* antagonism tests to evaluate capabilities for production of

79 inhibitory compounds, adhesion to the host or pathogen, and competition for nutrients
80 (Gram *et al.* 1999; Hjelm *et al.* 2004; Vine *et al.* 2004; Chabrillón *et al.* 2005).

81 The present study focused on two important opportunistic pathogens in salmonids,
82 namely *Flavobacterium columnare* and *Flavobacterium psychrophilum* (Durborow *et al.*
83 1998; Crump *et al.* 2001; Bernardet and Bowman 2006). Brook charr (*Salvelinus*
84 *fontinalis*), is a species that supports a promising aquaculture production in eastern
85 Canada, was retained as a host model (MPO 2005). The skin microflora was targeted
86 because isolates from other parts of the body (e.g. gut) are known to be inefficient to
87 inhibit growth of skin pathogens (Spanggaard *et al.* 2001). Skin mucus offers both
88 physical and biochemical barriers and is then the first line of defense against pathogens.
89 Many innate immune factors are released in epidermal fish mucus including proteases,
90 antibacterial agents, and other related immune compounds (Hjelmeland *et al.* 1983; Fast
91 *et al.* 2002; De Veer *et al.* 2007). These factors respond to stress and diseases. For
92 example, in *Salmo salar*, infection with sea lice induces an increase in related immune
93 compounds, namely lysozyme, alkaline phosphatase and activate proteolytic activities,
94 which in turn induce a part of the immune response to sea lice infection (Ross *et al.* 2000;
95 Fast *et al.* 2002; Easy and Ross 2009). Therefore, when investigating for direct inhibitory
96 mechanisms of probiotic candidates, it is important to distinguish between host immune
97 response and endogenous bacterial inhibitory effects.

98 Microbial communities were observed to be sensitive to various stressful environmental
99 conditions (Schimel *et al.* 2007). For example, corals bleaching is the direct consequence
100 of the global warming (Littman *et al.* 2010). In this respect, we hypothesized that stress
101 may unbalance the bacterial community structure of brook charr skin mucus which would

in turn trigger opportunistic infections. Therefore, our general aim was to test whether natural isolates of non-stressed skin mucus bacterial community exhibit competitiveness for nutrient against two pathogens *F.columnare* and *F.psychrophilum*.

The specific objectives were: *i)* to test the presence of inhibitory compounds in brook charr skin mucus*; ii)* to evaluate the competitiveness of eight microbial isolates from skin mucus against *F.columnare* and *F.psychrophilum* for nutrients, *iii)* to determine which defensive mechanism was exerted, i.e. competitive exclusion or synthesis of antimicrobial substances, iv) to confirm the *in vitro* effectiveness of our candidates on an *in vivo* experiment for aquaculture application.

EXPERIMENTAL PROCEDURE

SAMPLING OF *BROOK CHARR* BACTERIAL COMMUNITY

Sixteen fish families were raised in the Laboratoire Régional des Sciences Aquatiques (LARSA) at the Laval University. Among them, two families were collected from a stress experiment. A total of 2 individuals per family were sampled. One of them was previously exposed to stress physiological conditions. Skin mucus was sampled using a sterile razor blade and homogenized after addition of 9ml of sterile water.

IDENTIFICATION OF THE BACTERIAL ISOLATES

Fish mucus was diluted in sterile water from a 10 fold to a 10^{-7} dilution. Dilutions were spread on TSA and R2A media. Bacterial colonies were isolated and individualized by cross streaking and incubated at 20°C for 48H. A total of nine isolates was identified by 16S rDNA sequencing. Colonies were used as template DNA for PCR amplification of

123 the 16S rRNA gene using the universal bacterial primers 63F (5'-
124 CAGGCCTAACACATGCAAGTC-3') (Marchesi *et al.* 1998) and 907R (5'-
125 CCGTCAATTCMTTTRAGTTT-3') (Lane *et al.* 1985). PCRs were carried out in a
126 volume of 25 µL containing 0.2 mM dNTP (Promega), 0.3 µM each primer, 6.4 µg of
127 BSA, 1.25 mM of $MgCl_2$, 1X of Buffer and 0.4U of Taq DNA polymerase (Promega)
128 and performed in a Biometra T1 Thermocycler. The following amplification conditions
129 were applied: a first step of initial denaturation at 94 °C for 5 min followed by 28 cycles
130 of 94°C for 1 min, 55°C for 1min, 72°C for 90 sec and a final extension step at 72°C for
131 10 min. PCR products were analyzed by agarose gel electrophoresis [0.8% (w/v) agarose,
132 100 V] with ethidium bromide staining and visualized using an UV transilluminator.
133 Then, fragments were sequenced using the big Dye terminator V3 chemistry and ran on
134 an ABI 3130XL sequencer (Applied Biosystem, Foster City) at the Plateforme d'analyse
135 Biomoleculaire (IBIS, Laval University).

136 MEDIA USED FOR CULTURE AND COMPETITION ASSAY

137 Three general growth media, TSB (EMD), R2A (Difco) and TSA (Difco) were used for
138 isolation, identification, and culture of brook charr bacterial community isolates. Both
139 pathogens were grown on their respective general growth media, i.e. Anacker and ordal
140 (AO) (Anacker and Ordal 1959). This media was used for competition assays with
141 pathogens, *Flavobacterium columnare* (ATCC 49418) and *Flavobacterium*
142 *psychrophilum* (ATCC 49512) isolated from *Oncorhynchus kisutch* and *Salmo trutta*)
143 (Bernardet and Grimont 1989).

144 SCREENING FOR ANTAGONISTIC EFFECT IN AGAR DIFFUSION ASSAY

All host bacterial isolate strains were tested for antagonistic effects with well diffusion assays against *F. columnare* and *F. Psychrophilum*. Melted AO cooled to 45°C was inoculated with each pathogen to a final density of 10^6 cells.mL^{-1} agar and poured into Petri dishes (Gram and Melchiorsen 1996). A volume of 10 µL of probiotic candidate culture was added into a 3mm well punched in the solidified agar plates. Plates were incubated at 20°C and observed for zones of growth around the wells during 48 h (Spanggaard *et al.* 2001). Individual strains that exhibited competition capabilities were then mixed in three co-cultures: two co-cultures containing the same quantity of all strains with specific antagonistic effect against each pathogen *F.columnare* and *F.psychrophilum* (culture C and culture P) and one co-culture with all strains with antagonistic effect against the two pathogens (culture U). These three co-cultures (30 µL) were added into 3 mm wells punched in the solidified agar plates, and observed for zones of growth around the wells during 48 hr to check potential synergetic co-culture effects (Timmerman *et al.* 2004).

SCREENING FOR ANTAGONISTIC EFFECT IN BROTH CO-CULTURE ASSAY

All antagonistic effects observed in the diffusion agar assay were validated by a broth co-culture assay. Candidates (10^4 cells) were added to 1 mL of AO media in competition with 10^4 cells of the pathogen. These co-cultures were made in triplicates and incubated 48h at 20°C. Two mono-cultures of 10^4 cells of each pathogen acted as controls. Growth of both candidates and pathogens was observed by spreading the co-culture on AO agar plate along with morphological identification and counting.

SCREENING FOR ANTAGONISTIC EFFECT OF MUCUS PROTEINS

Forty-three fish were sampled and all mucus samples were mixed and sterilized with UV light during 25 min to minimizing degradation of proteins (Williams and Kraus 1963). 45 µL of mucus were added to 5 µL (500 cells) of pathogen culture in AO, spread on AO agar plate and incubated at 20°C for 48h days. After incubation, culture was spread on AO agar to observe the growth of pathogen. A volume of 45 µL of PBS mixed with 5 µL of pathogen culture acted as control.

PROBIOTIC TREATMENT OF FISH INFECTION

Seven strains selected for their antagonistic activity during the in vitro experiment were tested together in a co-culture. Four different fish families were used for the *in vivo* experiment (S1, S5, S9, S10). Those families were split in four tanks, two controls and two tests. All the individuals were juvenile and came from the "Station aquicole de l'Institut des Sciences de la Mer de Rimouski". Fish were raised at 12°C and oxygen tension was always higher than 12 mg/L. Each tank contain between 103 and 110 fish. Two weeks after transportation, the first symptoms appeared in all tanks and we started the addition of the mix of probiotics candidates. The probionts were added in test tank to reach the concentration of 10^5 cell ml^{-1} in water twice daily until the fish mortality stabilized. Dead fish and moribund were checked for clinical symptoms of columnaris disease.

STATISTICAL ANALYSIS

Usually, mortality data are often analysed by mean comparison with analysis of variance or Student's t-test. Furthermore it's commonly assumed that proportional hazard model or generalized model with a clog-log link are more appropriate to analyze mortality. In

our experiment, we observed low mortality so we focused on proportion of death in each replicates during 21 days using the generalized linear model approach with a logit link and a binomial distribution. The analysis was performed using the software R, version 2.12.2.

RESULTS

SCREENING OF THE ENDOGENOUS BROOK CHARR SKIN MICROBIOTA

A total of nine bacterial colonies were identified by 16S rDNA gene sequence analysis (Table 1). Among the nine strains, six were found on mucus culture isolated from both stressed and non-stressed fish. Actinobacteria (Microbacteriaceae, Nocardiaceae and Dietziaceae) was the predominant (6 strains) bacterial group that was isolated from skin mucus. Proteobacteria (gamma and alpha subclass) was the second group isolated (3 strains). Both of these groups belong to gram-negative bacteria. Most of these bacteria were isolated from skin on TSA, while Microbacteriaceae and Pseudomonadaceae were isolated with R2A only.

All strains except *Pseudomonas peli* were able to grow on AO agar and could then be tested as probiotic candidates against *F. columnare* and *F. psychrophilum* as those two pathogens only grow on AO agar.

SCREENING FOR ANTAGONISTIC EFFECT OF PROBIOTIC CANDIDATE IN AGAR DIFFUSION ASSAY

The eight strains were screened for antagonistic effects with an agar diffusion assay against the two pathogens, *F. columnare* and *F. psychrophilum* (Table 1). In all assays,

growth circles were observed, and no inhibitory zones involving antimicrobial compounds were found. Six strains (75% of the cultivable skin fish microflora: *Luteimonas aestuarii, Rhodococcus cercidiphylli, Microbacterium oxydans, Rhodococcus qingshengii, Sphingopyxis bauzanensis, Dietzia maris*) were more competitive (better growth) than *F. psychrophilum* after a 48h incubation period. All strains isolated from unstressed fish exhibited competitive capabilities against *F. psychrophilum*. Four strains (50% of the cultivable skin fish microflora: *Luteimonas aestuarii, Rhodococcus qingshengii, Leucobacter luti, Dietzia maris*) were more competitive than *F columnare*. The specific co-cultures (C and P) were still competitive against the two pathogens, but no synergetic effect was observed ($P > 0.05$). The co-culture U exhibited a stronger inhibitory effect ($p = 0.015$) when compared to the expected mean value of mono-cultures. This can be associated to the growth of *Microbacterium oxydans* in mono-cultures. Indeed, all the strains included in the co-culture had similar growth in mono-culture (1.5 mm ± 0.5 mm) except *Microbacterium oxydans*, which had an expensive growth in mono-culture (23 mm).

VALIDATION OF ANTAGONISTIC EFFECT IN BROTH CO-CULTURE ASSAY

In order to validate the presence of a competitive effect, the seven strains, which had exhibited antagonistic effects, were tested in broth co-culture assay with the pathogens. All candidates grew but no cells of pathogens were found after transfer on AO agar plates, although pathogen growth was clearly observed in controls plates (table 2). The antagonistic effect observed in broth co-culture thus further validates the probiotic potential of all the eight isolates tested in agar diffusion assays.

SCREENING FOR ANTAGONISTIC EFFECT OF MUCUS PROTEIN

Mucus of 43 fish was mixed, sterilized and spread on AO agar plate with 5 µl of each pathogen to test for the presence of antagonistic effect caused by the secretion of antibacterial peptides. Morphological analysis of colonies showed no contamination by bacterial strains coming from mucus or other sources. Only colonies of the two pathogens were found. Each pathogen showed a better growth with mucus complementation, especially for *F. psychrophilum* ($p < 2.2 \times 10^{-16}$).

IN VIVO ANTAGONISM AGAINST FLAVOBACTERIUM COLUMNARE

Seven strains with inhibitory activity against the two pathogens were mixed in order to be used in co-culture for the *in vivo* experiment. The dynamic of mortality was different between the two conditions (cf fig. 1). The first death occurred at day 4 in the control tanks but it occurred one day later in test tank. Then, the mortality stabilized at day 17 in the control and day 18 with the probiotic treatment. The probiotic addition significantly reduced the mortality in the test tank ($p < 0.001$). The four families responded differently to the infection and to the probiotic treatment ($p < 0.001$) (cf fig 2). The family S9 exhibited a very high sensitivity to infection (24% of death in control tanks), S1 and S10 families showed a medium sensitive response (12.9 and 12.8% of death in control tanks), and S9 family was strongly resistant (4.4% of death in control tanks). No significant difference was observed between duplicates ($p = 0.47$).

DISCUSSION

F. columnare and *F. psychrophilum* are known to be two majors pathogens in salmonids farming (Bernardet 1997). Actually, the only way to prevent and treat infections caused by these two pathogens consists in the addition of NaCl, formaldehyde or addition of antibiotics. However, various antibiotic resistance genes have rapidly invaded pathogen strains when fish were treated as described above (Schmidt *et al.* 2000; Thomas-Jinu and Goodwin 2004). To our knowledge, this study is the first that combines probiotic screening in skin microflora of brook charr, screening for the presence of inhibitory compounds in charr's skin mucus, and more importantly, *in vivo* validation.

Nine bacterial strains were isolated and identified by cultivable methods with TSA and R2A media. These strains represent 0.03 to 1.8 % of the total microbiota present in skin mucus of brook charr (Boutin *et al.*, unpublished). This result can be explained by the difficulty to cultivate environmental bacteria. Indeed, Amann *et al.* (1990) estimated that no more than 1% of the environmental bacterial communities are cultivable. In the present study, the predominant bacterial group isolated from brook charr skin microbiota was composed of three genera of Actinobacteria (Microbacteriaceae, Nocardiaceae, Dietziaceae). Actinobacteria were known to be an abundant group in soil (Madigan *et al.* 1996) but are also reported to be very abundant in freshwater (Glockner *et al.* 2000). This suggests that predominance of Actinobacteria in our mucus samples can be explained by the influence of surrounding water bacterial community on fish mucus (Cahill 1990).

Seventy-five percent of the skin isolates had antagonistic effects against *F. psychrophilum* and 50% of skin isolates against *F. columnare*. Results from broth culture

assays clearly indicate that all seven strains have antagonistic effects on the growth and the survival of the two pathogens. The results of the agar diffusion assays show that the mechanism involved in antagonistic effect is a competitive exclusion or a synthesis of non-diffusible antimicrobial. In this respect, no inhibition clearing was observed on agar plates but growth circles were present. The seven strains have grown quickly during the first 48h, despite the fact that pathogens have already colonized all the media.

At first sight, the inhibition of growth and survival along with the absence of inhibitory compounds in skin mucus, support the competitive exclusion hypothesis. This concept of suppression of pathogens by the development of resident bacteria was firstly suggested by Nurmi and Rantala (1973) in birds. The competitive exclusion exerted by probiotics against pathogens was also reported in fish, crustaceans or other aquatics organisms (Garriques and Arevalo 1995; Moriarty 1997; Gomez-Gil *et al.* 2000; Balcazar 2003; Balcázar *et al.* 2004; Vine *et al.* 2004). Competitive exclusion is the most promising mode of probiotic action because it involves many different processes and factors which are very important in microbial dynamics (Smith 1993). In order to become more competitive, pathogens need to evolve and gain new functions, each of them facing a single process implied in the competition. On the contrary, when competitors inhibit growth by secretion of a single antimicrobial agent, the pathogen needs to acquire only one specific resistance gene to this specific antimicrobial agent (Moriarty 1998). Furthermore, horizontal gene transfers mediated by plasmid vehicles favor quick and frequent acquisitions of new antimicrobial resistance (Lewin 1992; Courvalin 2006; Sørum 2006). However, in a competitive exclusion interaction, pathogen needs to evolve more than a single resistance gene. This mechanism of adaptation is slower than a single

297 plasmid transfer, therefore maintaining the *status quo* with the competing probiotic agent,
298 as formalized in the red queen theory (van Valen 1973).

299 In a second way, the survival inhibition in the broth assay act in favor of a non-diffusible
300 antimicrobial compound. We first thought about bacteriocin or other protein like
301 compound because some studies on these compound shown similar results with false
302 negative on agar diffusion assay (Schillinger and Lucke 1989; Bromberg *et al.* 2004;
303 Oliveira *et al.* 2008). Nevertheless, the target of bacteriocin is the cytoplasmic membrane
304 and Gram-negative bacteria like *Flavobacteria* do have a protective barrier provided by
305 the lipopolysaccharide of the outer membrane. However, some conditions can disrupt the
306 integrity of this barrier and increase the effectiveness of bacteriocin against gram-
307 negative bacteria (Stevens *et al.* 1991; Mortvedt-Abildgaard *et al.* 1995). The advantage
308 of bacteriocin and other protein-like compound is their sensitiveness to digestion enzyme
309 or protease activity. Indeed, those compounds are easily degradable and safe for human
310 consumption, which is not the case for antibiotics (Phillips *et al.* 2004; Courvalin 2006).
311 Further works to isolate and identify those compounds are necessary to clearly identify
312 the mechanisms involve in the in vitro antagonisms.

313 Our seven candidates fulfilled the three criteria for a subsequent *in vivo* validation
314 experiment. First, they were all isolated from resident community of skin mucus. Second,
315 they were able to adhere and colonize the mucus. Third, they all exerted antagonistic
316 effect against pathogen by competitive exclusion (Verschuere *et al.* 2000). So they were
317 mixed and test in an *in vivo* experiment. The addition of those probiotics significantly
318 decreased the fish mortality. This result indicates that there is a good correlation between
319 our *in vitro* experiment and the *in vivo* experiment, meanwhile it is not the case for many

320 studies (Reddy *et al.* 1994; Expert and Digat 1995; Gram *et al.* 2001). Nevertheless we
321 just test the effect in vivo against *Flavobacterium columnare* because the infection
322 occurred naturally after a stress due to the handling and not to a challenge against the
323 pathogen. Furthermore, the four families showed different intensity of response to the
324 treatment. A decrease of mortality occurred in all the families. Strikingly, the most
325 important decrease occurred in the most sensitive family. First, this result confirms
326 previous observations on *Salvelinus fontinalis*, showing that some families are more
327 sensitive than other to opportunistic infection (Bastien 2009). Second, this result is
328 further striking because it strongly suggests that host genotype controls the efficiency of
329 the probiotic effect on the pathogen. Knowing that microbiota composition is directly
330 influenced by the host genotype (Turnbaugh *et al.* 2007), we can therefore hypothesize
331 that some host genotypes are more tolerant to mutualism and let the probiotic colonize
332 their skin mucus. The hosted probiotic will in turn conferring a best protection to its host.
333 This mechanism is the basis of symbiosis and mutualism.

334 **CONCLUSION**

335 Our results indicate that isolation of host specific strains is easily manageable and cost
336 effective: Seven of the nine strains collected from unstressed fish exerted strong
337 exclusive competition against both *F. psychrophilum* and/or *F. columnare*, which
338 suggests they are all promising probiotic candidates. To conclude, agar diffusion assays,
339 combined with broth culture assay and the screening of inhibitory compound in mucus,
340 have clearly demonstrated that seven of our eight strains isolated from brook charr skin
341 mucus were able to exclude both *F. columnare* and/or *F. psychrophilum*. Nevertheless,
342 we were not able to identify clearly mechanisms involve in the exclusion. Two non-

343 exclusive hypothesis persist; i) Competition for nutrients, ii) Synthesis of bacteriocin or
344 other antimicrobial compound. The use of these seven probiotics *in vivo* clearly reduced
345 de mortality, therefore demonstrating the curative effect of these host specific strains.
346 These results show unambiguously that using of probiotic is a really manageable way to
347 efficiently prevent opportunistic infections. As a perspective, the efficiency of probiotic
348 action could be improved by combining a genetic selection program enhancing the
349 relationship between fish and probiotics. Further works on the impact of these probiotics
350 on the microbial load of fish and water are in progress and might explain the mechanism
351 involve in the antagonism observed in this study.

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TABLES AND FIGURES LEGENDS

Table 1. 16S rDNA gene sequence identification and capability to inhibit growth in nine indigenous bacterial isolates from mucus skin of brook charr. All strains were found in mucus from unstressed fish but only 6 were found in mucus from stressed fish. (+), isolates grew; (-), isolates did not grow**.**

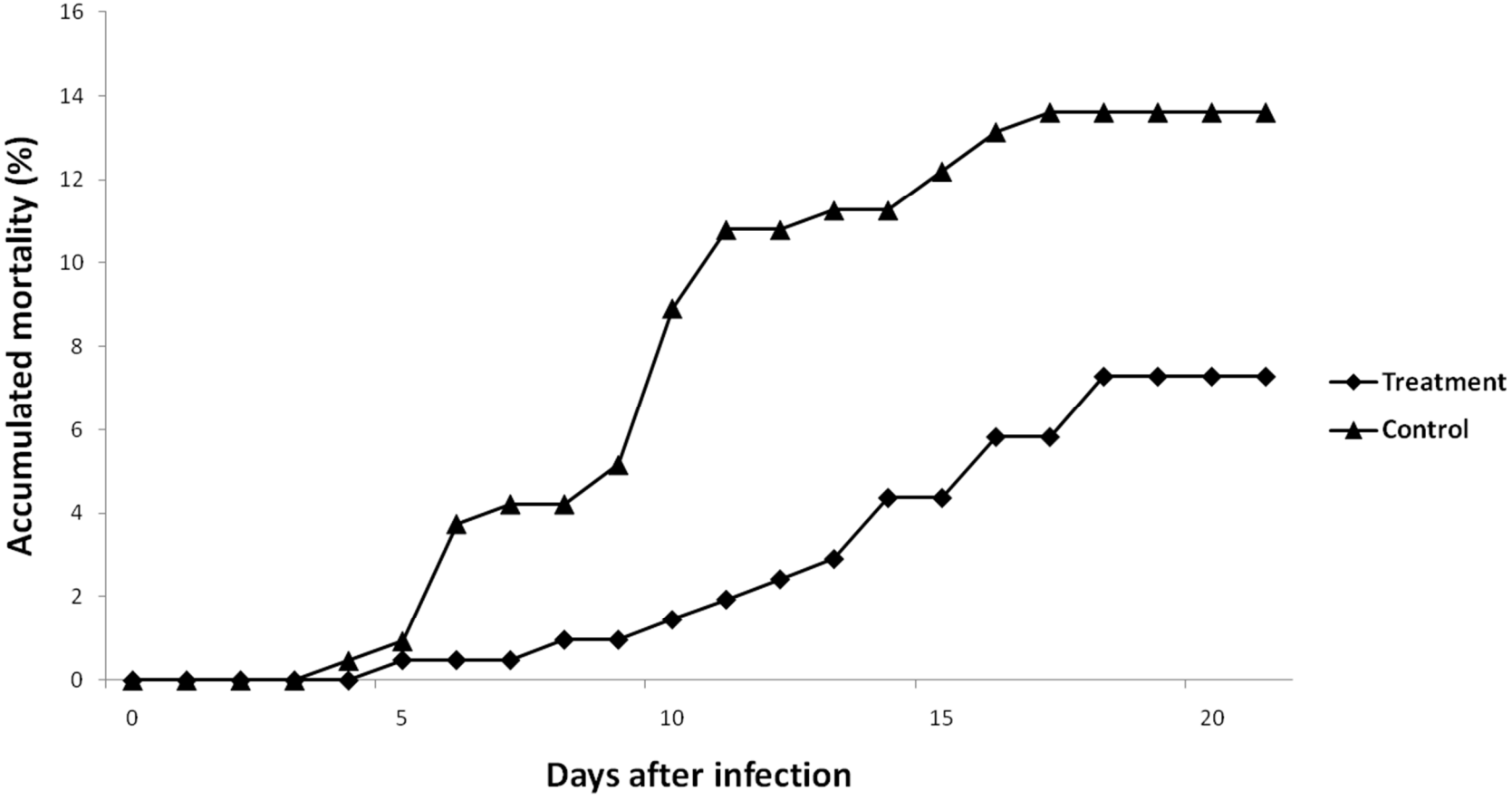
Table 2. Bacterial counts of broth co-culture assays after 48h incubation. Each assay was run in triplicate.

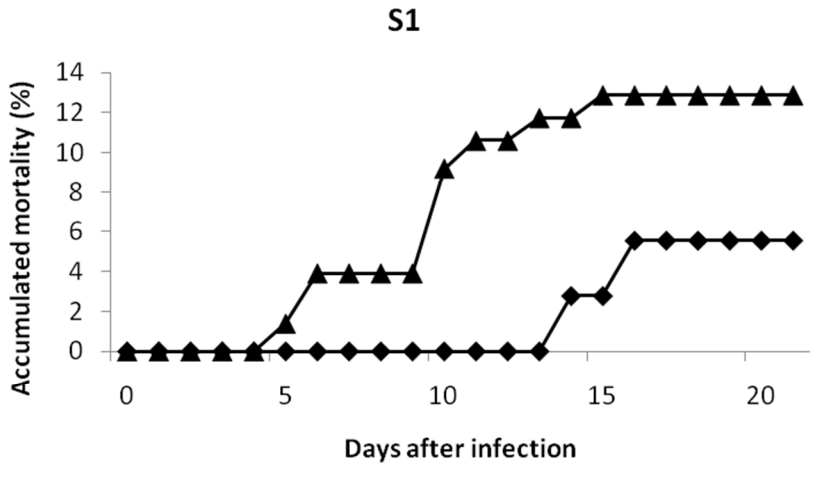
Figure 1. Accumulated mortality of brook charr (all family taken together) infected by *Flavobacterium columnare* and treated with probiotics candidates. Probiotic culture was added to the tanks during the infection.

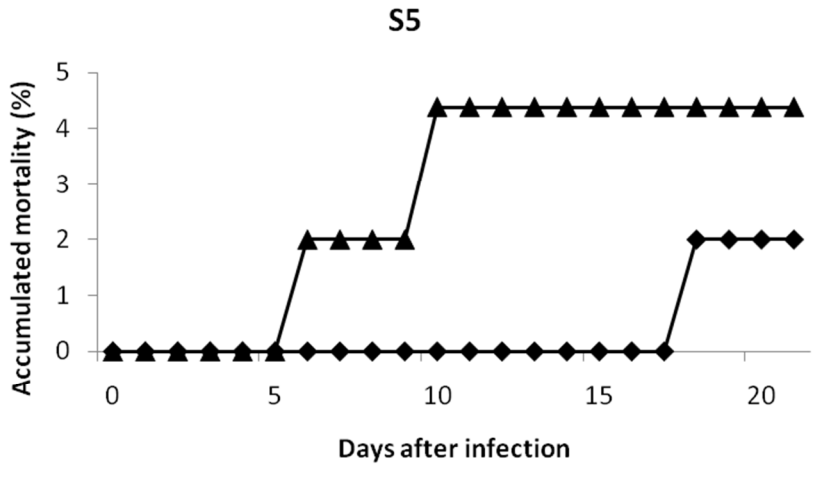
Figure 2. Accumulated mortality for the four families of brook charr infected by *Flavobacterium columnare* and treated with probiotics candidates. Probiotic culture was added to the tanks during the infection.

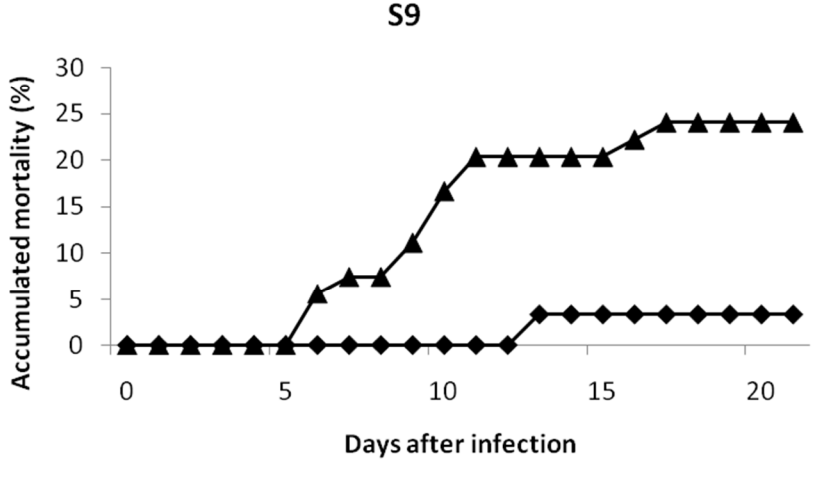
Isolates	Media	Growth on AO	Physiological condition of host	Bacterial group	Closest hit in GenBank	accession no.	Percentage similarity	No of sequenced base pair	Growth on *Flavobacterium columnare* culture	Growth on *Flavobacterium psychrophilum* culture
CP1	TSA	+	Unstressed	Gamma-proteobacteria	*Luteimonas aestuarii*	EF660758	98.899	1279	+	+
CP2	TSA, R2A	+	unstressed, stressed	Actinobacteria	*Microbacterium hatanonis*	AB274908	99.917	1215	-	-
CP3	TSA	+	unstressed, stress	Actinobacteria	*Rhodococcus cercidiphylli*	EU325542	99.439	1247	-	+
CP4	TSA, R2A	+	unstressed, stressed	Actinobacteria	*Microbacterium oxydans*	Y17227	99.762	1260	-	+
CP5	TSA	+	unstressed, stressed	Actinobacteria	*Rhodococcus qingshengii*	DQ090961	100.000	1248	+	+
CP6	TSA, R2A	-	Unstressed	Gamma-proteobacteria	*Pseudomonas pe*	AM114534	100.000	1264	NA	NA
CP7	TSA	+	Unstressed	Alpha-proteobacteria	*Sphingopyxis bauzanensis*	GQ131578	99.129	1122	-	+
CP8	TSA, R2A	+	unstressed, stressed	Actinobacteria	*Leucobacter luti*	AM072819	98.638	1254	+	-
CP9	TSA	+	unstressed, stressed	Actinobacteria	*Dietzia maris*	X79290	99.759	1249	+	+

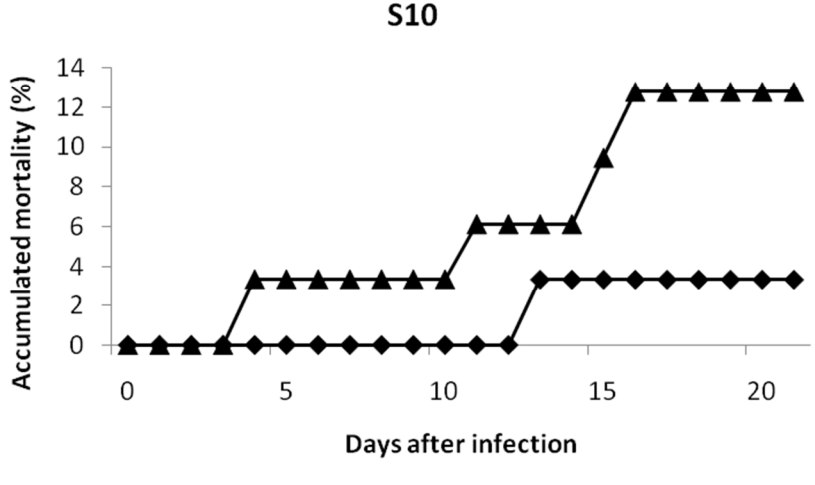
Isolates	Pathogens	Mean Count of i	Mean Count of Pathogens
CP1	*F.columnare*	4.84E+09	0.00E+00
CP1	*F.psychrophilum*	3.63E+10	0.00E+00
CP3	*F.psychrophilum*	5.53E+10	0.00E+00
CP4	*F.psychrophilum*	7.10E+10	0.00E+00
CP5	*F.columnare*	8.70E+10	0.00E+00
CP5	*F.psychrophilum*	6.00E+11	0.00E+00
CP7	*F.psychrophilum*	1.39E+11	0.00E+00
CP8	*F.columnare*	2.37E+11	0.00E+00
CP9	*F.columnare*	8.33E+10	0.00E+00
CP9	*F.psychrophilum*	7.73E+10	0.00E+00
NA	*F.columnare*	NA	5.00E+09
NA	*F.psychrophilum*	NA	3.00E+09


Accumulated mortality (%)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
Days after infection
Treatment
Control


S1
Accumulated mortality (%)
0
2
4
6
8
10
12
14
0
5
10
15
20
Days after infection


S5
Accumulated mortality (%)
0
1
2
3
4
5
0
5
10
15
20
Days after infection


S9
Accumulated mortality (%)
0
5
10
15
20
25
30
0
5
10
15
20
Days after infection


S10
Accumulated mortality (%)
0
2
4
6
8
10
12
14
0
5
10
15
20
Days after infection