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Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*

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ABSTRACT

Industrial fish production exposes fish to potentially 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 endogenous bacterial communities found in the mucus 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 assays and broth co-culture assays 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*. These 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 a decrease of mortality (54–86%) across fish families indicating that candidates from the host microbiota are potentially suitable for probiotic development. This would allow for the efficient (ability to adhere and colonize the host mucus) and durable management (antagonistic effect against pathogens which would be harmless for the host and safe for its environment) of opportunistic diseases in aquaculture.

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1. Introduction

Over the last decade, the aquaculture industry has greatly intensified productivity and is now a major economic activity in many countries (FAO, 2007). Massive production in fish farms may expose fish to stressful conditions, which in turn has the potential to induce infection by opportunistic pathogens (Wakabayashi, 1991). Infections are usually prevented and controlled by intrusive veterinary medicines and chemical substances (Rangdale et al., 1997). However, the beneficial effects of antimicrobial agents are counterbalanced by selection for

resistant pathogens (Nematollahi et al., 2003). Therefore, alternative strategies to prevent opportunistic infections in aquaculture are strongly needed. The development of probiotics appears to be one of the most promising ways to reach this goal (Merrifield et al., 2010).

The present study focused on two important opportunistic pathogens in Brook charr (*Salvelinus fontinalis*), namely *Flavobacterium columnare* and *Flavobacterium psychrophilum* (Bernardet and Bowman, 2006). Skin microflora was targeted as a potential source of probiotics because isolates from other parts of the body (e.g., gut) are known to be inefficient at inhibiting the growth of skin pathogens (Spanggaard et al., 2001). Microbial communities are sensitive to various stressful environmental conditions (Schimel et al., 2007). In this respect, we hypothesized that stress may imbalance the bacterial community structure of brook charr skin mucus and

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trigger opportunistic infections. Therefore, our general aim was to test whether natural isolates of non-stressed skin mucus bacterial community exhibit an antagonistic effect 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.

2. Experimental procedure

2.1. Sampling of brook charr bacterial community

Sixteen fish families derived from crosses between different parents were raised at the Laboratoire Régional des Sciences Aquatiques (LARSA) at Université Laval, in Quebec City, QC. Among them, two families were collected from a stress experiment. A total of two individuals per family were sampled. One of them was previously exposed to stressful physiological conditions (hypoxia and handling). Skin mucus was sampled using a sterile razor blade and homogenized after addition of 9 mL sterile water.

2.2. 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 (Difco). Bacterial colonies were isolated and individualized by cross streaking and incubated at 20 °C for 48 h. A total of nine isolates were identified by 16S rDNA sequencing. Colonies were used as template DNA for PCR amplification of the 16S rRNA gene using the universal bacterial primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and 907R (5'-CCGTC AATTCMTT-TRAGTTT-3') (Lane et al., 1985). PCRs were carried out in a volume of 25 µL containing 0.2 mM dNTP (Promega), 0.3 µM each primer, 6.4 µg of BSA, 1.25 mM of MgCl₂, 1× of Buffer and 0.4 U of Taq DNA polymerase (Promega) and performed in a Biometra T1 Thermocycler. The following amplification conditions were applied: a first step of initial denaturation at 94 °C for 5 min followed by 28 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 s and a final extension step at 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis [0.8% (w/v) agarose, 100 V]. Fragments were sequenced using the big Dye terminator V3 chemistry on an ABI 3130XL sequencer (Applied Biosystem, Foster City) at the Plateforme d'analyse Biomoléculaire (IBIS, Laval University).

2.3. Media used for culture and competition assay

Three general growth media, R2A (Difco) and TSA (Difco) were used for isolation, identification, and culture of brook charr bacterial community isolates. Both pathogens were grown on their respective general growth media, i.e., Anacker and Ordal (AO) (Anacker and Ordal,

1959). This media was used for competition assays with pathogens, *F. columnare* (ATCC 49418) and *F. psychrophilum* (ATCC 49512) isolated from *Oncorhynchus kisutch* and *Salmo trutta* (Bernardet and Grimont, 1989).

2.4. Screening for antagonistic effect in agar diffusion assay

All host bacterial isolate strains were tested for antagonistic effects using 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 Melchiorson, 1996). A volume of 10 µL of probiotic candidate culture was added into a 3 mm 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 exhibiting 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 h to check potential synergistic co-culture effects (Timmerman et al., 2004).

2.5. 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 48 h 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.

2.6. Screening for antagonistic effect of mucus proteins

Forty-three fish were sampled and all mucus samples were mixed and sterilized with UV light for 25 min to minimize degradation of proteins (Williams and Kraus, 1963). Forty-five microliter 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 48 h. 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.

2.7. Probiotic treatment of fish infection

Seven strains selected for their antagonistic activity during the *in vitro* experiment were tested together in a co-culture for logistical reasons. Four different fish families (i.e., resulting from different parental crosses), all selected on the basis of zootechnical traits of interest (growth and

reproduction) were used for the *in vivo* experiment (S1, S5, S9, S10). Each family was equally split up into four tanks: one pair of test tanks and one pair of control tanks. Although each tank pair shared the same water recirculation system, fish in separated tanks could not transmit pathogens by skin contact. Therefore, tanks supplied with the same water recirculation system were deemed as test pseudo-replicates. Test and control tank pairs were separated in two different units (i.e., supplied with independent water recirculation systems). All of the individuals were juveniles (mean size: 12.2 ± 1.4 cm; mean weight: 16 ± 5.8 g) and came from the “Station aquicole de l’Institut des sciences de la mer de Rimouski”. Fish were raised at 12 °C and oxygen concentration was always higher than 12 mg/L. Each tank contained between 103 and 110 fish. Two weeks after transport to LARSA, the first symptoms of columnaris disease appeared in all tanks and we started the addition of the mix of probiotics candidates. The probiotics were added in the two test tanks to reach the concentration of 10^5 cell/mL in water twice daily until the fish mortality stabilized. Dead fish and moribund were checked for clinical symptoms of columnaris disease. The moribund fish were euthanized with MS-222 as required by the “Comité de Protection des Animaux de l’Université Laval (CPAUL, <http://www.vrr.ulaval.ca/deontologie/cpa/index.html?accueil>).

2.8. Statistical analysis

We compared the percentage of death between test and control during 21 days using a generalized linear model approach with a logit link and a binomial distribution. The analysis was performed using the software *R*, version 2.12.2.

3. Results

3.1. 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 in mucus cultures isolated from both stressed and non-stressed fish. Actinobacteria (Microbacteriaceae, Nocardioceae and Dietziaceae) was the predominant (six strains) bacterial group that was isolated from skin mucus. Proteobacteria (gamma and

alpha subclass) was the second group isolated (three strains). Both of these groups belong to gram-negative bacteria and 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.

3.2. 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 (exhibited better growth) than *F. psychrophilum* after a 48 h incubation period. All strains isolated from unstressed fish exhibited competitive capabilities against *F. psychrophilum*. Four strains (50% of the cultivable mucosal 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 ± 0.5 mm) except *Microbacterium oxydans*, which had an extensive growth in mono-culture (23 mm).

3.3. Validation of antagonistic effect in broth co-culture assay

In order to validate the presence of a competitive effect, the seven strains exhibiting antagonistic effects were tested in broth co-culture assays with the pathogens. All candidates grew but no cells of pathogens were found after

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.

Isolates	Media	Physiological condition of host	Bacterial group	Closest hit in GenBank	Percentage similarity	Growth on <i>Flavobacterium columnare</i> culture	Growth on <i>Flavobacterium psychrophilum</i> culture
CP1	TSA	UnStressed	Gamma-proteobacteria	<i>Luteimonas aestuarii</i>	98.899	+	+
CP2	TSA, R2A	UnStressed, stressed	Actinobacteria	<i>Microbacterium hatanonis</i>	99.917	–	–
CP3	TSA	UnStressed, stressed	Actinobacteria	<i>Rhodococcus cercidiphylli</i>	99.439	–	+
CP4	TSA, R2A	UnStressed, stressed	Actinobacteria	<i>Microbacterium oxydans</i>	99.762	–	+
CP5	TSA	UnStressed, stressed	Actinobacteria	<i>Rhodococcus qingshengii</i>	100.000	+	+
CP6	TSA, R2A	UnStressed	Gamma-proteobacteria	<i>Pseudomonas peli</i>	100.000	NA	NA
CP7	TSA	UnStressed	Alpha-proteobacteria	<i>Sphingopyxis bauzanensis</i>	99.129	–	+
CP8	TSA, R2A	UnStressed, stressed	Actinobacteria	<i>Leucobacter luti</i>	98.638	+	–
CP9	TSA	UnStressed, stressed	Actinobacteria	<i>Dietzia maris</i>	99.759	+	+

Table 2

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

Isolates	Pathogens	Mean count of isolates	Mean count of pathogens
CP1	<i>F. columnare</i>	4.84×10^9	0
CP1	<i>F. psychrophilum</i>	3.63×10^{10}	0
CP3	<i>F. psychrophilum</i>	5.53×10^{10}	0
CP4	<i>F. psychrophilum</i>	7.1×10^{10}	0
CP5	<i>F. columnare</i>	8.7×10^{10}	0
CP5	<i>F. psychrophilum</i>	6×10^{11}	0
CP7	<i>F. psychrophilum</i>	1.39×10^{11}	0
CP8	<i>F. columnare</i>	2.37×10^{11}	0
CP9	<i>F. columnare</i>	8.33×10^{10}	0
CP9	<i>F. psychrophilum</i>	7.73×10^{10}	0
NA	<i>F. columnare</i>	NA	5×10^9
NA	<i>F. psychrophilum</i>	NA	3×10^9

transfer on AO agar plates, although pathogen growth was clearly observed in control 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.

3.4. Screening for antagonistic effect of mucus protein

Mucus of 43 fish was mixed, sterilized and spread on AO agar plate with 5 μ L 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 addition, especially for *F. psychrophilum* ($p < 2.2 \times 10^{-16}$).

3.5. In vivo antagonism against *F. columnare*

Seven strains with *in vitro* inhibitory activity against the two pathogens were mixed in order to be used in co-culture for the *in vivo* experiment for logistical reasons. Mortality differed between the two conditions (cf Fig. 1). The first death occurred on day 4 in the control tanks but it occurred one day later with the treatment. The mortality rate stabilized at day 17 in the control and day 18 with the

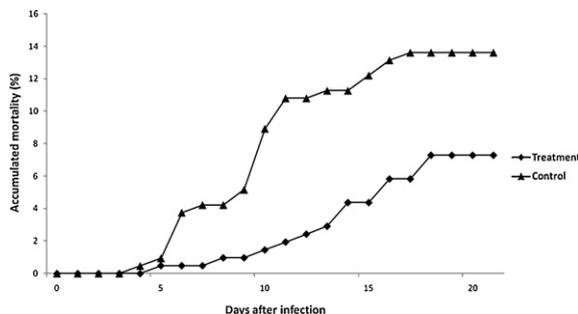


Fig. 1. Accumulated mortality of brook charr (all family taken together) infected by *Flavobacterium columnare* and treated with probiotics candidates. Probiotic culture was added to the tanks during the infection.

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 an intermediate response (12.9% and 12.8% of death in control tanks), and S9 family was strongly resistant (4.4% of death in control tanks) to columnaris disease. No significant difference was observed between duplicates ($p = 0.47$).

4. Discussion

F. columnare and *F. psychrophilum* are known to be two major pathogens in salmonid 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 with antibiotics (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 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 0.03–1.8% 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 are 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 and 50% of the skin isolates had antagonistic effects against *F. psychrophilum* and *F. columnare*, respectively. 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 grew quickly during the first 48 h, despite the fact that pathogens had 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 first suggested by Nurmi and Rantala

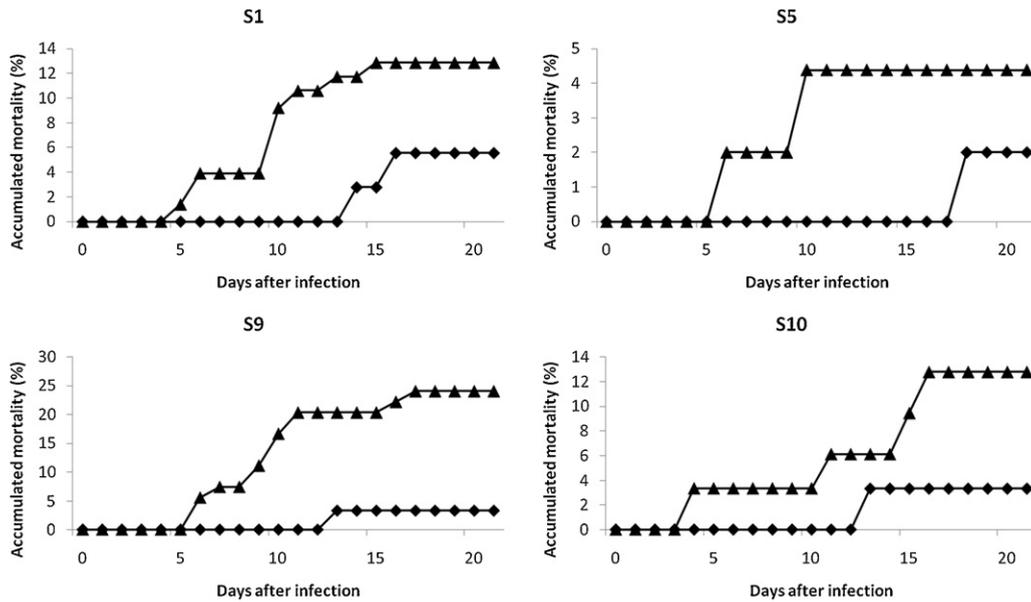


Fig. 2. Accumulated mortality for the four families of brook charr infected by *Flavobacterium columnare* and treated with probiotics candidates. Probiotic culture was added to the tanks during the infection.

(1973) in birds. The competitive exclusion exerted by probiotics against pathogens was also reported in fish, crustaceans, and other aquatic organisms (Balcázar 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 to gain new functions. Each of these new functions 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 (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 plasmid transfer, therefore maintaining the *status quo* with the competing probiotic agent, as formalized in the red queen theory (van Valen, 1973).

The survival inhibition in the broth assay suggests a non-diffusible antimicrobial compound (Oliveira et al., 2008). The target of bacteriocin is the cytoplasmic membrane, but Gram-negative bacteria like *Flavobacteria* do have a protective barrier provided by the lipopolysaccharide of the outer membrane. However, some conditions can disrupt the integrity of this barrier and increase the effectiveness of bacteriocin against gram-negative bacteria (Stevens et al., 1991; Mortvedt-Abildgaard et al., 1995). The advantage of bacteriocin and other protein-like compounds is their sensitivity to digestion enzymes or proteases activity. Indeed, those compounds are easily degradable and safe for human consumption, which is not

the case for antibiotics (Phillips et al., 2004; Courvalin, 2006). Further works to isolate and to identify those compounds are necessary to clearly identify the mechanisms involved in the *in vitro* antagonisms.

Our seven candidates fulfilled the three criteria for a subsequent *in vivo* validation experiment. First, they were all isolated from the resident community of skin mucus. Second, they were able to adhere and to colonize the mucus. Third, they all exerted antagonistic effects against pathogens by competitive exclusion for nutrient and/or synthesis of antimicrobial compounds (Verschuere et al., 2000). The addition of those probiotics in water tanks significantly decreased the fish mortality, which indicates a good agreement between our *in vitro* experiment and the *in vivo* experiment in contrast to many previous studies (Reddy et al., 1994; Gram et al., 2001). However, we just tested the effect *in vivo* against *F. columnare* because the infection occurred naturally after a stress due to the handling and not to a challenge against the pathogen itself. Furthermore, the four fish families showed different intensity of response to the treatment. A decrease of mortality occurred in all the families. Strikingly, the most important decrease occurred in the most sensitive family. This last result confirms previous observations on *S. fontinalis*, showing that some families are more sensitive than others to opportunistic infections (Bastien, 2009) and also strongly suggests that host genotype controls the efficiency of the probiotic effect on the pathogen. Knowing that microbiota composition is directly influenced by the host genotype (Turnbaugh et al., 2007), we can therefore hypothesize that some host genotypes are more tolerant to mutualism and let the probiotic colonize their skin mucus. The hosted probiotic will in turn confer a best protection to its host. This mechanism is the basis of symbiosis and mutualism.

5. Conclusion

Our results indicate that isolation of host specific strains is easily manageable and cost effective in fish. To this respect, these probiotic strains exhibited high survival rate after freezing at -80°C with half volume of glycerol (data not shown). Seven of the nine strains collected from unstressed fish exerted strong exclusive competition against both *F. psychrophilum* and/or *F. columnare*, which suggests they are all promising probiotic candidates. Agar diffusion assays, combined with broth culture assays and the screening of inhibitory compounds in mucus, have clearly demonstrated that seven of our eight strains isolated from brook charr skin mucus were able to exclude both *F. columnare* and/or *F. psychrophilum*. Nevertheless, we were not able to clearly identify the mechanisms involved in the exclusion. Two non-exclusive hypotheses persist: (i) competition for nutrients; (ii) synthesis of bacteriocin or other antimicrobial compounds. The use of these seven probiotics *in vivo* clearly reduced the mortality, therefore demonstrating the curative effect of these host specific strains. These results show unambiguously that using of probiotics is a really manageable way to efficiently reduce mortality due to opportunistic infections. Moreover, the efficiency of probiotic action could be improved by combining a genetic selection program enhancing the relationship between fish and probiotics. Further works on the impact of these probiotics on the microbial load of fish and water are in progress in order to more-specifically examine the antagonistic mechanisms observed in this study.

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