

1 **Probiotic treatment by indigenous bacteria decreased mortality without disturbing the**
2 **natural microbiota of *Salvelinus fontinalis*.**

3 Running title: Probiotic does not induce dysbiosis

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Abstract

Since the rise of new generation sequencing (NGS), host-bacteria interactions are viewed as a complex interactive network. This study focused on the influence of a probiotic treatment on the natural microbiota of Brook charr (*Salvelinus fontinalis*), especially on the disturbance of the natural microbiota (dysbiosis). The results indicated that an indigenous probiotic strain (identified as *Rhodococcus* sp.) colonized neither the fish skin nor the water following the probiotic treatment. Instead, the probiotic strain was only detected in the biofilm of the test tank. Nevertheless, a substantial beneficial effect of the probiotic treatment was observed through the decrease of *F. psychrophilum* in the treated tank water. This study clearly shows that the indigenous strain chosen for the probiotic treatment did not disturb the natural fish skin microbiota, but acted directly through the production system to control the growth of the pathogen, and, as a consequence, to enhance fish survival.

Introduction

During the last decades, the interest on host-bacteria interactions has greatly increased. Indeed, this field has improved significantly considering both, theoretical and technical aspects due to the development of new culture-independent methods such as metagenomic and metatranscriptomic (Costello *et al.* 2009; Roberts *et al.* 2010; Robinson *et al.* 2010; Gonzalez *et al.* 2011; Rumbaugh and Kaufmann 2012). Thus, host-pathogenic bacteria interaction is no longer understood as two-component system involved in an “evolutionary arms race” but rather as an integrative system in which the host harbors a consortia of cooperating bacteria, as suggested in the Black queen theory (Van Valen 1973; Darch *et al.* 2012; Morris *et al.* 2012). Those theoretical advances are also correlated with the assessment of new methods for the prevention of bacterial infections (Verschuere *et al.* 2000; Merrifield *et al.* 2010). Indeed, the use of probiotics is now considered as a valuable tool in the struggle against pathogens in agriculture and aquaculture, as well as for improving human health (Fuller 1995; Balcázar *et al.* 2004; Balcazar *et al.* 2006; Wang *et al.* 2008).

The FAO/WHO defined a probiotic agent as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”(FAO/WHO 2001). 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 may 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 may be more efficient to isolate it from the host’s endogenous bacterial community (Balcazar *et al.* 2006). However, information about the probiotics influence on the natural microbiota equilibrium is still scarce. For therapeutic use, probiotics are often added in greater concentration relative to the abundance naturally found in hosts (Vine *et al.* 2004). Therefore, there is a pressing need to test whether probiotic treatments have any disturbing effect on the homeostasis of both endogenous microbiota and surrounding environmental bacterial community.

In this study, we focused on the treatment of a cold-water disease (CWD) affecting *Salvelinus fontinalis* by using a mix of probiotics. Cold water disease is caused by a bacterial agent, *Flavobacterium psychrophilum* (Starliper 2011). This bacterium has been recovered from a broad geographic range and is responsible for the rainbow trout (*Oncorhynchus mykiss*) fry syndrome and CWD, two important infectious diseases in farmed fish (Wiklund *et al.* 2000). These diseases affect especially the early life-stages and it is well documented that stressful conditions and injuries facilitate infections (Madetoja *et al.* 2000; Bader *et al.* 2003; Starliper 2011). The probiotics used in this study, recently isolated from the natural microflora of brook charr, belongs to the rare biosphere on natural fish skin microbiota and has already proven their efficiency through *in vitro* and *in vivo* experiments (Boutin *et al.* 2012). Specifically, we used 454-pyrosequencing to monitor the natural microflora of the fish skin and the surrounding water during the *in vivo* experiment to ensure that those probiotics disturbed neither the natural microflora of fish skin nor the bacterial community of the surrounding environment.

Methods

Ethics statement

We raised fish and did the experiment according to the guidelines required by the “Comité de Protection des Animaux de l’Université Laval (CPAUL, <http://www.vrr.ulaval.ca/deontologie/cpa/index.html?accueil>).

Probiotic treatment of fish infection

Seven bacterial strains isolated from brook charr skin and selected for their antagonistic activity during the *in vitro* experiment done by Boutin *et al.* (2012) were tested together in a co-culture in Tryptic soy broth. Four different fish families (i.e. resulting from different parental crosses) selected on the basis of zootechnical traits of interest (growth and late sexual maturity) were used for the *in vivo* experiment (for complementary information see Boutin *et al.* 2012). In the present paper, we focused on two specific families (S5, S9) because they exhibited the greater variations in terms of mortality during the infection and were considered either as resistant (S5) or sensitive (S9) (Boutin *et al.* 2012).

Sampling

Fish from the four families were divided among 4 tanks: 2 control and 2 test tanks (25 fish per family per tank). Ten individuals from each family and from each tank were randomly sampled at three different time periods after the occurrence of infections: 1) before treatment with the probiotic, 2) the last day of the probiotic treatment, 3) two weeks after the probiotic treatment. Mucus samples were collected with sterile swab on the surface of the fish (Livia *et al.* 2006). Samples were put into sterile micro-centrifuge tubes containing lysis buffer (Tris 50 mM, EDTA 40 mM, Sucrose 0.75 g) and stored in a -80°C freezer until DNA extraction. Skin mucus and gut samples from each fish that died during the probiotic treatment were sampled. At each sampling time, water level was lowered by 5 cm to allow sampling of the biofilm by swabbing the wall of the tank. Original water level was restored after sampling.

For water bacterial community sampling, a two-step filtration was realized using peristaltic filtration equipment (Masterflex L/S Pump System with Easy-Load II Pump Head, Cole-Parmer) cleaned up with HCl 5% and rinsed with Milli-Q water before each filtration. The first step was the conditioning of the filtration set up using two liters of the rearing water without filters. The last step was to collect duplicates of water from test and control units, by filtering a total of four liters of water over a series of filters beginning with a 3.0 µm followed by a 0.22 µm x 47 mm nitrocellulose membrane (Advantec). Immediately after each filtration process, filters were placed into cryotubes containing 1 mL of sterile lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 mM sucrose) and then stored at -80°C until DNA extraction.

DNA extraction

DNA was extracted from mucus and water using a modified salt-extraction protocol (Aljanabi and Martinez 1997). During the first lysis step, 22.6 µL of lysozyme (40mg/mL) were added to the sample which were then incubated for 45 min at 37°C. Secondly, 22.6 µL of proteinase K (20mg/mL) and 90 µL of SDS 20% were added to the lysate, followed by incubation at 55°C over night with agitation. The aqueous phase was transferred into a clean Eppendorf tube containing 600 µL of NaCl 6M, mixed and centrifuged 20 min at 16000 g. The supernatant was transferred again into a clean Eppendorf tube containing one volume of cold isopropanol, mixed, and stored 30 minutes at -20°C. The mixture was centrifuged 20 min at 16000 g, and the

supernatant was thrown away. The pellet was washed with cold ethanol 70%, air dried, and finally resuspended in 25 μ L of sterile MilliQ H₂O. Subsequently, DNA integrity and quantity were controlled using a Nanodrop instrument (ND-1000, Nanodrop).

Pyrosequencing

Each DNA sample was PCR amplified using Takara Ex taq premix (Fisher). All PCR reactions were performed in a final volume of 50 μ L containing 25 μ L of Premix Taq, 1 μ M of each primer, 500 ng of template and sterile MilliQ H₂O to up to 50 μ L. To achieve the PCR amplifications, a general reverse primer (R519) combined with B primer (Roche) was used in combination with a unique tagged forward primer (F63-targeted) combined with A primer (Roche) (Marchesi *et al.* 1998; Turner *et al.* 1999). PCR conditions were as follows: after a denaturing step of 30 sec at 98°C, samples were processed through 30 cycles consisting of 10 sec at 98°C, 30 sec at 55°C and 30 sec at 72°C. The final extension step was done at 72°C for 4 min 30 sec. Following amplification, samples were purified using AMPure Beads (Beckman Coulter Genomics). Samples were adjusted to 100 μ L with EB (Qiagen), 63 μ L of beads were added. Samples were mixed and incubated for 5 min at RT. Using a Magnetic Particle Concentrator (MPC), the beads were pelleted against the wall of the tube and the supernatant was removed. The beads were washed twice with 500 μ L of 70% ethanol and incubated for 30 sec each time. The supernatant was removed and beads were allowed to air dry for 5 min. Tubes were removed from the MPC and 24 μ L of EB were added. Samples were vortexed to resuspend the beads. Finally, using the MPC, the beads were pelleted against the wall once more and supernatant was transferred to a new clean tube. Samples were quantified with Nanodrop and mixed equally before being sent to the Plateforme d'Analyses Biomoléculaires (Institut de Biologie Intégrative et des Systèmes, Université Laval) for sequencing on a 454 GS-FLX DNA Sequencer with the Titanium Chemistry (Roche), according to the procedure described by the manufacturer.

Sequence analysis

The data were analyzed in two steps. First, CLC Genomics Workbench 3.1 (CLC Bio, Aarhus, Denmark CLC work bench BIO®) was used to trim sequences for quality and recover the primers' sequences and tags. In a second step, pre-processing and analysis were performed using the MOTHUR software (Schloss *et al.* 2009). We screened all the dataset to extract the sequences

reaching 300 bp to ensure an accurate taxonomic assignment at the genus level. We used the Operational Taxonomic Unit-based method described by Costello *et al.* (2009). The index retained to assess the quality of pyrosequencing was the sequence coverage index (Good's coverage estimator). This index is a non-parametric index used to estimate the quality of a dataset (Esty 1986). All sequences were clustered into OTU using a 97% identity threshold and OTU were classified from phylum to genus using the program MOTHUR with the default setting. To visualize similarities between mucus communities and water communities, distances between communities were computed using Unifrac weighted. Distances were then represented using dendrograms based on ThetaYC (weighted) index because it takes into account the relative abundance of each OTU (Yue and Clayton 2005). We also used a Principal Coordinates Analysis (PCoA) using an eigenvector-based approach (Joliffe and Morgan 1992) to represent multidimensional data in as few dimensions as possible to visualize the distances between communities.

Detection of *Flavobacterium sp.* and *Rhodococcus sp.*

Each sample was tested for the presence of *F. psychrophilum*, *F. columnare* and *Rhodococcus sp.* using a diagnostic PCR approach. The method used to detect *F. psychrophilum* from fish tissues and water samples was developed by Wiklund *et al.* (2000). The primers developed by Bader *et al.* (2003) to detect *F. columnare* and *Rhodococcus sp.* were used in combination with the PCR method developed to targets the *catA* gene (Táncsics *et al.* 2008). The specificity of those primers was tested by analyzing three strains of *Rhodococcus* isolated from fish skin mucus. The skin and gut of dead fish were also tested during the experiment and all dead fish showed symptoms of CWD.

Results

The probiotic addition significantly reduced the mortality in test tanks ($p < 0.001$). The family S9 exhibited a very high sensitivity to infection (24% mortality in control tanks) whereas the S5 family was more resistant (4.4% mortality in control tanks) as detailed previously (Boutin *et al.* 2012). Fish that died during the experiment exhibited symptoms imputable either to *Columnaris* disease or CWD (i.e. fin erosion, necrosis...). The PCR approach was used to accurately identify the causative agent. Only *F. psychrophilum* was successfully detected in 87% of the skin from

dead fishes and all water samples, except for control tanks at the end of the experiment (table 1). In the gut from dead fish, the pathogen was detected in 43.4% of our samples. Gut samples from control and test tanks were not equally infected; 60% of the dead fish from control tanks were positive on the PCR whereas 30 % of the dead fish from test samples were positive, although this difference was not significant ($p= 0.159$). *F. psychrophilum* was also found at the initial step of the experiment in samples from one duplicate of the family S5 and S9 in test tanks. After two weeks of treatment (control or test), *F. psychrophilum* was found in all the samples. Finally, two weeks after the probiotic treatment, *F. psychrophilum* was still present in all control samples but only in 50% of the test samples. Using pyrosequencing, it was noted that the genus *Flavobacterium* was weakly represented in the bacterial community (0.7%). This pathogen was not found in the mucus samples, except in one sample from the family S9 in a test tank at the end of the probiotic treatment. In the water, *Flavobacterium* was also poorly represented. It was never found in test tanks. In control tanks, it was absent at the first sampling but reached 3.9 % at the end of the placebo treatment to finally decreased at 0.3% at the end of the experiment.

A total of 117 260 reads was obtained from the 31 samples through 454 pyrosequencing analysis. After the filtering process of short (<300 bp) and bad quality sequences, 61 881 reads were kept (52% of the dataset). The relatively low rate of retrieved sequences is due to the strong threshold of quality. Usually, the recommended length threshold for taxonomic assignation was 150 bp (Costello *et al.* 2009). According to this threshold (i.e. filtering process of short (<150 bp) sequences), 96 931 sequences (82.6% of the dataset) were retrieved. Although all analyses done with this dataset gave consistent results for spatial (PCoA) and phylogenetical (Unifrac) relationships between samples, this low threshold of filtering process greatly increased the uncertainty of the taxonomic assignation at the genus level due to shorter sequences (Okubo *et al.* 2012). This results about short reads and their sufficiency was already discussed (Liu *et al.* 2007). To combine a good genus assignation and an accurate community analysis, we choose a threshold of 300 bp as recommended in other study (Liu *et al.* 2008). In order to focus on the more biologically relevant taxa, OTUs represented by less than ten reads were discarded for the analysis where taxonomic assignation was important (relative abundance in figure 3-1) but were kept for the other analysis (PCoA and Unifrac analysis). The resulting dataset was distributed among 38 genera and 5 groups or phyla. The Good's coverage estimations ranked between 69% and 100%.

The results from pyrosequencing indicated that only one genus was detected in the mix of probiotics, namely *Rhodococcus* (100% of the reads from the probiotic sample). The genus *Rhodococcus* was also detected by pyrosequencing in one sample from the family S9 in a test tank at the end of the treatment. There were two strains of *Rhodococcus* in the probiotic mix. Among the three *Rhodococcus* strains isolated from fish skin mucus, only one strain from the two used as probiotic in our experiment was detected by PCR. This *Rhodococcus* strain was detected with the PCR method only in the biofilm of test tanks at the end of the addition of probiotic, but not in any fish or water samples (table 1). Furthermore, we validated this taxonomic assignment by culturing this bacterium from the mix of probiotic and re-sequenced it. The strain was assigned to *Rhodococcus* genus with a read length of 1500 bp.

The bacterial community of fish skin mucus was quite stable except for the family S9 sample at the first sampling in the control unit (CAS91) (figure 1). Samples from mucus were dominated by the occurrence of the genus *Sphingomonas*, which reached a mean of 91.6% of the community. The other major genera were *Pseudomonas*, *Pseudoxanthomonas*, *Kerstersia*, *Dichelobacter* and *Propionibacterium* (2.7%, 1.4%, 0.9%, 0.5%, and 0.4%). Bacterial communities from the water were more dynamic during the experiment even in control tanks in which no probiotic were added. Bacterial communities from the water samples also showed more diversity than fish skin microbiome. In general, the most abundant genus was still *Sphingomonas* (47.1%), followed by *Legionella* (16.5%), *Smithella* (15.6%), *Microvirga* (4%), *Dinoroseobacter* (2.2%), *Curvibacter* (1.8%), *Hydrocarboniphaga* (1.8%), *Kestersia* (1.6%), *Pseudomonas* (1.4%), and *Kordiimonas* (1.2%).

The Principal Coordinates Analysis (PCoA) shows that our data is highly dimensional (30 dimensions) but the first and second axes represent 44.6% and 16.2% of the variation (60.8% total) for the theta YC distances. Furthermore, the R-squared between the original distance matrix and the distance between the points in 2D PCoA space was 0.978. As visualized in the PCoA plot, all the samples from mucus cluster in the same groups excepted once again for the sample coming from the family S9 at the first sampling in the control unit (CAS91) (figure 2). Considering the first sampling time, water samples are very close from mucus samples (TEAUA1 and CEAUA1). The bacterial communities from water samples in control tanks for sampling times 2 and 3 (CEAUA2 and CEAUA3) are outliers to the cluster and formed a second cluster

with the water sample in test tanks for sampling time 2. The dendrogram representing the phylogenetic distances between the bacterial communities shows exactly the same results than the PCoA (figure 3). All the samples from fish mucus are phylogenetically close and related to the water samples from the first sampling time (CEAU1 and TEAU2). The only exception was the CAS91 sample and the water samples of the second and third sampling times. The probiotic mix, as it was composed of only one genus that was not recovered in the other samples, clusters as an outgroup.

Discussion

The aim of this study was to monitor the effect of a probiotic treatment on both the natural microbiota of Brook charr skin mucus and the surrounding water microflora. Using 454-pyrosequencing, the variation in abundance for the different bacterial strains was screened to test whether the treatment using probiotic candidate (*Rhodococcus sp.*) would disturb either the fish skin microbiota and / or the surrounding natural microflora.

The analysis of bacterial communities present in fish mucus showed that bacterial communities were stable through the three sampling times and among samples from different families. This may be explained by the high abundance of the genus *Sphingomonas*, which represents 91.6% of the bacterial communities. This genus is widely distributed in freshwater environments and has already been isolated from environments with high concentrations of organic nutrients, such as blood and sputum (Hsueh *et al.* 1998). It is therefore not surprising to detect this bacterial genus in fish skin mucus. *Sphingomonas* is also known for consuming a wide range of organic compounds and to exhibit remarkable biodegradative and biosynthetic capabilities (Balkwill *et al.* 2006). Such metabolic abilities are correlated with the domination of *Sphingomonas alaskensis* in marine picoplankton (Eguchi *et al.* 2001; Vancanneyt *et al.* 2001). Therefore, a high competitiveness for nutrients could explain how *Sphingomonas* present in skin mucus would become a protective barrier against other environmental bacteria. The benefic effect of *Sphingomonas* for the host could also be related to both competition for nutrients and interference competition. Indeed, another species of the same genus, *Sphingomonas paucimobilis*, is known to inhibit the growth of fungi in plants (White *et al.* 1996). We can hypothesize that Brook charr co-evolved with this bacterial genus to ensure protection by the bacteria in exchange of nutrients offered by the host (Sachs *et al.* 2004).

The proximity between bacterial community present in mucus and water samples should also be noticed. Prior to probiotic treatment, the PCoA analysis and the dendrogram showed a great similarity between the two. This is in accordance with the hypothesis that skin microbiota results from the colonization by strains inhabiting the surrounding water (Cahill 1990). Here, the results showed that this proximity was disturbed by the appearance of the disease and the increase of *F. psychrophilum* abundance in control tanks.

Surprisingly, the probiotic strain was neither detected in fish skin mucus nor in water samples such that the presence of the probiotic strain was detected by PCR only in the biofilm of test tanks, at the end of the treatment. Its absence in fish skin mucus can be explained by the inability of our probiotic to out-compete the bacteria already present in the mucus. This strain was isolated from skin mucus of Brook charr, thus demonstrating its ability to live on mucus and its competence for adhesion. However, the probiotic strain was isolated from another Brook charr family where *Sphingomonas* was not the most abundant genus present. Hypothetically, this probiotic strain may not be co-adapted to *Sphingomonas*, and in turn unable to colonize the mucus.

Aside of this, the absence of the probiotic in water suggests either a low survival of the probiotic strain in open water or a quick sedimentation or a biofilm formation. Furthermore, the probiotic strain was not detected in the gut from dead fish in test tanks either. This suggests that this *Rhodococcus* strain was not able to colonize the gut. The survival differences recorded between tests and the control tanks were significant (diminution of 47% death, $p < 0.001$) correlated with the addition of this probiotic strain in test tanks (Boutin *et al.* 2012). Surprisingly, the results indicate that this beneficial effect was not correlated with a colonization of skin mucus by probiotic strains that were isolated from fish skin mucus. In this respect, no community change was observed in the skin mucus microbiota between the control and test tank samples. Interestingly, in the surrounding water of control tanks, we observed an increase of *F. psychrophilum* abundance that was significantly correlated to fish mortality (Pearson correlation, $p < 0.001$). Such finding makes sense because the disease symptoms encountered in this study were clearly associated to the CWD.

Furthermore, shedding of *F. psychrophilum* by infected fish has previously been shown to be correlated with mortality (Madetoja *et al.* 2000). In test tanks, no such increase was observed

after the addition of the probiotic strain, so we can conclude that probiotic controlled the growth of *F. psychrophilum* in the surrounding water, but not on skin mucus as we first hypothesized. This conclusion is also supported by the fact that the third water sample from test tanks (TEAUA3) tightly clustered with the mucus samples and the initial water samples in the dendrogram and the PCoA, whereas the water sample from the control tank was differentiated from the initial sample. This result suggests that the probiotic mitigate the fish skin mucus colonization by the pathogens by controlling the growth of *F. psychrophilum* in the water.

It was recently discovered that pathogens from the genus *Flavobacterium* can increase their survival in the system by colonizing the biofilm of the tank, by this means staying available for infection longer (Sundell and Wiklund 2011; Cai and Arias 2012). As hypothesized in the *in vitro* study, the mechanism of action is probably an exclusion via a competition for the nutrients and/or a synthesis of antimicrobial compounds (Verschuere *et al.* 2000). Furthermore, the proportion of generalized infection (i.e. infection in the gut by *F. psychrophilum*) tends to be lower with addition of probiotic (60% of infection in the control samples versus 30.8% with the treatment). Such antagonistic action would in turn favor the resilience of the skin mucus microbiota. However, it remains unclear why we observed that the beneficial effect of this probiotic strain had different impacts on fish mortality depending on the family (Boutin *et al.* 2012).

Some families are more or less sensitive to the treatment and consequently, this result does not fit with the direct effect on surrounding water. The most likely hypothesis is that the differences between those families are inherent to the genetics of the host, the inter-individual specific microbiota and the physiological response to a low amount of pathogens. It is already known that *S. fontinalis* families differ in their response to a given stress (Crespel *et al.* 2011). Also, the infection dynamics of *F. psychrophilum* are not well known (Madetoja *et al.* 2000; Bernardet and Bowman 2006). Apparently, the pathogen needs stressful condition to become infectious and injuries may also facilitate infections (Madetoja *et al.* 2000; Starliper 2011). Finally, a recent study revealed that genetic background is an important factor in the susceptibility to *F. psychrophilum* and particularly differences in the host transcriptome response (Langevin *et al.* 2012).

In conclusion, the probiotic strain *Rhodococcus sp.* used in this experiment did not disturb the natural microflora of fish skin. Our results indicate that the beneficial effect on fish survival

(diminution of 47% death) was most likely associated to an improvement of the surrounding water quality by a control of the pathogenic abundance of *F. psychrophilum* to a non infectious level in water and by avoiding biofilm formation by the pathogen. These results indicate that the probiotic strain used is harmless for the fish and is a promising candidate for aquaculture. This first analysis of the influence of this probiotic strain on natural microflora of Brook charr associated with the previous study (Boutin *et al.* 2012) showing differential effect on survival clearly shows that probiotics interact with the pathogen but also with the host and his microbiota because each family responded differently to the treatment. However, it remains unclear how this beneficial effect operates and how the host can respond differently if there is no colonization. This phenomenon is probably imputable to a genetic basis of the host-microbiome interaction. To provide a better understanding of this question, we need to understand the biology of this strain by analyzing further its antimicrobial functions against *F. psychrophilum in vitro* and understand the genetic basis of resistance to *this bacterium*.

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Table 1 Detection of *Flavobacterium psychrophilum* and *Rhodococcus* probiotic strain on samples from skin and gut of Brook charr, water, biofilms and biofilters. Presence of the bacteria was assessed by PCR methods and represented as percentage of positive samples.

Samples	Condition	Family	<i>F. psychrophilum</i>	<i>F. columnare</i>	<i>Rhodococcus sp.</i> (probiotic strain)
Dead fishes skin (n=10)	treatment	S9	80 %	0 %	0 %
Dead fishes skin (n=3)	treatment	S5	100 %	0 %	0 %
Dead fishes skin (n=8)	placebo	S9	87.50 %	0 %	0 %
Dead fishes skin (n=2)	placebo	S5	100 %	0 %	0 %
Dead fishes gut (n=10)	treatment	S9	20 %	0 %	0 %
Dead fishes gut (n=3)	treatment	S5	66.70 %	0 %	0 %
Dead fishes gut (n=8)	placebo	S9	62.50 %	0 %	0 %
Dead fishes gut (n=2)	placebo	S5	50 %	0 %	0 %
fish skin mucus (n=60)	treatment	S9	66.70 %	0 %	0 %
fish skin mucus (n=60)	treatment	S5	66.70 %	0 %	0 %
fish skin mucus (n=60)	placebo	S9	66.70 %	0 %	0 %
fish skin mucus (n=60)	placebo	S5	66.70 %	0 %	0 %
water (n=3)	treatment		66.70 %	0 %	0 %
water (n=3)	placebo		100 %	0 %	0 %
Biofilter (n=3)	treatment		0 %	0 %	0 %
Biofilter (n=3)	placebo		0 %	0 %	0 %
Biofilm (n=3)	treatment		0 %	0 %	33 %
Biofilm (n=3)	placebo		0 %	0 %	0 %

Figures legends

Figure 1. Bacterial composition and structure at the genus level of fish skin mucus, water samples and probiotic solution (Prob).

Figure 2. PCoA analysis of bacterial analysis of fish skin mucus, water samples and probiotic solution.

Figure 3. Dendrogram analysis based on Theta YC index of fish skin mucus, water samples and probiotic solution.





