



# Walleye Autochthonous Bacteria as Promising Probiotic Candidates against *Flavobacterium columnare*

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Walleye (*Sander vitreus*) is the second most fished freshwater species in Canada. While much sought by anglers, walleye also supports substantial commercial fisheries. To cope with the recent decline of wild walleye populations, fish farmers produce juveniles for lake stocking. However, walleye breeding is particularly tedious, mostly due to high disease susceptibility at larval and juvenile developmental stages. The main threat is the columnaris disease, which is caused by *Flavobacterium columnare*, an opportunistic bacteria. As *F. columnare* strains exhibit increasing antibiotic resistance, there is a strong need to develop efficient and sustainable alternative strategies to control columnaris disease. Bacterial probiotics have been shown to mitigate infections either by enhancing host immune response or by inhibiting pathogen growth. Being successfully assessed in many fish/pathogen combinations, we developed a tailored probiotic strategy for walleye to prevent and treat columnaris disease. Thirty-seven endogenous bacterial strains were isolated from healthy walleye's skin and gut, were tested *in vitro* against *F. columnare*. Significant antagonistic effect against *F. columnare* was measured for 2 out of 37 endogenous strains. These two probiotic strains were identified as *Pseudomonas fluorescens*. The antagonistic effect of these two successful probiotics was further validated *in vivo* during a 2-month stress trial: groups receiving probiotic treatments showed on average 53.74% survival improvement.

## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Aquatic Microbiology,  
a section of the journal  
Frontiers in Microbiology

**Received:** 24 April 2017

**Accepted:** 03 July 2017

**Published:** 18 July 2017

### Citation:

Seghouani H, Garcia-Rangel C-E, Füller J, Gauthier J and Derome N (2017) Walleye Autochthonous Bacteria as Promising Probiotic Candidates against *Flavobacterium columnare*. *Front. Microbiol.* 8:1349. doi: 10.3389/fmicb.2017.01349

**Keywords:** probiotics, *Sander vitreus*, *Flavobacterium columnare*, walleye diseases, autochthonous bacteria

## INTRODUCTION

Walleye (*Sander vitreus*) is a fecund piscivorous species usually found in moderately productive lakes. Native from North America, its geographical distribution ranges from the east of United States, to the north of St. Lawrence River in eastern Quebec, Canada (Colby et al., 1979; Wilson and Nagler, 2006). Largely known as an effective predator, walleye is one of the most economically important sport and commercial species in Canada (Bernatchez and Giroux, 1991; DFO, 2007, 2012). However, in the last decades, wild walleye populations encountered significant declines, due to overfishing (Sullivan, 2003; Hunt et al., 2011). To cope with the rarefaction of this species, fish farmers started producing juveniles for lake stocking. Noteworthy, walleye breeding is particularly tedious, because optimal rearing conditions are still very challenging, mostly in terms of nutrition (Huntingford, 2004) but also because of high disease susceptibility at early developmental stages (Suomalainen et al., 2005). The most prevalent threat is the columnaris disease, which is mainly caused by *Flavobacterium columnare*, that naturally inhabits both fish microbiota and in environmental microbial communities.

*Flavobacterium columnare* is described as one of the most important bacterial diseases of freshwater fish species (Arias et al., 2004), affecting wild and cultured fish [e.g., Arctic charr, *Salvelinus alpinus* (L.), Perch, *Perca* sp. (L.), Atlantic salmon, *Salmo salar* (L.); Austin and Austin, 2007]. For instance, highly virulent strain of *F. columnare* was able to trigger death within 24 h in coho salmon fry, *Oncorhynchus kisutch* (Walbaum) (Rucker et al., 1953; see in Austin and Austin, 2007). Several studies have indicated the potential for *F. columnare* to survive for extended periods of time in water (Kunttu et al., 2009, 2012). Under laboratory conditions, *F. columnare* maintains its infectious property for more than 5 months (Kunttu et al., 2012). Welker et al. (2005) confirmed that the disease can be transmitted horizontally and indirectly through the water column without essentially being in contact fish-to-fish. When surviving outside the host, *F. columnare* inhibits virulence gene expression in order to save energy before colonizing another fish host with compromised immune system (Kunttu et al., 2009). Indeed, the occurrence of this opportunistic disease is directly related to stress, elevated temperatures, crowding, etc. (Suomalainen et al., 2005). Columnaris disease symptoms occur internally or externally (gill or skin lesions), and appear as dark-gray or yellow lesions or ulcers (Hartman, 2009). As aquaculture intensifies, overcrowding, low water quality and intensive handling increase physiological stress and physical injury, which in turn favors opportunistic pathogens (Derome et al., 2016a,b). Under such a condition, walleye larvae and juveniles become highly susceptible to columnaris disease, which causes substantial economic loss to fish farmers.

In recent decades, prevention and control of diseases in cultured animals focused research and budgets on antibiotics and chemotherapeutic agents, which are still extensively employed. To date, there are multiple evidences that intensive use antibiotics inevitably leads not only to the emergence of drug-resistant pathogens and other microorganisms, but also to the release of active molecules in the environment, both of which represent a significant risk for public health (Miranda and Zemelman, 2001; Radu et al., 2003; World Health Organization [WHO], 2014). Therefore, there is an urgent need to develop efficient and sustainable methods to control and prevent opportunistic disease such as columnarise, to meet the increasing demand for environment friendly aquaculture. Overall, such alternative methods are expected to warrant a microbiologically healthy environment to enhance fish production and economic profits (Díaz-Rosales et al., 2009). The use of probiotics to increase disease resistance and improving the overall health of terrestrial animals, has long been established as efficient, innocuous, and sustainable (Parker, 1974; Sissons, 1989; Rolfe, 2000; Scharek et al., 2007; Boutin et al., 2013; Foureaux et al., 2014; Hai, 2015). The competition between probiotic bacteria and pathogens was reported in many fish and other aquatic species (Balcázar et al., 2000, 2004, 2007). Probiotic development usually bears on two strategies: allochthonous and autochthonous. The allochthonous strategy aims to test probiotic properties of candidates that were isolated from another host organism, whereas the autochthonous strategy targets the host microbiota

to isolate promising probiotic candidates (PC), in order to ensure both efficiency against the pathogen and innocuity for the host. PC isolated from host associated microbial community (i.e., microbiota) have been shown to be efficient in fish and other vertebrates such as Solea, *Solea senegalensis* (Kaup) (García de La Banda et al., 2010), Brook trout, *Salvelinus fontinalis* (Mitchill) (Boutin et al., 2012, 2013), Zebrafish, *Danio rerio* (Hamilton) (Rane and Markad, 2015), and Pigs (Hou et al., 2015). Indeed, the host microbiota, which is composed with numerous microbial strains that closely interact with each other, is a dynamic system that evolves through fish development (reviewed in Llewellyn et al., 2014; Zac Stephens et al., 2015). It is now widely acknowledged that resident bacteria contribute to host disease resistance *via* two kind of mechanisms: (1) specifically targeting pathogens either by nutritional competition, synthesis of antimicrobial compounds, or competitive exclusion from epithelial surfaces (Bermudez-Brito et al., 2012; Kamada et al., 2013); (2) mechanisms targeting the host immune signaling pathways control (Kamada et al., 2013). Regarding resistance against columnaris and other skin diseases in fish, skin mucus is playing a major role as a physical and chemical barrier (Rottmann et al., 1992). More specifically, skin microbiota associated strains have been reported to protect their host against pathogens by competitive action for adhesion sites (Vine et al., 2004; Chabrilón et al., 2005; Boutin et al., 2013; Ige, 2013), production of organic acids and other antimicrobial compounds such as bacteriocins and siderophores (Yan et al., 2002). Therefore, autochthonous skin bacteria are relevant targets to develop efficient probiotic strains against opportunistic skin disease such as columnaris. Also, as gut microbiota is a reservoir of numerous bacterial symbionts that were proved to be efficient against opportunistic pathogens (reviewed in Gomez et al., 2013), and more specifically against *Flavobacterium* (Burbank et al., 2012; Ghosh et al., 2016), those bacterial strains were also considered as PC in this work.

The goal of the present study was to develop an autochthonous probiotic strategy against columnaris disease in walleye. To do so, 37 bacterial candidates were isolated from healthy adult walleye skin and gut microbiomes in order to screen *in vitro* their antagonistic properties *vis-à-vis* *F. columnare*. The two candidates that demonstrated highest efficiency against *F. columnare* were further validated *in vivo* to assess both their innocuity and ability to decrease mortality rates in walleye during a stress trial.

## MATERIALS AND METHODS

### *In Vitro* Experiments

#### Walleye Bacteria Sampling

Autochthonous bacteria were isolated from both skin and gut of healthy walleye (*S. vitreus*) from the Station Aquicole des Trois-Lacs (Asbestos, QC, Canada). Skin mucus samples were recovered by scraping the skin surface between the opercula and the caudal fin with a sterile razor blade. Gut mucus samples were recovered by scraping the intestine epithelial layer with a

sterile Q-tip. Then, skin and gut mucus samples were diluted and homogenized 1:9 with sterile phosphate-buffered saline (1×, pH 7.4). Both pathogen and potential PC were grown on the same general growth media, i.e., Anacker and Ordal known as AO (Anacker and Ordal, 1959). The mucus dilutions were spread on fresh growth media (AO) by single-step streaking with a sterile inoculating loop. Agar plates were incubated at 20°C for 48–72 h. Individual colonies were sampled with an inoculating loop and streaked in three steps on the corresponding fresh growth media, and incubated as described above, and then stored at 4°C as solid pre-cultures. Bacterial stock cultures were prepared from pure solid culture by resuspending bacteria in excess in liquid growth medium supplemented with 15% w/v glycerol, and by storing immediately at –80°C.

### Screening of Antagonistic Bacteria with Agar Diffusion Assays

The 37 autochthonous PC were screened on the basis of antagonism against *F. columnare* strain by diffusion of antimicrobial compounds through agar using same growth media (AO). In aseptic conditions, liquid cultures of *F. columnare* were prepared by resuspending bacteria from a solid culture in liquid AO medium up to an optical density at 600 nm (OD<sub>600</sub>) of 0.67. Liquid PC cultures were also prepared in a similar manner. Bacterial lawns of PC and *F. columnare* were prepared by streaking the whole surface of fresh agar media with a sterile cotton swab dipped in liquid bacterial strain culture. Before incubation, sections with a radius of 03 mm was excised from PC solid cultures and laid equidistantly upside down on *F. columnare* solid cultures. Wells were then incubated at 17°C. Inhibition surfaces around the wells were measured by scanning individually each day over a 9-day time course at a resolution of 23.6 pixels per mm, then the inhibition surfaces around the PC sections were measured using the image processing software ImageJ (NCBI, NIH)<sup>1</sup>. The radiuses were measured in the same manner using ImageJ. To obtain the final inhibition surface, the PC section area was subtracted from the inhibition area (Dheilly, 2014). Then, the PC showing inhibition radius were selected for *in vivo* experiment. All manipulations were executed in triplicates (Sharon et al., 2011).

### Bacterial Strain Identification

The best two PC were identified to the genus level by sequencing the 16S rRNA gene. After DNA isolation using the Dneasy Blood and Tissue Kit (Qiagen), polymerase chain reaction (PCR) amplification of the 16S rRNA gene was undertaken using the universal set of bacterial primers 331F (5'-TCCTACGGGAGGCAGCAGT-3') (Nadkarni et al., 2002) and 1389R (5'-AGGCCCGGAACGTATTCAC-3') (Woo et al., 2001). PCRs were conducted in volume of 50 µL using a Biometra T1 Thermocycler, using a following amplification conditions: initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C 1 min, 72°C for 1 min 30 s and a final extension step at 72°C for 10 min. Gel electrophoresis [2% (w/v) agarose, 100 V] was used to visualize

the PCR products. Fragments were sequenced using the Big Dye Terminator V3 chemistry on an ABI 3130XL sequencer (Applied Biosystems, Foster City, United States) at the Plateforme d'Analyses Génomiques (IBIS, Université Laval, Quebec, Canada).

### In Vivo Experiment

Walleye juveniles (2 cm, ~1 g) were obtained from the Station Aquicole des Trois-Lacs (Asbestos, QC, Canada). Upon arrival, fish were acclimated in 1 m<sup>3</sup> indoor tanks for 2 months. All fish were held under natural photoperiod conditions, constant temperature of 21°C, and fed daily with commercial fish food (Corey Aquafeeds). After acclimation, a total number of 324 fishes were distributed randomly between six independent recirculating 50 L tanks: each experimental group (PC1, PC2, control) was duplicated. Each tank was independent in terms of filtration and water recirculation using external filter (550 L/h).

It has been clearly demonstrated that in fish farms, *F. columnare* originates from environmental water, farm environment; then, handling practices are the principal cause triggering disease outbreaks (Pulkkinen et al., 2010; Kunttu et al., 2012). As physiological stress was identified as the most efficient disease triggering factor in intensive aquaculture (Iwama, 2011), our stress protocol aimed to mimic recurrent transfers occurring in farm conditions.

The intensity of thermal stress and mechanical stress were less extreme from previous studies (Nakano et al., 2014; Blanco Garcia et al., 2016). A combination of mechanical and thermal stresses was applied as follow: fish were captured and released into a 20-L bucket where temperature was 6°C below tank's temperature. After a 10-min exposure to low temperature, fish were put back into their respective tanks. This stress protocol was repeated after each sampling.

Two PC isolated from walleye skin mucus and selected for their *in vitro* antagonistic activity were selected for this *in vivo* experiment. Probiotic formulations were administered twice a day (8 am and 8 pm), the mean count of isolate at each administration was 6.5 × 10<sup>8</sup> colony forming unit (CFU). Moribund fish were collected daily and euthanized by overdose of MS-222 (250 mg/L). Then, dead fish were stored at –80°C for future analysis. All experiments were conducted at the Laboratoire de Recherche en Sciences Aquatiques (LARSA – Université Laval) and carried out in accordance with the LARSA guidelines approved by the “Comité de Protection des Animaux” (CPA).

### Detection of *F. columnare* in Fish Samples by Polymerase Chain Reaction

The detection of *F. columnare* was performed using the experimental procedure of Michel et al. (2002) with some modifications. Five muscle samples were taken directly from lesion and from different fish. Samples were then placed into microtubes containing 400 µL of distilled water. Using an electric homogenizer (Heidolph DIAX 100, Schwabach, Germany), the slurry was crushed and homogenized then treated in 40 µL of 40 mM Tris–ethylenediaminetetraacetic acid (EDTA) and 10 µL

<sup>1</sup><http://imagej.nih.gov/ij/>

of 1% proteinase K. The mixture was incubated 20 min at 60°C and then 5 min at 100°C. After a 15 s centrifugation at 13,000 g, the supernatant was then stored at 4°C for PCR analysis.

As a negative control, two samples were used: healthy fish muscle and a pure culture of *F. psychrophilum* mixed with a healthy fish muscle tissue. As positive control, a pure culture of *F. columnare* mixed with a healthy fish muscle tissue. A DNeasy Blood and Tissue Kit (Qiagen) was used for DNA extraction on series of dilutions.

## Polymerase Chain Reaction

Using a species-specific primer for *F. columnare* Col-72F (5'-GAAGGAGCTTGTTCCCTTT-3') and Col-1260R (5'-GCCTACTTGCCTAGTG-3') as describe by Triyanto et al. (1999). A PCR reaction was performed in final volume of 50 µL, using 1 µL of Q5® High-Fidelity DNA Polymerase (M0491), 10 µL Q5 Reaction Buffer, 10 µL Q5 High GC Enhancer, 1 µL of dNTPs 10 mM, 2.5 µL of primers 10 mM, and 3 µL of template DNA samples. PCR conditions were applied as follows: samples denaturation 30 s at 98°C, then processed through 35 cycles consisting of 30 s at 98°C, 30 s at 58°C, 30 s at 72°C and 2 min at 72°C for final extension. The final products were visualized in UV light after electrophoresis in 2% agarose gel.

Furthermore, the resulting PCR products were sequenced using an Applied Biosystems ABI 3130XL DNA analyzer at the Plate-forme d'Analyses Génomiques (IBIS, Université Laval, Quebec, Canada).

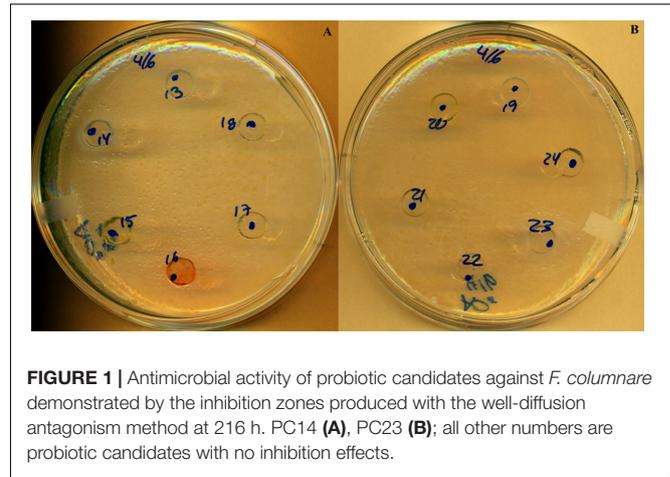
## Statistical Analysis

Survival times were calculated as the time of experiment started, until death. Deaths and mortality were reported daily, and stratified by tanks and treatment. The mortality proportions between treatments during 60 days were compared by chi-square tests. We used Kaplan–Meier methods, log rank test to describe survival curves and Cox's proportional hazards multivariate regressions were used to calculate the hazard ratios for the effect of treatment on mortality. All statistical analyses were performed using the software Rstudio, version 0.98.1102.

## RESULTS

### *In Vitro* Screening against *F. columnare*

Among the 37 bacterial strains issued from skin mucus and the 12 bacterial strains issued from gut epithelial layer that were initially tested with agar diffusion assays against *F. columnare*, PC14 and PC23 exhibited a growth circle with an inhibition zone of respectively 5 and 3 mm diameter (Figure 1 and Table 1). The plates were monitored over a 9-day period and scanned at different time (48, 120, and 216 h). The 16S rDNA gene sequence analysis showed that these two successful PC were closely related to *Pseudomonas fluorescens*, a Gram-negative bacteria, belonging to the Gammaproteobacteria subclass and shared 99% identity between each other. The closest hit in GenBank for CP14 and CP23 was *P. fluorescens* with 99.25% of average nucleotide identity (A506 complete genome accession number: NC\_017911.1).



**FIGURE 1** | Antimicrobial activity of probiotic candidates against *F. columnare* demonstrated by the inhibition zones produced with the well-diffusion antagonism method at 216 h. PC14 (A), PC23 (B); all other numbers are probiotic candidates with no inhibition effects.

### Antagonistic Effects against *F. columnare* in the *In Vivo* Experiment

Fish mortalities occurred within 24 h following the stress trial. Mortality events increased further until the end of the experiment for the control group. Columnaris disease symptoms were clearly identified on 40% of moribund and dead individuals.

At the end of experiment, mortality rate was defined for each PC. PC14 and PC23 exhibited a mean mortality rate of 6.42 and 10.07%, respectively (Figures 2, 3), which was significantly lower ( $p < 0.01$ ) for PC14 than what was observed in the control group 13.88%. There were no significant differences among duplicates for both treatment and control groups ( $p = 0.62$ ,  $p = 0.81$ , and  $p = 0.72$ , respectively; Figure 4). Thus, the administration of PC14 reduced consistently and significantly the mortality across duplicates.

### PCR Analysis

The resulting PCR from the use of the specific primers Col-72F and Col-1260R was effective for all samples (Table 2). A band of 1200 bp was clearly identified for all samples with columnaris symptoms, whereas absent in all samples without columnaris symptoms. The sequencing of PCR products confirmed the presence of *F. columnare* in fish samples with columnaris disease symptoms (Table 2).

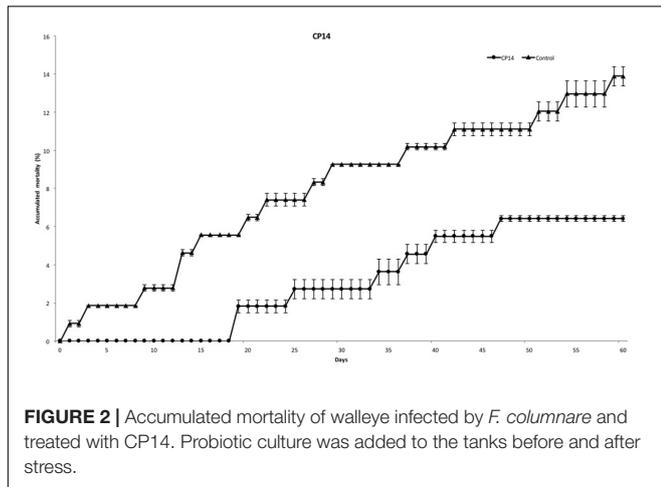
## DISCUSSION

Fish recruit bacterial strains to build up their microbiota directly from the environmental water microbial community, however, microbiota assemblages are very specific to the corresponding body surface, and highly differentiated from environmental bacterial communities (Apun et al., 1999; Diler et al., 2000; Austin, 2006; Llewellyn et al., 2014, 2015; Zac Stephens et al., 2015; Sylvain et al., 2016). Many studies showed that during their long co-evolution, microbial communities and their hosts have established mutualistic interactions for many physiological aspects, providing major beneficial molecules and services to their host such as enzyme synthesis, vitamins, metabolites,

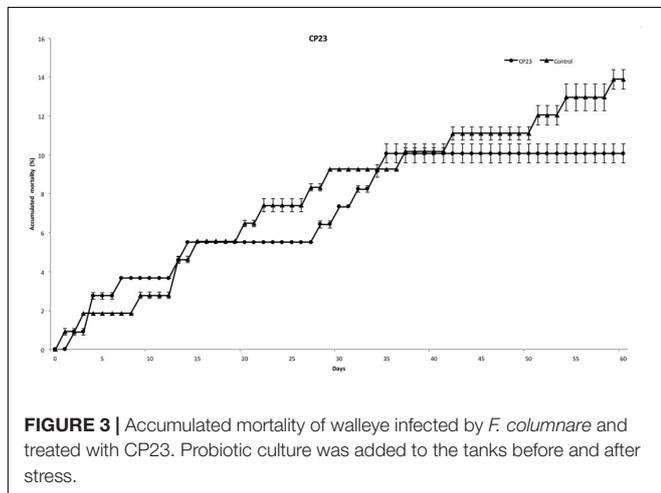
**TABLE 1** | Autochthonous bacteria from walleye exhibiting diffusible inhibitory effect on agar at 216 h against *F. columnare*.

Isolates	Media	Closest hit in GenBank	Percentage similarity	Sampling site	Inhibitory effect
CP14	AO	<i>P. fluorescens</i>	99.45%	Skin mucus	+++
CP23	AO	<i>P. fluorescens</i>	99.25%	Skin mucus	++

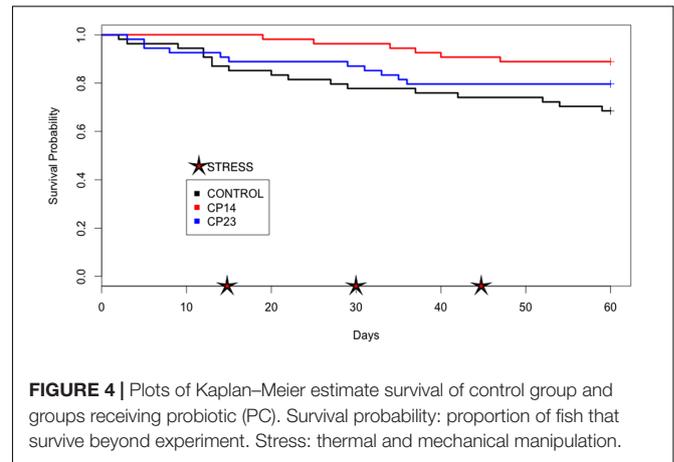
Inhibition diameter around culture wells carved in bacterial laws of *F. columnare* at  $t = 216$  h; +++, 3–5 mm wide; ++ more than 5 mm wide.



**FIGURE 2** | Accumulated mortality of walleye infected by *F. columnare* and treated with CP14. Probiotic culture was added to the tanks before and after stress.



**FIGURE 3** | Accumulated mortality of walleye infected by *F. columnare* and treated with CP23. Probiotic culture was added to the tanks before and after stress.



**FIGURE 4** | Plots of Kaplan–Meier estimate survival of control group and groups receiving probiotic (PC). Survival probability: proportion of fish that survive beyond experiment. Stress: thermal and mechanical manipulation.

**TABLE 2** | PCR detection of *F. columnare* in samples.

Sample	Description	PCR detection	Percentage similarity	NCBI reference sequence
Water	Control	+	99.81	NZ_CP018912.1
Water	CP14 tank	+	99.95	NZ_CP018912.1
Water	CP23 tank	+	100.00	NC_016510.2
Muscle	Healthy fish	–	NA	NA
Muscle	Infected fish	+	100.00	NC_016510.2
Muscle	Healthy fish + <i>F. columnare</i>	+	100.00	NC_016510.2
Bacteria	<i>F. columnare</i>	+	100.00	NC_016510.2

+, *F. columnare* detected; –, *F. columnare* not detected; percentage of similarity of the closest hit of *F. columnare* in GenBank.

antimicrobial compounds, immune system development, and maturation (Rawls et al., 2004; Salinas et al., 2006; Nayak, 2010; Tremaroli and Bäckhed, 2012).

The present study aimed to take advantage of beneficial host microbiota properties to develop an autochthonous probiotic strategy against columnaris disease in walleye. Among the 49 isolated strains screened *in vitro* for their potential antagonistic properties vis-à-vis *F. columnare*, two PC produced clear inhibition circles on *F. columnare* lawns (PC14 and PC23). Such result suggests their antagonistic effect against *F. columnare* is likely due, at least in part, to a diffusible antimicrobial compound. These two successful PC were further validated *in vivo* to test both their innocuity vis-à-vis *S. vitreus* and their ability to

decrease mortality in a stress trial. According to our results, the antagonistic properties of these two PC strains measured *in vitro* were potentially maintained *in vivo* by improving significantly fish survival (+53%) under a context of stress trial which, according to both PCR analysis and sequence identification, triggered columnaris disease. However, it is not clear if the same mechanisms of action were involved in both *in vitro* and *in vivo* contexts. Overall, the successful administration of these two probiotic strains to walleye is coherent with previous studies that observed both *in vitro* and *in vivo* beneficial effects (Gram et al., 2001; Boutin et al., 2012). Gram and Ringø (2005) proposed that an effective probiotic should be identified by its capability to reduce the incidence of disease with a decrease of mortality. More recently, Boutin et al. (2012) confirmed that a positive effect of probiotic is represented by significant decrease of mortality. Still, it is premature to state whether the antibacterial

properties observed *in vitro* are the sole mechanism that favored fish survival.

The two successful PC were closely related to *P. fluorescens*, belonging to the Gammaproteobacteria subclass. Interestingly, antagonistic properties against pathogenic bacteria and fungi were frequently documented in other aquatic *Pseudomonas* species and have been suggested to present a high interest as autochthonous PC for aquaculture (Sugita et al., 1996; Gram et al., 2001; Nayak, 2010). Furthermore, some authors concluded that the recurrent presence of *Pseudomonas* on fish skin represents potentially a promising probiotic strains for fish (Bly et al., 1997; Gram et al., 1999). For instance, *Pseudomonas aeruginosa* and *P. aeruginosa* YC58 improved the survival of two varieties of oysters (*Pinctada mazatlanica* and *Crassostrea corteziensis*; Aguilar-Macías et al., 2010; Campa-Cordova et al., 2011). Other *Pseudomonas* were successfully tested against different pathogenic organisms *in vitro* such as *Aeromonas hydrophila* (Eissa and El-Ghiet, 2011; Samal et al., 2014) and *Vibrio midae* (Silva-Aciaries et al., 2010). Two studies showed the beneficial effect of *P. fluorescens* as a promising PC to control pathogens in two distantly related fish species: rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Gram et al., 1999) and Nile tilapia, *Oreochromis niloticus* (L.) (Eissa et al., 2014). In our study, annotation of the two PC 16S rDNA partial sequence (<1000 nucleotides) indicated that both of them shared 99% of identity with *P. fluorescens* A506. This strain is registered as BlightBan® A506 and has been commercialized as a microbial pest control agent against a *Erwinia amylovora*, a pathogen that affect apples and pear trees (Health Canada Pest Management Regulatory Agency, 2011).

The *in vivo* probiotic effect of PC from our study was efficient in promoting fish survival in a context of *F. columnare* disease, which occurred after fish handling and thermal stress. However, regarding the current data, it is not possible to conclude whether the important mortality decrease observed in this experiment was only due to the antibacterial effect attributed to the *Pseudomonas* strain. Indeed, the *Pseudomonas* genus encompasses numerous strains, those own diverse mechanisms of action: some are producing bioactive agents such as bacteriocins, pyocin, and phenazinen (Tinh et al., 2007), other strains are triggering bacterial cell membrane lysis, or are producing inhibitors of fatty acid synthesis pathway such as acetyl-CoA, and nitrous oxide (Freiberg et al., 2004; Isnansetyo and Kamei, 2009).

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The time scale of a probiotic administration experiment and mode of supplementation are an important criterion affecting the establishment of the probiotic bacteria, their persistence, and even their influence on host immune response. Studies showed that application of probiotic directly to the rearing water play a significant role to health benefits of fish, but also to the rearing environmental (Boyd and Massaut, 1999; Zhou et al., 2010).

The significant improvement of fish survival obtained after 2 months of probiotic administration suggests that autochthonous probiotic strategy is a promising avenue in aquaculture industries. As many studies showed the effectiveness of (allochthonous/autochthonous) probiotics *in vivo* to decrease mortality and even prevent disease in many species: shrimp, *Litopenaeus vannamei* (Boone) (Kongnum and Hongpattarakere, 2012), Brook trout (Boutin et al., 2012), and Nile tilapia (Villamil et al., 2012; Eissa et al., 2014). Our work confirms further how efficiently endogenous probiotic can be developed “*de novo*” to decrease mortality in a context of fish farming industry stressing conditions. Overall, the use of endogenous probiotics in aquaculture provides a straightforward tool to both efficiently and sustainably increase survival rates in aquaculture.

## AUTHOR CONTRIBUTIONS

HS performed *in vivo* experiment, data analysis, and writing manuscript. C-EG-R performed *in vitro* and *in vivo* experiment. JF worked with C-EG-R on *in vitro* experiment. JG brought fish and helped with *in vitro* experiment. ND revised the manuscript and supervised the work.

## FUNDING

This research was funded by the CRSNG ENGAGE Grant # 463106-14 to ND.

## ACKNOWLEDGMENTS

The authors are thankful to Station Piscicole Trois Lacs Inc., the staff of the “Regional Laboratory of Aquatic Sciences” for their help during fish rearing, and the Animal Protection Committees.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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