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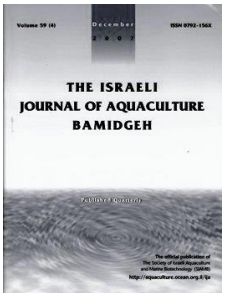

Isolation and Characterization of Potential Probiotic Bacteria from Rainbow Trout *Oncorhynchus mykiss*, (Walbaum) Rearing Units Against Bacterial Pathogens

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Abstract

Bacteria were studied for potential probiotic activity against vibriosis, yersiniosis and lactococcosis in rainbow trout. A total of 79 bacterial strains were isolated from rainbow trout rearing water, and screened for antagonistic activity against *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae* using a well-diffusion agar assay. *Vibrio* spp. showed inhibitory activity against *V. anguilarum* and *L. garvieae*, while *Aeromonas* spp. displayed antagonistic activity against *L. garvieae*. Antagonistic *L. garvieae* strains displayed inhibitory activity against all pathogens. Antagonistic strains were characterized for enzymatic activity (protease, lipase) and hydrophobicity. *Vibrio* sp. A12, and *Aeromonas* sp. A5, G1, were found to have enzymatic properties and hydrophobicity. *L. garvieae* strains showed weak hydrophobicity and did not display enzymatic activity. Phenotypic characteristics of antagonistic strains were determined by conventional API 20NE and API STREP rapid identification systems. Antagonistic *L. garvieae* strains were confirmed as *L. garvieae* by PCR using species-specific primers. Candidate probiotic strains were tested for pathogenicity in rainbow trout by intraperitoneal injection. Following a challenge, *L. garvieae* strains caused mortality and were eliminated from further study. As a result, *Aeromonas* spp. and *Vibrio* spp. were identified as probiotic candidates with the potential to control vibriosis and lactococcosis.

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Introduction

The use of probiotics has been increasingly viewed as an alternative to antibiotic treatment in reducing the incidence of disease in aquaculture (Balcázar et al., 2006). Potential probiotic strains exhibit different modes of action and properties. These include antagonism to pathogens due to a lowering of localized pH, the production of antibacterial substances, competition for nutrients and adhesion sites, their ability to produce metabolites (like vitamins) and enzymes, colonization or adhesion properties, and the enhancement of the immune system (Ganguly et al., 2010; Balcázar et al., 2006; Ali, 2006; Swain et al., 2009; Merrifield et al., 2010; Aguirre-Guzmán et al., 2012).

Probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. The pre-selection of candidate probiants has often led to the discovery of effective probiotic bacteria (Verschuere et al., 2000). The selection for probiotic candidate organisms was based on in vitro antagonism (Vershuere et al., 2000), as well as on the results of adhesion, colonization, and growth in intestinal mucus (Irianto and Austin, 2002; Vine et al., 2004). The ability of some strains to adhere to mucus, gastrointestinal tract, epithelial cells, and other tissues, is generally a required characteristic in probiotic selection because it is associated with bacterial colonization (Verschuere et al., 2000; Farzanfar, 2004; Crittenden et al., 2005). The evaluation of probiotic adhesion may be determined by a hydrophobicity test using Congo Red stain (Sharma et al., 2006; Leyva-Madrigal et al., 2011).

Probiotic microorganisms have the ability to release chemical substances with bactericidal or bacteriostatic effects on pathogenic bacteria. In general, the antibacterial effect is due to one or more of the following factors: production of antibiotics, bacteriocins, siderophores, enzymes (lysozymes, proteases) and/or hydrogen peroxide, as well as alteration of the intestinal pH due to the generation of organic acids (Verschuere et al., 2000).

There has been a growing interest in the administration of probiotics through the use of beneficial microorganisms to prevent infection by pathogenic micro-organisms and reduce the incidence of fish diseases (Irianto and Austin 2002; Brunt & Austin, 2005; Balcázar et al., 2006; Aly et al., 2008). In vitro evaluation prior to in vivo testing is a good indicator of probiotic effectiveness for controlling furunculosis, lactococcosis, streptococcosis and yersiniosis in rainbow trout (Irianto and Austin 2002; Brunt & Austin 2005; Balcazar et al. 2006; Capkin & Altinok 2009).

The aims of this study were to isolate potential probiotic bacteria from exogenous microbiota of rainbow trout, screen all isolates for in vitro antagonistic effects to bacterial pathogens, and determine enzymatic activity and hydrophobicity of the isolates for the control of bacterial diseases caused by *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae*.

Materials and Methods

Isolation and identification of potential probiotic bacteria. Bacteria were isolated from the rearing water of juvenile, adult, and broodstock rainbow trout using the spread plate method. They were kept at 25^{o}C for 2 days on Triptic Soy Agar (TSA, Merck) and Plate Count Agar (PCA, Merck). Representative colonies from these plates were sub-cultured onto fresh medium for purity, and identified by Gram staining, catalase reaction (3 % H_2O_2), motility in TSB, oxidase reaction, ability to metabolize glucose by oxidation and/or fermentation in OF basal medium supplemented with 1.5% glucose and API 20 NE, API STREP rapid identification systems (bioMérieux SA, Marcy l'Etoile, France). For long-term preservation, cultures were frozen at -80^{o}C in trypticase soy broth (TSB, Merck) with 15% (v/v) glycerol.

DNA extraction and PCR amplification. Genomic DNA of isolates was extracted from pure subcultures using a commercial kit (Qiagen, GmbH) according to manufacturer instructions. Molecular identification of isolates was carried out for *L. garvieae* using specific PCR with the oligonucleotide primers of pLG-1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3') (Zlotkin et al., 1998). The PCR assay was performed with a 25-µl volume for each sample containing DEPC-treated water, 1xPCR Buffer, 1.5 mM of $MgCl_2$, 0.2 mM each dNTP, 1.0 U Taq polymerase (Fermentas), 1µM of

each primer and 5 µl template DNA. Thirty five cycles of amplification were performed in a Thermo PxE 0.2 thermal cycler (Thermo Scientific) after initial denaturation of DNA at 95°C for 3 min. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1.5 min, with a final extension at 72°C for 10 min following the last cycle. The expected size of PCR products was 1100 bp. All the PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide and then visualized using an UV transilluminator. *L. garvieae* ATCC 43921 reference strain was used as a positive control.

Inhibitory activity of the isolates. All bacteria isolated from the samples were tested for antagonistic activity by a well diffusion agar assay (WDAA) against bacterial fish pathogens, *V. anguillarum, Y. ruckeri* and *L. garvieae*. Each of the pathogens were grown in 4mL TSB for 1 d at 25°C, and 10 µl of each culture was mixed into 10 mL of melted TSA (43.5–44°C). After solidifying and drying for 15–20 minute, wells were punched (diameter = 3 mm) and 10 µl of a 2 day old potential probiant culture (approx. 10^8–10^9 CFU mL^{-1}) grown in TSB at 25°C was added to wells in triplicate. Plates were incubated at 25°C for one day and observed for clearing zones around the wells. Strains causing clearing zones in the WDAA were tested once more in TSA to ensure that the antagonistic activity was stable after storage and sub-culture (Hjelm et al., 2004).

Hydrophobicity test using Congo red stain (CRS). A hydrophobicity test using Congo red stain (CRS) to determine the hydrophobicity of the bacteria was carried out using plates prepared in TSA with 0.03% Congo red. The test was performed in triplicate. Congo red was added after sterilization of TSA. Using the cross-streak method each isolate was spread on plates, and incubated at 25°C for 24 h. Red colonies were considered positive (hydrophobic) and white or colorless colonies were considered negative (non-hydrophobic) (Sharma et al., 2006).

Extracellular enzymatic activity. Lipase and protease production of potential probiotics were tested using tributyrin agar and skim milk agar in triplicate, respectively. A clear zone surrounding the colonies indicated lipolytic and proteolytic activity.

Pathogenicity of candidate probiotics. Candidate probiotics were grown to log phase in 20 ml of TSB at 25°C and subsequently harvested by centrifugation at 1600 g for 15 min at 15°C. The supernatant was poured off and the pellet was re-suspended with PBS. Subsamples were taken using the drop plate method (Chen et al., 2003). Duplicate groups of 10 fish (30g) were injected intraperitoneally (IP) with 0.1 ml of each candidate probiotic bacteria resulting in doses between $3.075x10^7$ and $2.05x10^8$ CFU/fish (Irianto and Austin,2002). Control groups were injected with 0.1 ml PBS. Re-isolation of the bacteria was performed from the kidney liver and spleen onto TSA, from all mortalities as well as a subset of fish from each tank after a period of 28 days (Burbank et al., 2012).

Results

Bacterial isolation and characterization. Antagonistic activity of 79 bacterial isolates against *V. anguillarum*, *Y.ruckeri* and *L. garvieae* was tested by the well- diffusion agar assay from rearing water of juvenile, adult, and broodstock rainbow trout. Eleven of the isolates showed inhibitory activities against these pathogens. *Vibrio* spp. and *Aeromonas* spp. were isolated from the rearing water of adult rainbow trout, while *L. garvieae* strains were isolated from adult and juvenile rearing water. *Vibrio* spp. showed inhibitory activity against *V. anguilarum* and *L. garvieae.* *Aeromonas* spp. displayed antagonistic activity against *L. garvieae*. Candidate probiotic *L. garvieae* strains displayed inhibitory activity against all pathogens. *Vibrio sp.* A12 and *Aeromonas* sp. A5, G1 were found to have enzymatic activities and hydrophobicity. Enzymatic activities were not determined in *L. garvieae* strains. *L. garvieae* A7 showed strong hydrophobicity. Other *L. garvieae* strains were found weakly hydrophobic (Table 1).

Phenotypic characteristics of antagonistic *Aeromonas* spp., *Vibrio* spp. and *L. garvieae* strains are represented in Tables 2 and 3.

Table-1: Inhibition zones(mm) against pathogens, enzymatic activities and hydrophobicity of candidate probiotic bacteria

	Inhibitory activity (zone diameter) against pathogen seeded in TSA					
Isolates	*Y. ruckeri*	*V. anguillarum*	*L. garvieae*	Protease activity	Lipase activity	Hydrophobicity
Vibrio sp. A3	-	12mm	10mm	-	-	+
Vibrio sp. A8	-	-	15mm	-	-	-
Vibrio sp. A12	-	8mm	-	+++	++	+
Aeromonas sp. A5	-	-	10mm	++	+++	+
Aeromonas sp.G1	-	-	13mm	-	+++	+
Aeromonas sp. A4	-	-	13mm	-	-	+
L. garvieae A7	-	-	10mm	-	-	+
L. garvieae H9	8mm	-	-	-	-	+(weak)
L. garvieae H10	8mm	-	-	-	-	+(weak)
L. garvieae H11	9mm	-	-	-	-	+(weak)
L. garvieae H12	-	10mm	-	-	-	+(weak)

Table-2: Phenotypic characteristics of antagonistic *Aeromonas* spp. and *Vibrio* spp.

	Aeromonas sp. A5	*Aeromonas* sp. G1	*Aeromonas* sp. A4	*Vibrio* sp. A3	*Vibrio* sp. A8	*Vibrio* sp. A12
Gr staining	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
O/F Test	F	F	F	F	F	F
O/129	R	R	R	S	S	S
Nitrate reduction[a]	+	-	-	+	+	+
Indole production[a]	+	-	-	+	+(weak)	-
Glucose acidification[a]	+	-	-	-	+	-
Arginine dihydrolase[a]	+	-	-	-	+	-
Urea hydrolysis[a]	-	-	-	-	-	-
Esculin hydrolysis[a]	+	-	-	-	+	-
Gelatin hydrolysis[a]	+	-	-	+	+	+
p-Nitropyhenyl-ßD Galactopyranoside[a]	+	+	+	-	+	-
Glucose assimilation[a]	+	+	+	+	+	-
Arabinose assimilation[a]	+	-	+	-	+	-
Mannose assimilation[a]	+	+(weak)	+	+	+	-
Mannitol assimilation[a]	+	+(weak)	+	+	+	-
N-Acetyl Glusomine[a]	+	+	+	+	+	+
Maltose Glusomine[a]	+	+	+	+	+	-
Gluconate Glusomine[a]	+	+	+	+	+	-
Caprate Glusomine[a]	+	-	+	-	-	-
Adipate Glusomine[a]	-	-	-	-	-	-
Malate Glusomine[a]	+	+	+	+	+	-
Citrate Glusomine[a]	+	-	-	+	-	-
Phenyl acetate Glusomine[a]	-	-	-	-	-	-

[a]: performed by API 20 NE, R: Resist, S: Sensitive, F: Fermentative

In PCR assay targeting the 16S rRNA gene, PCR products of the expected sized (1100 bp) were observed in all *L. garvieae* isolates obtained from adult and juvenile rainbow trout rearing water and reference strain (Figure1).

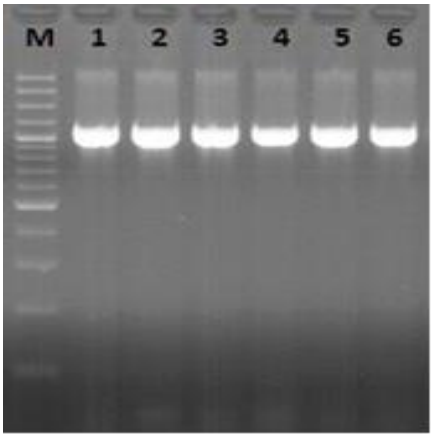

Figure 1**.** *L. garvieae* specific PCR, 1100 bp. M; Marker (100-3000 bp), 1; *L. garvieae* ATCC 43921, 2-6; *L. garvieae* isolates.

Table-3: Phenotypic characteristics of antagonistic *L.garvieae* strains

	A7	*H9*	*H10*	*H11*	*H12*
Gr staining	+	+	+	+	+
Shape	c	c	c	c	c
Motility	-	-	-	-	-
Oxidase	-	-	-	-	-
Catalase	-	-	-	-	-
O/F Test	F	F	F	F	F
VP[b]	+	+	+	+	+
Hippurate hydrolysis[b]	+	+	+	+	+
Aesculin hydrolysis[b]	+	+	+	+	+
Pyrrolidonyl arylamidase[b]	-	-	-	-	-
α-Galactosidase[b]	-	-	-	-	-
β-Glucuronidase[b]	-	-	-	-	-
β-Galactosidase[b]	-	-	-	-	-
Alkaline phosphatase[b]	-	-	-	-	-
Leucine arylamidase[b]	+	+	+	+	+
Arginine dihydrolase[b]	+	+	+	+	+
Acid production from:					
Ribose[b]	-	+	+	+	+
L-Arabinose[b]	-	-	-	-	-
Mannitol[b]	-	-	-	-	-
Sorbitol[b]	-	-	-	-	-
Lactose[b]	-	-	-	-	-
Trehalose[b]	+	+	+	+	+
Inulin[b]	-	-	-	-	-
Raffinose[b]	-	-	-	-	-
Starch[b]	-	-	-	-	-
Glycogen[b]	-	-	-	-	-

[b]:performed by API Strep; c:Coccus; F: Fermentative

Pathogenicity of candidate probiotics

Of the 11 isolates evaluated, *L. garvieae* strains caused immediate mortality when injected into fish (Table 4). No mortality was observed in any fish from the non-injected control group. *L. garvieae* strains were isolated from the kidney, liver and spleen. No overt disease signs or mortalities were observed following injection of *Vibrio* spp. and *Aeromonas* spp. strains. *L. garvieae* strains induced mortalities after the challenge as compared to the control group.

Table 4 Isolate pathogenicity (% mortality) towards rainbow trout following an intraperitoneal injection between $3.075x10^7$ and $2.05x10^8$ CFU/fish

Isolate	Mortality (%)
Vibrio sp. A3	0
Vibrio sp. A8	0
Vibrio sp. A12	0
Aeromonas sp. A5	0
Aeromonas sp. G1	0
Aeromonas sp. A4	0
L. garvieae A7	45
L. garvieae H9	35
L. garvieae. H10	25
*L. garvieae.*H11	35
L. garvieae H12	40
Control(PBS)	0

Discussion

Probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals (Balcázar et al., 2006). In the present study, we obtained a pool of bacterial isolates from rainbow trout rearing water and characterized those with inhibitory activity against *V. anguillarum, Y. ruckeri* and *L. garvieae*. Our study demonstrated that 11 of the screened bacteria (79) were antagonistic towards these pathogens.

Nonpathogenic strains of known pathogenic bacteria like *Vibrio* spp., *A. sobria,* and *A. hydrophila*, showing antibacterial activity against bacterial fish pathogens have also been used as a probiotic bacteria in fish culture and shrimp culture (Irianto and Austin, 2002; Brunt et al., 2007; Mujeeb Rahiman et al., 2010). There is an antagonistic effect of *Aeromonas sobria* GC2 against *Aeromonas salmonicida*, *Lactococcus garvieae*,

Streptococcus iniae, *Vibrio anguillarum*, *Vibrio ordalii* and *Yersinia ruckeri* (Brunt et al., 2007). Similarly in our study, *Aeromonas* spp. strains displayed inhibitory activity against *L. garvieae*. *Aeromonas media* displayed antagonistic activity against *A. caviae, A. hydrophila, A. salmonicida, A. veronii* var. *sobria, V. anguiilarum, Photobacterium damsela,* eight species of *Vibrio,* and *Y. ruckeri* (Gibson et al. 1998). Unlike these, in the present study, *Aeromonas* spp. strains did not show an inhibitory effect on *Y.ruckeri* and *V. anguillarum.* These differences are most likely attributed to different strains of *Aeromonas* species.

In the present study *Vibrio* spp. showed inhibitory activity against *V. anguilarum*. A strain of *Vibrio alginolyticus* was effective in reducing disease caused by *Aeromonas salmonicida* and *Vibrio anguillarum* and *V. ordalii* (Austin et al., 1995). Similarly, five strains of *V. tubiashii* or *V. tubiashii*-like bacteria isolated from Manila clam, *Ruditapes philippinarum,* were able to inhibit the growth of *V. tapetis* (Castro et al., 2002). Furthermore, the antibacterial abilities of intestinal bacteria isolated from juveniles and larvae of Japanese flounder (*Paralichthys olivaceus*) reported that 53.3% of *Vibrio* spp. inhibited the growth of *Pasteurella piscicida* (Sugita and Ito, 2002). There are few studies on the screening of probiotic bacteria against *L. garvieae* (Brunt & Austin, 2005; Balcazar et al., 2007; Perez-Sanchez et al., 2011). The majority of these studies are associated with the screening of lactic acid bacteria. This study demonstrates the inhibitory activity of *Vibrio* spp. against *L. garvieae*.

The production of extracellular enzymes such as proteases and lipases in probiotic bacteria improves the nutrition of the host (Balcázar et al., 2006; Farzanfar, 2006). The inhibitory effect of probiotic bacteria against bacterial pathogens is probably due to one or more of the following factors: production of antibiotics, bacteriocins, siderophores, enzymes (lysozymes, proteases), and/or hydrogen peroxide (Verschuere et al., 2000). In this study, Aeromonas sp. A5, and *Vibrio* sp. A12 strains showed protease and lipase activity, while *Aeromonas* sp. G1 displayed only proteolytic activity. Inhibitory effects of these candidate probiotics against *V. anguillarum* and *L. garvieae* may be due to the production of these enzymes. Extracellular enzymatic activity has been used as a selection criterion for potential probiotics and it has been found that lactic acid bacteria with proteolytic activity have a beneficial effect against white spot syndrome virus (WSSV) in whiteleg shrimp, *Litopenaeus vannamei* (Leyva-Madrigal et al., 2011).

A positive hydrophobicity result indicates that the bacteria have the ability to bind nonspecifically to the epithelium of the intestine by hydrophobic interactions (An, and Friedman, 2000; Rinkinen, 2004). A positive hydrophobicity result indicates that lactic acid bacteria can bind nonspecifically to the epithelium of the shrimp intestine by hydrophobic interactions (Leyva-Madrigal et al., 2011). In the present study *L. garvieae* A7, *Vibrio* spp., and *Aeromonas* spp. strains showed strong hydrophobicity, while non-pathogenic strains of *L. garvieae* H9, H11, H12, and A11, showed weak hydrophobicity. Therefore, it is plausible that strong hydrophobicity features of *Vibrio* spp. and *Aeromonas* spp. strains may still be viable candidates for probiotics.

It is important to note that the use of probiotic bacteria belonging to genera with pathogenic species is common in aquaculture. Some of these genera are *Pseudomonas* (Alavandi et al., 2004; Chythanya et al., 2002; Gram et al., 1999), *Vibrio* (Alavandi et al., 2004; Austin et al., 1995), *Aeromonas* (Gibson et al., 1998), and *Streptococcus* (Lara-Flores et al., 2003). Similarly, of the 11 isolates evaluated, *L. garvieae* strains were observed to cause mortality when injected into fish. This mortality was not surprising after injection of *L. garvieae* strains since mortality caused by lactococcosis has long been established, especially after injection of large quantities of bacteria (Kubilay et al., 2008). Candidate probiotic bacteria, *Vibrio* spp. and *Aeromonas* spp. strains did not cause direct mortality in rainbow trout in the present study. Five *A. sobria* and three *A. cavieae* strains isolated from rainbow trout intestines were not pathogenic (Burbank et al., 2012).

This study has identified *Aeromonas* sp. A5, G1, and *Vibrio* sp. A12 strains, as having antagonistic and enzymatic activity, hydrophobicity, and as being safe for rainbow trout. These findings provide a starting point from which to evaluate the effectiveness of these candidate probiotics. This is a critical step in determining whether such probiotic bacteria are capable of reducing mortality caused by *V. anguillarum* and *L. garvieae* and could lead to an alternative treatment method for vibriosis and lactococcosis. However, these

candidate probiotic strains need further study to explore their probiotic effects (cellular and humoral immune responses) in vivo. The positive results obtained from in vivo studies suggest that probiotic bacterial strains may possibly be used commercially as feed additives to reduce mortality. The use of probiotics in rainbow trout culture could prevent antibiotic, and other chemical drug residue contamination of the environment, and inhibit the establishment of resistant pathogenic bacteria.

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