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The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)

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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH - Kibbutz Ein Hamifratz, Mobile Post 25210, ISRAEL

Phone: + 972 52 3965809

http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_68.2016.1317, 8 pages

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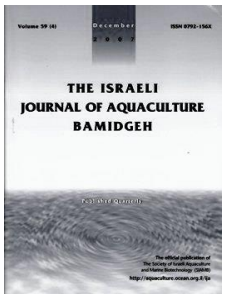

In Vitro Testing of Potential Probiotic Bacteria against *Vagococcus salmoninarum* in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum 1792)

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Keywords: *Oncorhyncus mykiss;* probiotic, *Vagococcus salmoninarum;* aquaculture; *Lactococcus garvieae; Lactococcus lactis*

Abstract

In this study, probiotic properties of endogenous microbiota of rainbow trout against *Vagococcus salmoninarum* isolated during an outbreak of vagococcosis in a trout farm in the Mediterranean region were evaluated. The candidate probiotic bacteria were isolated from rainbow trout intestines. A total of 157 isolates were obtained and screened for antagonistic activity against *V. salmoninarum* via the Well Diffusion Agar method. Six isolates were determined for antagonistic activity against *V. salmoninarum*. Conventional microbiological tests and API 20 Strep tests (bioMe´rieux) were used for further phenotypic characterization of all six antagonistic isolates. For molecular identifications of isolates, *L. garvieae* specific PCR and 16S rRNA gene sequence analysis were used. Antagonistic strains were identified including TUB/2013/V47 (*L. garvieae*), TUB/2013/V27 (L.*garvieae*), TUB/2013/V10 (*L. garvieae*), TUB/2013/V2 (*L. garvieae*) TUB/2013/V1 (*L. lactis*) and TUB/2013/V4 (*L. lactis*). The strains were then tested for hydrophobicity, bile salts and acid tolerance and antimicrobial activity. All isolates were congo red-positive, indicating the presence of hydrophobic structures in their cell walls. It was determined that whole antagonistic strains were resistant to low pH conditions and 0.6-1.5% bile concentrations. Antimicrobial test results showed that most of the strains are susceptible *in vitro* to amoxicillin/clavulanic acid ampicillin, doxycycline, erythromycin and florfenicol, which are frequently used in aquaculture. As a result, it was found that strains have *in vitro* probiotic properties (hydrophobic, tolerant to bile salts and low pH conditions). Further study is needed to explore their *in vivo* probiotic effects against vagococcosis.

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Introduction

Streptococcosis has been defined as a complex of diseases caused by agents belonging to different genera and species of gram-positive cocci. Streptococcal infections can be divided into two major groups: warm water infections (caused by *Lactococcus garvieae*, *Streptecoccus iniae*, *S. agalactiae* and *S. parauberis* pathogenic for both cultured freshwater and marine fish at water temperatures above 15 °C); and coldwater infections (caused by streptococcal species such as *Vagococcus salmoninarum* pathogenic for salmonids at water temperatures below 12°C) (Eldar & Ghittino, 1999; Ghittino et al., 2003).

Vagococcosis caused by *V. salmoninarum* is an important bacterial disease in the European rainbow trout industry, with mortality rates of 20-80% in sub adult or adult fish. Outbreaks occur at water temperatures of 10-12^{o}C and are associated with post-spawning stress in broodstock (Michel et al., 1997; Ruiz-Zarzuela et al., 2005). *V. salmoninarum* has been reported in rainbow trout in Australia (Schmidtke and Carson, 1994), France (Michel et al.,1997), Italy (Ghittino et al. 2004), Spain (Ruiz-Zarzuela et al., 2005), and Turkey (Didinen et al., 2011; Tanrikulu et al., 2014; Ozcan et al., 2014).

Antibiotic treatment of vagococcosis in the field has not been effective, although *V. salmoninarum* isolates are susceptible to several drugs such as ampicillin, amoxycillin, erythromycin, oxytetracycline and doxycycline tested *in vitro* (Michel et al,. 1997; Ruiz-Zarzuela et al., 2005; Didinen et al., 2011). Treatments with antibiotics were effective only for short periods (5-7 days) and continuous delivery was necessary to reduce mortality, followed by a probable enhancement of antibiotic resistance (Ruiz-Zarzuela et al., 2005). Vaccination has also not been successful (Michel et al., 1997; Ruiz-Zarzuela et al., 2005). It is necessary to determine and develop effective protection against *V. salmoninarum*.

Probiotic lactic acid bacteria (such as *Lactococcus* spp., *Pediococcus* spp. or *Lactobacillu*s spp.) have been proposed as biological control agents in aquaculture (Merrifield et al., 2010). These bacteria have been used as probiotics in aquaculture due to their low or no virulence properties, outcompeting harmful bacteria by producing antimicrobial substances, adherence capacity to colonize the digestive tract and competing with pathogens for adhesion sites (Farzanfar, 2006). Reports about screening and use of lactic acid bacteria against lactococcosis in rainbow trout have been documented (Balcázar et al. 2007;2008; Vendrell et al., 2008; Pérez-Sánchez et al., 2011a; Pérez-Sánchez et al., 2011b; Didinen et al., 2016). There is only one study about screening of LAB against *V. salmoninarum in vitro* conditions (Balcázar et al. 2007).

The aim of this study was to isolate candidate probiotic bacteria from the intestines of rainbow trout and determine *in vitro* probiotic properties, their inhibitory activity against *V. salmoninaru*, pH, and bile tolerance that enables them to survive in the gastrointestinal tract of the host, hydrophobicity that enables intestinal colonization, and antibiotic resistance.

Materials and Methods

Isolation and initial screening of potential probiotic bacteria. Bacteria were isolated from the hindgut of 20 healthy rainbow trout (250 g) using the spread plate method. They were kept at 25^{o}C for 2 days on Triptic Soy Agar (TSA, Merck). Each isolate was stored at -80°C in Triptic Soy Broth with 15% (v/v) glycerol. *V. salmoninarum* was previously isolated during a natural vagococcosis outbreak in rainbow trout (Didinen et al. 2011). All potential probiotic bacteria were tested for antagonistic activity against *V. salmoninarum* by a well diffusion agar assay (WDAA). The pathogen was grown in 4mL TSB for 1 d at 25°C, and 10 µl of each culture was mixed into 10 mL of melted TSA. After solidifying and drying for 15-20 minutes, wells were punched (diameter = 3 mm) and 10 µl of a 2 day old potential probiotic bacteria culture (approx. 10^8-10^9 CFU/mL) grown in TSB at 25°C was added to the wells in triplicate. Plates were incubated at 25°C for one day and clearing zones were measured 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. A hydrophobicity test using Congo red stain to determine the hydrophobicity of the bacteria was carried out using plates prepared in TSA with 0.03%

Congo red. 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).

Bile and pH tolerance. Candidate probiotic bacteria was prepared in PBS suspensions as 10^7 CFU/ml. Bile samples were collected from fish and stored at -20°C until used. The bacterial suspension was inoculated in sterile PBS (control), or in sterile PBS containing 0.6-1.5% bile. The samples were incubated for 1.5 hours at 25°C. Following incubation, samples were serially diluted in sterile PBS and counts of viable bacteria were determined by plate count using TSA (Balcázar et al., 2008; Pérez-Sánchez et al., 2011a). Tolerance at different pH conditions was determined using a suspension (10^7 CFU/ml) in PBS adjusted with a pH 2-7 by addition of HCl for 1.5 h at 25°C (Balcázar et al., 2008; Pérez-Sánchez et al., 2011a).

Sensitivity to antibiotics. Antibiotic susceptibilities of antagonistic bacteria were performed by disc diffusion in Mueller-Hinton agar. The antibiotic sensitivity discs included amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), chlortetracycline (30 µg), clindamycin (2 µg), doxycycline (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), oxolinic acid (2 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim/ sulfamethoxazole (1.25/23.75 µg), tylosin (150 µg) and vancomycin (30 µg). Agar plates were incubated at 25°C for 48 h. The diameters of the growth inhibition zones were measured and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations.

Phenotypic Characterization of Candidate Probiotic Bacteria. All antagonistic strains (n=6) were initially characterized by gram staining, motility, cytochrome oxidase, catalase and O/F tests. Further biochemical characteristics were determined using API 20 STREP test (bioMe´rieux), according to the manufacturer's instructions.

Genetic Analysis of Candidate Probiotic Bacteria: DNA extraction. Genomic DNA of bacterial isolates was extracted using the commercial DNA extraction kit (Thermo Scientific, GeneJET Genomic DNA Purification Kit) following manufacturer instructions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm.

L. garvieae specific PCR. L. garvieae specific PCR with the oligonucleotide primers of pLG-1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3') was used for the genetic identifications of isolates (Zlotkin et al., 1998).

PCR reactions were carried out using DEPC-treated water, 1xPCR Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1.0 U Taq polymerase, 1µM of each primer, and 5 µl templates DNA in a total volume of 25 µL. PCR amplification consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide (2 µg/ml) and visualized by an UV transilluminator. Bands at molecular size of approximately 1100 bp were considered as positive for *L. garvieae*. *L. garvieae* strain ATCC 43921 was used as a positive control and distilled water as negative control in molecular analysis.

16S rRNA Sequence Analysis. 16S rRNA sequence analysis was used to identify isolates which could not be identified via *L. garvieae* specific PCR assay. Additionally, one representative *L. garvieae* strain was also sequenced. A large fragment of the 16S rDNA gene (approximately 1,500 bp) was amplified using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). PCR reactions were carried out in 50 µl containing 10 µl template DNA, 1XPCR buffer, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 2 pmol of each primer, 1.5 U of Taq DNA polymerase, and DEPC-treated water. The PCR protocol was: 93°C for 3 minutes followed by 35 cycles at 94°C for 1 min, at 56°C for 1 min, at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were sent to a commercial sequencing company (Macrogen, Seoul, Korea) for purification and sequencing in both directions using 785F

(GGATTAGATACCCTGGT), and 907R (CCGTCAATTCMTTTRAGTTT) primers. After sequencing analysis, the obtained sequence data were compared with previously published data for identification with the Basic Local Alignment Search Tool (BLAST) via GenBank.

Statistical analysis. Data (pH and bile tolerances of bacteria) were assessed by one-way analysis of variance ANOVA and Duncan's multiple range test were used to determine the significant variation ($p<0.05$). All statistics were performed using SPSS for Windows version 15.0 (SPSS, Chicago, USA).

Results

Isolation and identification of the isolates. Six of 157 isolates obtained from rainbow trout intestine exhibited inhibitory activity against *V. salmoninarum* (Table 1). Phenotypic characteristics of antagonistic isolates are represented in tables 2 & 3.

Table 1. Antagonistic activity of isolates from rainbow trout against

V. salmoninarum	
Isolate code	*Inhibition zone (mm)*
TUB/2013/V47	9
TUB/2013/V27	10
TUB/2013/V10	8
TUB/2013/V2	16-20
TUB/2013/V4	22-24
TUB/2013/V1	18

Table 2. Phenotypic characteristics of antagonistic isolates by conventional methods and API 20 STREP

	TUB/2013/ V47	*TUB/2013/ V27*	*TUB/2013/ V10*	*TUB/2013/ V2*	*TUB/2013/ V4*	*TUB/2013/ V1*
Gr staining	+ coccus	+ coccus	+ coccus	+ coccus	+ coccus	+ coccus
Shape	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Catalase	F	F	F	F	F	F
O/F Test	+	+	+	+	+	+
VP[b]	-	-	-	-	+	-
Hippurate	+	+	-	+	+	+
Aesculin	+	+	-	+	-	-
Pyrrolidonyl	-	-	-	-	-	-
α-Galactosidase[b]	-	-	-	-	-	-
β-Glucuronidase[b]	-	-	-	-	-	-
β-Galactosidase[b]	-	-	-	-	-	-
Alkaline	+	+	+	+	+	-
Leucine	+	+	+	+	+	+
Acid production						
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 20 Strep; F: Fermentative

Table 3. Tolerance of antagonistic strains at different bile concentrations for 1.5 h at 25°C (log cfu/ml, SD*)

pH	*L. garvieae* V47	*L. garvieae* V27	*L. garvieae* V10	*L. garvieae* V2	*L. lactis* V4	*L. lactis* V1
7	7.47±0.67	7.59±0.86	7.66±0.55	7.55±0.37	7.74±0.77	7.84±0.44
3	7.69±0.46	7.76±0.67	7.43±0.76	7.51±0.69	7.81±0.48	7.67±0.56
2	7.84±0.77	7.82±0.45	7.34±0.34	7.62±0.38	7.48±0.56	7.56±0.73

*Bacterial levels were determined by plate counts on TSA. Data are presented as mean (standard deviations)

Genetic Analysis of Candidate Probiotic Bacteria. For genetic identification of isolates, *L. garvieae* specific PCR and 16S rRNA, gene sequence analysis was used. Accordingly *L. garvieae* specific PCR assay results, isolates TUB/2013/V2, TUB/2013/V10, TUB/2013/V27 and TUB/2013/V47 were identified as *L. garvieae* (Fig.1), but two other isolates (TUB/2013/V1 and TUB/2013/V4) were not identified as *L. garvieae*. The 16S rRNA gene region of these two isolates and representative strain of *L. garvieae* TUB/2013/V47 were amplified and sequenced. 16S rRNA gene sequence data have been deposited in GenBank databases (www.ncbi.nlm.nih.gov/genbank/) under accession numbers KT428593, KT428592 and KT428591, respectively. Based on the morphological, physiological, and sequence data, isolates TUB/2013/V1 and TUB/2013/V4 were identified as *Lactococcus lactis.*

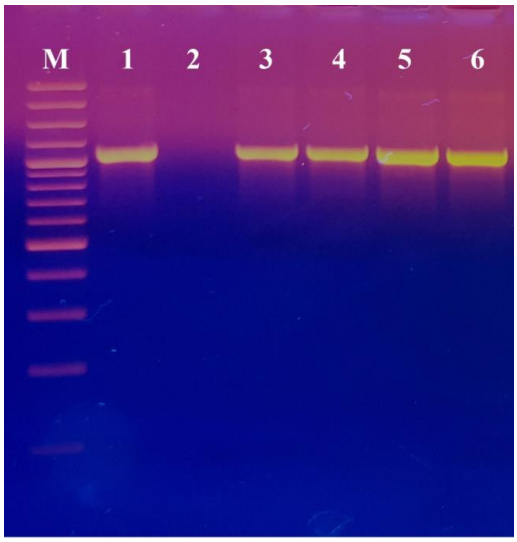

Fig.1 *L. garvieae* specific PCR (1100 bp). M; Molecular Marker (100 bp Plus DNA Ladder),
1: *L. garvieae* ATCC 43921,
2: Negative control (distilled water),
3: TUB/2013/V2,
4: TUB/2013/V10,
5: TUB/2013/V27,6;TUB/2013/V47.

Hydrophobicity, pH and bile tolerance, and sensitivity to antibiotics of antagonistic strains. All isolates were congo red-positive, indicating the presence of hydrophobic structures in their cell walls. All strains showed resistance to bile salts (0.6-1%) and low pH (2-3) (Tables 3 & 4). Antibiotic sensitivities of the antagonistic strains are given in Table 5. *L. garvieae* TUB/2013/V47, TUB/2013/V27 and *L. lactis* strains were sensitive to amoxicillin/clavulanic acid, ampicillin, doxycycline, erythromycin, and florfenicol which are often used in aquaculture.

Table 4. Tolerance of antagonistic strains at different pH conditions for 1.5 h at 25 °C (log cfu/ml, SD*)

% bile	*L. garvieae* V47	*L. garvieae* V27	*L. garvieae* V10	*L. garvieae* V2	*L. lactis* V4	*L. lactis* V1
0	6.95±0.36	6.41±0,08	6.57±0.16	6.82±0.41	6.82±0.44	7.44±0.65
0.6	6.88±0.58	6.97±0.41	6.66±0.48	7.05±0.56	7.14±0.3	6.75±0.49
1	6.89±0.51	6.91±0.54	6.63±0.38	7.18±0.50	6.51±0.45	6.63±0.32
1.5	6.94±0.52	7.22±0.39	6.96±0.42	7.21±0.55	7.21±0.6	6.94±0.47

*Bacterial levels were determined by plate counts on TSA. Data are presented as mean (standard deviations)
R: resistant, I: moderately susceptible, S: susceptible

Table 5. Antibiotic susceptibilities of antagonistic strains

	L. garvieae V47	*L. garvieae* V27	*L. garvieae* V10	*L. garvieae* V2	*L. lactis* V4	*L. lactis* V1
Amoxicillin/ clavulanic acid	S	S	S	S	S	S
Ampicillin	S	S	S	S	I	S
Chloramphenicol	R	I	I	I	I	I
Chlortetracycline	S	R	S	S	S	S
Clindamycin	R	R	S	R	S	S
Doxycycline	S	S	R	S	S	S
Enrofloxacin	I	S	I	R	R	R
Erythromycin	S	S	I	S	I	S
Florfenicol	S	S	S	S	S	S
Flumequine	R	R	R	R	R	R
Gentamicin	R	R	I	I	S	I
Kanamycin	R	R	I	R	I	I
Nalidixic acid	R	R	R	R	R	R
Nitrofurantoin	S	S	R	R	S	R
Oxolinic acid	R	R	R	R	R	R
Penicillin	S	S	S	S	S	S
Streptomycin	R	R	R	R	I	R
Tetracycline	I	S	S	S	S	R
Trimethoprim/ Sulfamethoxazole	R	R	R	R	R	R
Tylosin	R	R	R	R	R	R
Vancomycin	S	S	R	R	S	I

R: resistant,I: moderately susceptible, S: susceptible

Discussion

In this study, we screened potential probiotic bacteria taken from the intestine of rainbow trout and tested its efficacy against vagococcosis. Our study demonstrated that 6 (3.82%) of the screened bacteria (n=157) isolates were antagonistic to *V. salmoninarum*. There is an antagonism between bacterial pathogens and intestinal microflora of fish and this normally provides a protective barrier or protective function for a microbiota (Gómez and Balcázar, 2008; Cain and Swan, 2010). In the present study the inhibitory effects of *L. garvieae* V2, V10, V47, V27 and *L. lactis* V1, V4 strains against *V. Salmoninarum* were determined.

Antagonistic activity against fish pathogens of lactic acid bacteria isolated from fish have been reported in previous studies (Joborn et al., 1997; Nikoskelainen et al., 2001; Balcázar et al., 2007; Balcázar et al., 2008; Kim and Austin 2008; Pérez-Sánchez et al., 2011a). *L. plantarum*, *L. lactis* and *Leuconostoc mesenteroides* strains isolated from rainbow trout intestine were antagonistic against *L. garvieae (*Pérez-Sánchez et al. 2011). An antagonistic effect was found against *L. garvieae* of *L. lactis subsp. cremoris* DSM 20069 isolated from the intestines of healthy salmonid fish (Balcázar et al. 2007). In addition, *L. lactis subsp. lactis* and M17 strains, *Lactobacillus sakei* and *Weissella viridescens* showed inhibitory effects against *L. garvieae (*Didinen et al. 2016). However, there is only one study on screening of LABs against *V. salmoninarum* under *in vitro* conditions where inhibitory activity of *Lactobacillus curvatus* (CLFP150) isolated from the intestine of rainbow trout against *V. salmoninarum* was reported (Balcázar et al. 2007).

The ability to adhere to intestinal epithelial cells is considered important for potential probiotic adherence. The positive hydrophobicity results in this study show the adhesion of bacteria to the intestinal epithelial cells and their ability to survive throughout the gastrointestinal tract (An and Friedman, 2000; Nikoskelain et al., 2001). The more hydrophobic LAB strains were found to display stronger adhesive capability to intestinal epithelial cells (Pan et al. 2006). *Lactobacillus plantarum* and *L. lactis* strains isolated from rainbow trout displayed high hydrophobicity (Pérez-Sánchez et al. 2011a). In addition, *L. lactis subsp. lactis* M17 and *Lactobacillus sakei* strains from rainbow trout intestines were hydrophobic (Didinen et al., 2016). Similarly, in the present study all *L. garvieae* and *L. lactis* strains were also hydrophobic and can potentially survive throughout the gastrointestinal tract (e.g. resistance to bile salts, low pH). The

concentration of bile in the intestine of salmonid fish has been estimated to range between 0.4-1.3% (Balcázar et al., 2008). *L. lactis, L. plantarum,* and *L. mesenteroides* exhibited tolerance to 1.0 % bile and pH 3 (Pérez-Sánchez et al. 2011a). *L. lactis, L. plantarum* and *L. fermentum* can survive at pH 2.5 and at 2.5-10 % bile for 1.5 h (Balcázar et al. 2008). *Lactobacillus sakei* 2-3 showed tolerance to pH 3 and 2.5 % bile (Didinen et al. 2016). Similarly, all LAB strains were found tolerant to pH 2-3, and 0.6-1.5% bile concentrations in this study.

Conclusions

As a result of this study, 6 strains amongst 157 isolates from rainbow trout intestines showed an ability to suppress growth of *V*. *salmoninarum* under *in vitro* conditions. The antagonistic strains identified included TUB/2013/V47 (L. *garvieae*), TUB/2013/V27 (L.*garvieae*), TUB/2013/V10 (*L. garvieae*), TUB/2013/V2 (*L. garvieae*) TUB/2013/V1 (*L. lactis*) and TUB/2013/V4 (*L. lactis*). These strains were able to survive low pH and 0.6-1.5% bile concentrations, showed good adherence characteristics (hydrophobic) and were susceptible to amoxicillin/clavulanic acid ampicillin, doxycycline, erythromycin and florfenicol commonly used in aquaculture (relatively safe in terms of transfer of antibiotic resistance). Results suggest that *L. garvieae* (V47, V27, V10, V2) and *L. lactis* (V1 and V4) strains can be used to limit vagococcosis in cultured fish. However, these strains should be further studied *in vivo* to explore their probiotic effects.

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