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Characteristics of Bacterial Isolates from the Gut of Freshwater Fish, *Labeo rohita* that May be Useful as Potential Probiotic Bacteria

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Abstract In this study, we aimed to evaluate the in vitro probiotic characteristics of three bacteria, *Lactobacillus plantarum* VSG3, *Pseudomonas aeruginosa* VSG2, and *Bacillus subtilis* VSG1, isolated from the gut of *Labeo rohita*. The bacterial isolates tolerated low pH and high bile concentrations in the fish well. The bacterial adhesion capacity to fish intestinal mucosa revealed that the three potential probiotic isolates had a significantly higher adhesion capacity compared to the pathogenic strains tested. *L. plantarum* VSG3 exhibited the best adhesion capacity (19.1 %) to the intestinal mucosa. Among the isolates, *L. plantarum* VSG3 and *P. aeruginosa* VSG2 showed strong antibacterial activities against fish pathogens as measured in spent culture liquids. Moreover, all the isolates were susceptible to each tested antibiotic, which ensured their inability to exhibit antibiotic-resistance properties. Considering these promising results, selected strains should be further studied to determine their probiotic effects in vivo in fish.

Keywords Probiotic · Tolerance · Adhesion · Antibacterial activity

Introduction

Disease is a major problem in the fish farming industry, which currently is the fastest growing food-protein producing sector [1]. Bacterial infections are one of the most important causes of disease problems in Indian aquaculture [2]. *Aeromonas hydrophila* is the most common fish pathogen, and it can be spread easily through accidental abrasions [3]. This bacterium causes haemorrhagic septicaemia, characterised by the presence of ulcers, abscesses, exophthalmia, abdominal distension, small superficial lesions, and local haemorrhages, particularly in the gills and opercula [3]. Traditional disease control and prevention strategies employ vaccines, antibiotics, and chemotherapeutics. However, extensive application of chemotherapeutic agents leads to the development of antibiotic-resistant strains and causes environmental hazards. Moreover, the use of antibiotics can adversely affect the health status of fish [4, 5]. This situation has promoted the use of probiotics as a significant alternative to chemotherapeutics [6].

Probiotics are viable cell preparations that have beneficial effects on the health of a host by improving its intestinal balance via improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activities, growth-promoting factors, and an increased immune response [7]. Probiotics include lactic acid bacteria (LAB), *Bacillus* spp., *Pseudomonas* spp., and other gram-negative bacteria [5]. They have been applied in aquaculture for disease control, enhancing immune response, replacing the use of antimicrobial compounds, providing nutrients and enzymatic contributions, and improving water quality [8]. Earlier studies have demonstrated that probiotic application of *Lactobacillus plantarum* [9], *Bacillus subtilis* [10], and *Pseudomonas aeruginosa* [11] can enhance the growth, immune responses, and disease resistance in fish.

Selection of probiotics is very important because inappropriate microorganisms can lead to undesirable effects in the host. An ideal probiotic strain, irrespective of its source, should also be able to colonise, establish, and multiply in the host gut [7]. The key selection criteria for

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probiotics include many functional aspects—tolerance to gastric acidity and bile toxicity, adhesion to the intestinal epithelium, and the ability to modulate the immune response of the host [12, 13]. Recently, our research group isolated three potential probiotic bacteria (*L. plantarum* VSG3, *Pseudomonas aeruginosa* VSG2, and *B. subtilis* VSG1) from the gut of the tropical freshwater fish *Labeo rohita* [14]. Different cellular components of these isolates exhibited strong inhibition towards *Aeromonas hydrophila* [14]. Moreover, before performing in vivo tests, it is important to choose microorganisms on the basis of their probiotic properties in vitro [15]. Therefore, the present research was undertaken to investigate the in vitro probiotic properties of VSG1, VSG2, and VSG3 in several fish pathogens by examining their adhesion to fish intestinal mucosa, characterising their antimicrobial profile, and determining their tolerance to low pH and high fish bile concentrations.

Materials and Methods

Bacterial Strains and Radiolabelling

The potential probiotic strains *L. plantarum* VSG3, *P. aeruginosa* VSG2, and *B. subtilis* VSG1 were previously isolated from the gut of *L. rohita* [14]. The pathogenic strains, *Aeromonas hydrophila* MTCC 1739, *Aeromonas salmonicida* MTCC 1522, *Vibrio harveyi* MTCC 3438, and *Vibrio alginolyticus* MTCC 4439, were collected from Microbial Type Culture collection (MTCC), Chandigarh, India. All the strains were reactivated and grown in tryptone soya broth for 12 h at 35 °C. Titrated thymine ([methyl-1,2-^{3}H] thymine; 10 μL/mL, 117 ci/mmoL; Amersham International, UK) was added to the medium for radioactive labelling [16]. After cultivation, bacterial broths were centrifuged at 20,000 rpm for 10 min, washed twice with phosphate buffer solution (pH 7.2) and resuspended in the same buffer. Bacterial suspensions were adjusted to $A_{600} = 0.25 \pm 0.05$, which corresponded to 10^{7}–10^{8} cfu/mL.

Assay of Acid and Bile Tolerance

Acid tolerance of the isolates was determined in phosphate buffer solution (PBS) adjusted to pH 1.0–5.0 by adding 1 M HCl. The initial cell density was adjusted to 10^{7} cfu/mL, and the cells were incubated at different pH values for 1.5 h at 35 °C. Viable cell counts were determined using a plate counting method involving tryptone soya agar (TSA).

For bile tolerance, the bacterial suspension (10^{7} cfu/mL) was inoculated in sterile PBS containing 2–6 % (v/v) fish bile and incubated for 1.5 h at 35 °C. A control was maintained in PBS devoid of bile. Otherwise, the test was performed in a similar manner as those for acid tolerance.

Mucus Collection and Preparation

Mucus samples were collected from the skin and the intestine of rohu, immediately after sacrifice following the method described by Balcázar et al. [16]. Collected mucus was homogenised in PBS. Mucus preparations were centrifuged twice at 12,000 rpm for 10 min at 4 °C to remove particulates and cellular materials followed by filtration of the final supernatant through a 0.45-μm Millipore filter (Millipore, Bedford, USA).

In Vitro Adhesion Assay

Adhesion of radioactively labelled bacteria (both bacterial isolates and fish pathogens) was determined using the method of Nikoskelainen et al. [13], with slight modifications. Briefly, 100 μL of skin or intestinal mucus was immobilised in polystyrene microtitre plate wells during overnight incubation at 4 °C. The wells were then washed twice with 250 μL of PBS to remove excess mucus, and a suspension of 100 μL of radioactively labelled bacteria (10^{7} cfu/mL) was added to each well. After incubation for 1 h at 35 °C, the wells were washed with PBS to remove unbound bacteria. Bound bacteria were released and lysed via incubation at 60 °C for 1 h with 1 % sodium dodecyl sulphate (SDS) in 0.1 M NaOH. Adhesion was measured by quantifying the amount of radioactivity recovered after adhesion relative to radioactivity in the bacterial suspension added to the immobilised mucus. Experiments were performed in triplicate to determine intra-assay variations.

Similarly, the adhesion of bacteria to polystyrene was determined as an indicator for cell surface hydrophobicity.

Antimicrobial Profile

To measure antimicrobial activity, probiotic bacteria culture filtrates were prepared by filtration-sterilisation (0.45 μM Millipore filter) of supernatants obtained via centrifugation from overnight cultures of the isolates. Filtrates were neutralised (set to pH 6.8) with 5 N sodium hydroxide (NaOH). Tryptone soya agar plates were flooded with 100 μL of pathogenic bacteria, air-dried, and then 6-mm-diameter wells were punctured in each plate. The prepared supernatants were placed into respective wells (100 μL) and incubated for 24 h at 35 °C. An agar-containing well filled with tryptone soya broth was used to determine the inhibitory activity of the medium.

Susceptibility of probiotic isolates to amoxicillin, ampicillin, cephalexin, cloxacillin, penicillin G, chloramphenicol, tetracycline, ciprofloxacin, gentamycin, and erythromycin was

determined in TSA plates with octadiscs (HiMedia, Mumbai, India).

Results

Acid and Bile Tolerance

The results of acid tolerance are summarised in Table 1. None of the bacteria showed resistance at pH 1.0 or pH 1.5 after a 1.5-h exposure, except *P. aeruginosa* VSG2, which showed a slight tolerance at pH 1.5 ($10^{0.062}$ cfu/mL). All strains showed a gradual increase in survival from pH 2.0 to pH 5.0.

All assayed bacteria exhibited tolerance to fish bile (2–6 %, v/v). Significantly lower survivals were observed after a 1.5-h incubation in 5 and 6 % bile (v/v) compared to the control and other bile concentrations tested. With an increase in bile concentrations, a significantly lower growth was observed (Table 2). However, at 6 % bile (v/v) concentrations, *B. subtilis* VSG1, *P. aeruginosa* VSG2, and *L. plantarum* VSG3 showed concentrations of $10^{3.97}$, $10^{5.3}$, and $10^{4.87}$ cfu/mL, respectively.

In Vitro Adhesion Assay

Our results reveal that the probiotic strains were highly capable of adhering fast to intestinal mucus (12.9 ± 0.24 % to 19.1 ± 0.51 % adhesion) but less to skin mucus (7.5 ± 0.33 % to 10.23 ± 1.4 % adhesion; Table 3). These observations were further confirmed by assessing their adhesion to polystyrene. All the tested bacteria adhered less to polystyrene compared to intestinal mucosa. However, all the pathogens showed poor (1.76 ± 0.08 % to 4.4 ± 0.26 % adhesion) adhesion to the mucus, except *A. hydrophila*, which showed 5.9 ± 0.43 % adhesion to the intestinal mucus.

Table 1 Tolerance of potential probiotic isolates at various pH conditions for 1.5 h at 35 °C

pH tested	Log CFU/ml		
	L. plantarum VSG3	*B. subtilis* VSG1	*P. aeruginosa* VSG2
1.0	ND	ND	ND
1.5	ND	ND	0.062 ± 0.007^{a}
2.0	0.29 ± 0.02^{a}	0.17 ± 0.03^{a}	0.73 ± 0.061^{a}
2.5	0.96 ± 0.055^{b}	0.76 ± 0.06^{b}	1.32 ± 0.032^{b}
3.0	1.87 ± 0.061^{c}	1.89 ± 0.04^{c}	2.15 ± 0.031^{b}
4.0	3.07 ± 0.14^{d}	3.33 ± 0.07^{d}	4.18 ± 0.083^{c}
5.0	5.06 ± 0.12^{e}	4.7 ± 0.05^{e}	6.2 ± 0.058^{d}

Bacterial counts were determined by plate counts on TSA plates. Data are represented as mean ± S.E.M ($n = 3$). Mean values in same row with different superscripts vary significantly ($P < 0.05$). *ND* not detected

Table 2 Tolerance of potential probiotic isolates at various fish bile concentrations for 1.5 h at 35 °C

Bile (%; v/v)	Log CFU/ml		
	L. plantarum VSG3	*B. subtilis* VSG1	*P. aeruginosa* VSG2
0	6.44 ± 0.046^{a}	5.96 ± 0.068^{a}	7.19 ± 0.02^{a}
2.0	5.71 ± 0.075^{b}	5.22 ± 0.041^{b}	6.7 ± 0.02^{b}
4.0	5.54 ± 0.061^{b}	4.89 ± 0.023^{c}	6.59 ± 0.07^{c}
5.0	5.19 ± 0.064^{c}	4.44 ± 0.029^{d}	6.2 ± 0.06^{d}
6.0	4.87 ± 0.11^{d}	3.97 ± 0.014^{e}	5.3 ± 0.02^{e}

Bacterial counts were determined by plate counts on TSA plates. Data are represented as mean ± S.E.M ($n = 3$). Mean values in same row with different superscripts vary significantly ($P < 0.05$)

Table 3 Adhesion of bacterial strains to fish mucus and polystyrene

Organism	% Adhesion[a]		
	Intestinal mucus	Skin mucus	Polystyrene
L. plantarum VSG3	19.1 ± 0.51^{a}	8.1 ± 0.26^{a}	9.03 ± 0.4^{a}
B. subtilis VSG1	12.9 ± 0.24^{b}	7.5 ± 0.33^{b}	12.7 ± 0.35^{b}
P. aeruginosa VSG2	14.9 ± 0.12^{c}	10.23 ± 1.4^{c}	9.4 ± 0.317^{c}
A. hydrophila MTCC1739	5.9 ± 0.43^{d}	2.96 ± 1.4^{d}	4.76 ± 0.352^{d}
A. salmonicida MTCC1522	3.03 ± 0.18^{e}	4.4 ± 0.26^{e}	4.16 ± 0.176^{e}
V. harveyi MTCC3438	1.96 ± 0.17^{f}	2.9 ± 0.15^{f}	5.9 ± 0.11^{f}
V. alginolyticus MTCC4439	3.23 ± 0.24^{g}	1.76 ± 0.08^{g}	3.8 ± 0.14^{g}

[a] Percentage of radioactivity recovered from wells (immobilised mucus or polystyrene) compared to radioactivity of the added bacteria. Mean values in same row with different superscripts vary significantly ($P < 0.05$). Results are expressed as mean ± S.E.M ($n = 3$)

Antimicrobial Profile

Inhibitory activities of the potential probiotic isolates in the form of cell-free spent broth against fish pathogens are shown in Table 4. Among the isolates, *L. plantarum* VSG3 exhibited the strongest inhibitory activities against the pathogens; although the extent of inhibition varied (ranged from 7 to 12 mm inhibition). *P. aeruginosa* VSG2 inhibited the growth of all pathogens and produced an inhibition zone ranging from 5 to 8 mm. *B. subtilis* VSG1 failed to show antimicrobial effects against *V. alginolyticus* MTCC4439 but exhibited moderate antimicrobial activity (2–5 mm inhibition zone) against other pathogens tested.

All the isolates were susceptible to all tested antibiotics, and they produced more than a 10-mm zone of inhibition.

Table 4 Antimicrobial activities of cell-free spent broth of potential probiotic isolates against fish pathogens

Fish pathogens tested	Inhibition zone (mm) produced by		
	B. subtilis VSG1	*P. aeruginosa* VSG2	*L. plantarum* VSG3
A. hydrophila MTCC1739	++	+++	++++
A. salmonicida MTCC1522	+	++	++++
V. harveyi MTCC3438	+	+++	+++
V. alginolyticus MTCC4439	–	+++	+++

–, no inhibition zone; +, ≥2 mm inhibition zone; ++, ≥5 mm inhibition zone; +++, ≥7 mm inhibition zone; ++++, ≥9 mm inhibition zone

Discussion

The pH of gastric juice is the main factor that determines the survival of bacteria that pass from the stomach to the intestine [16]. In the present study, none of the bacteria exhibited resistance to pH 1.0, but displayed gradual increase in the resistance from pH 2.0 to pH 5.0. These results are in agreement with those obtained from previous studies where lactic acid bacteria (e.g. *L. plantarum*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *B. subtilis*, *Lactobacillus casei*, etc.) showed viability at low pH values [16–18]. Acid tolerance of bacteria is important not only for withstanding gastric stresses, but it is also a requirement for their use as dietary adjuncts [19].

Bile plays a fundamental role in specific and non-specific defence mechanisms in the gut; the magnitude of its inhibitory effects is determined primarily by the concentrations of bile salts [19]. Bile salts are toxic for living cells because they disrupt the structure of cell membranes. Tolerance to bile is considered as one of the essential properties required for probiotic bacteria to survive in the small intestine [18]. In the present study, all the potential probiotic strains could grow in the presence of fish bile (2–6 %, v/v); with an increase in bile concentration, an obvious decline in growth was observed. The bile concentration used in the present study was relatively higher than that encountered in the small intestine of fish. In agreement with our study, three lactic acid bacterial strains isolated from fish intestine, viz. *L. lactis*, *L. plantarum*, and *L. fermentum*, showed high tolerance to fish bile (2.5–10 % v/v) [16]. The acid and bile salt tolerance study divulged excellent assorted properties among probiotic bacteria. Therefore, these bacteria may be expected to survive in stomach and intestinal juice, which may contribute to increased shelf life.

Adhesion to the intestinal mucosa of the host is generally considered to be an important selection criterion for a probiotic organism because it leads to closer host-microbe interactions [20]. We found that three potential probiotic strains *L. plantarum* VSG3, *P. aeruginosa* VSG2, and *B. subtilis* VSG1 adhered in large numbers to the intestinal mucosa (12.9–19.1 % adhesion (Table 3) compared to the skin mucosa (7.5–10.23 % adhesion). Our results are in complete agreement with previous studies, which demonstrated that LAB isolated from fish intestine tended to adhere more to intestinal mucosa than to skin mucosa [16, 21]. We assume that this higher adhesion may be due to the presence of specific receptors for these strains on the intestinal mucosa since they were actually isolated from the gut of rohu.

All the pathogens showed poor (1.76 ± 0.08 %–4.4 ± 0.26 % adhesion) adhesion to skin mucosa as well as to intestinal mucosa (1.96–5.9 %). Among the pathogens, *Aeromonas* spp. adhered slightly better to both types of mucosa. This suggests that they have the ability to bind to both types of mucosa, which may help them in spreading virulence. The intestine is a site of colonisation and a possible route of infection for pathogens such as *A. salmonicida* [21], as well as a target for protective treatments such as feed containing probiotic bacteria [22]. These observations have been further confirmed by evaluating the adhesion of the bacteria to polystyrene. All the bacteria tested in our study adhered less to polystyrene compared to intestinal mucosa, except *B. subtilis* VSG1. This suggests that specific interactions may be involved in the binding. Previous reports have suggested that the microbial adhesion process of LAB involves passive forces, electrostatic interactions, hydrophobic interactions, steric forces, lipoteichoic acids, and specific structures such as external appendages covered by lectins [23].

The most important property by which probiotic bacteria exert their protective and beneficial physiological effects is through antagonistic activity against pathogenic bacteria [15]. In our study, neutralised supernatant from *L. plantarum* VSG3 and *P. aeruginosa* VSG2 exhibited strong inhibitory activity against the fish pathogens tested, while *B. subtilis* VSG1 showed moderate inhibition. This is in agreement with the findings of previous studies, which have demonstrated inhibitory activity of potential probiotic bacteria against various fish pathogens [14–17, 21]. In addition, several bacilli have been reported to establish their antimicrobial properties against various gram-positive and gram-negative pathogenic bacteria [13, 16, 17]. The inhibitory activity observed cannot be due to the acidity of the culture, since a neutralised supernatant was utilised (pH 6.8). Earlier studies have suggested that the inhibitory activity of probiotic bacteria may be due to the production of antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins, etc. [15, 21].

A key property of probiotic strains is that they should not carry any transmissible antibiotic-resistant genes.

Ingestion of such bacteria is undesirable, as horizontal gene transfer to recipient bacteria in the gut could lead to the development of new antibiotic-resistant pathogens [24]. Results from the present study show that all three potential probiotic isolates are susceptible (≥10 mm of zone of inhibition) to β-lactum antibiotics (ampicillin, cephalexin, and penicillin), gram-positive spectrum antibiotics (erythromycin), broad spectrum antibiotics (chloramphenicol, tetracycline), aminoglycosides (gentamycin), and other antibiotics tested. Antibiotic susceptibility of various probiotic bacteria has been demonstrated previously [14, 15, 17, 21]. Vulnerability against antibiotics is considered to be the most important probiotic characteristic [17]. Moreover, the susceptibility of all potential probiotic isolates to each antibiotic tested ensures its inability to transfer antibiotic resistance. It is worth mentioning that *P. aeruginosa* VSG2 used in this study was found to be safe for mammals [11].

In conclusion, three strains, *L. plantarum* VSG3, *P. aeruginosa* VSG2, and *B. subtilis* VSG1, presented interesting probiotic characteristics, especially greater pH and bile tolerance, in vitro adhesion to intestinal mucus, and suppressed pathogen growth under in vitro conditions. Moreover, all the isolates were susceptible to each tested antibiotic. These results collectively suggest that VSG1, VSG2, and VSG3 show promising properties important for potential probiotics. However, the selection of probiotic strains is always difficult because all the desirable properties hardly ever converge in the same strain. Therefore, these isolates should be further studied using challenge experiments in fish to determine whether they can function as probiotics in real-life situations.

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