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RESEARCH PAPER

Diversity and Probiotic Potentials of Putative Lactic Acid Bacteria for Application in Freshwater Aquaculture

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Abstract

In order to study the diversity of putative lactic acid bacteria (LAB) in freshwater fish, 76 strains of LAB were isolated from intestines and identified by phenotypic tests and 16S rDNA gene sequencing. Phenotypic characterization of the isolates allowed the identification of 18 clusters at 78% similarity level by Hierarchical cluster analysis. Functional evenness index (E value) a measure of phenotypic diversity, was found to be quite high (0.7 approximately) in most of the samples. 16S rDNA gene sequencing identified the isolates as different strains of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus fermentum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus brevis*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Weissella paramesenteroides*, *Weissella cibaria*, *Enterococcus faecium* and *Enterococcus durans. Lactobacillus plantarum* was found to be the dominating strain and LAB occurred more frequently in hindgut. Most of the strains showed good survival in acid and bile tolerance tests and antimicrobial activity against fish pathogen *Aeromonas hydrophila.* Different bacteriocin producing genes were detected in several strains by PCR. Selective strains with probiotic attributes could be potential candidates for freshwater aquaculture practices.

Keywords: Lactic acid bacteria, freshwater fish, phenotypic diversity, 16S rDNA gene, probiotic.

Introduction

A macro-organism and its microflora are always in a state of dynamic equilibrium. The alimentary tract of fish consists of complex, densely populated, highly diversified, microbial ecology which plays the main role in the immunobiological activity of the fish and has an important influence on its health and disease (Denev *et al*., 2009). Of particular significance is the indigenous, residential i.e. wall microflora and its important constituent part-the lactoflora or the group Lactic acid bacteria (LAB).

LAB is a group of Gram-positive rod and coccus-shaped organisms that have less than 55 % mol G+C content in their DNA. They are non-spore forming, non-motile, micro-aerophilic and produce lactic acid as their major end product during the fermentation of carbohydrates. LAB are generally associated with habitats rich in nutrients such as, various food products (milk, meat, vegetables), fermented, decaying material and the mucosal surfaces of the gastrointestinal tract of animals (Salminen and Wright, 2004). They are the most common type of microbes used as probiotics which are safely applied in medical and veterinary functions (Rauta *et al*., 2013). Also, with the demand for environmental friendly aquaculture, microbial interventions of such indigenous LAB in form of probiotic treatments is on rise as they may provide broad spectrum and greater non-specific disease protection (Balcazar *et al*., 2008). There is a general consensus that the efficacy of probiotics is highest in the host species from which they are isolated because such strains perform better as they have already adhered to the gut wall of the fish and are well adapted to compete with the pathogens (Picchietti *et al*., 2009). The strategy of isolating probiotics from the gut of mature animals and then use in immature animals of the same species has been successfully applied in fish (Picchietti *et al*., 2009; Perez-Sanchez *et al*., 2011). Studies have shown putative LAB in form of dietary probiotics resulted in better feed utilization and growth performance in fish (Carnevali *et al*., 2006; Giri *et al*., 2014; Beck *et al*., 2015). It also enhanced the non specific immune responses and conferred protection against potential fish pathogens (Balcazar *et al*., 2007; Picchietti *et al*., 2009; Perez-Sanchez *et al*., 2011; Beck *et al*., 2015). However, such applications require exhaustive studies in digestive tract lactoflora. LAB are taxonomically, metabolically and genetically diverse (Salminen and Wright, 2004). A polyphasic approach which takes

into account phenotypic and genotypic characteristics is required for proper study of diversity of such microorganisms.

Identifying probiotic characteristics of these putative LAB strains by *in vitro* studies forms the basis for selection of functional probiotics for future *in vivo* applications. To be a good or highly potential probiotic, LAB must tolerate and survive extremely stressed conditions like low pH and high concentrations of bile salts available in upper parts of the gastrointestinal tract (GIT) so as to reach the hindgut in an active and functional form and exert their beneficial properties (Karimi Torshizi *et al*., 2008). Moreover, the good probiotics should have other abilities such as antagonistic activity against pathogenic bacteria in order to enhance health promotion of the host (Ghosh *et al*., 2014). Beside the wide range of substances such as organic acids, hydrogen peroxide and antifungal compounds with antimicrobial activity, LAB also produces certain proteinaceous compounds called bacteriocins. These are ribosomally synthesized peptides exhibiting antimicrobial activity directed, in most cases, against bacteria closely related to the producer microorganism (Stephens *et al*., 1998). Bacteriocin production is a desirable probiotic trait that enables the establishment and persistence of the producing strains within the GIT and as such, would offer potential alternatives to traditional antibiotics with respect to controlling pathogens within the gut (O Shea *et al*., 2011). There are four genes associated with bacteriocin production which are organized in operons. These genes are the structural gene encoding a pre bacteriocin, an immunity gene (involved in affording immunity to the producing strain), a gene encoding an ABC transporter and a gene encoding an accessory protein essential for the externalization of the bacteriocin (Stephens *et al*., 1998).

The present study was undertaken with the aim to elucidate the frequency and diversity of LAB in separate segments of the GIT of freshwater fish. Taxonomic grouping by phenotype based clustering and identification based on 16S rDNA gene sequence of the isolates were determined. Probiotic potentials such as acid and bile tolerance capacity, antagonistic activity against fish pathogen and presence of bacteriocin producing genes in different isolates were also evaluated.

Materials and Methods

Isolation

Live freshwater fish Rohu (*Labeo rohita*), Catla (*Catla catla*), Mrigal (*Cirrhinus mrigala*), Silver carp (*Hypopthalmichthys molitrix*) and Grass carp (*Ctenopharyngodon idella*) (n=5 of each specimen) of average weight 1.1±0.56 kg, were used in the study. Samples were collected at regular interval from five different locations within 60 km radius of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India. Fish were sacrificed and the whole intestine from each sample was removed under aseptic conditions. After extracting and discarding fecal material, the intestine was flushed thoroughly with sterile saline (0.85% w/v) to remove non-adherent bacteria. It was further divided into three segments namely foregut, midgut and hindgut and the segments were homogenized separately in a mortar and pestle by using sterile normal saline solution (1:10; wt:vol). Subsequently, dilution series of each homogenate was prepared and from appropriate dilution, 0.1 mL was pour plated on *Lactobacillus* MRS (de Man, Rogosa and Sharpe) agar (de Man *et al*., 1960) (Himedia, India) plates. The plates were incubated anaerobically at 37 ^{o}C for 48 h. Individual colonies were selected on the basis of morphology, Gram staining and catalase activity. Each of Gram positive and catalase negative isolates were transferred into *Lactobacillus* MRS broth and incubated at 37 ^{o}C for 48 h. The isolates were kept in *Lactobacillus* MRS broth containing 20% (v/v) glycerol at -80 ^{o}C. Further analysis was carried out from the stored cultures.

Phenotypic Characterization and Identification

A set of 26 tests (including morphology, Gram staining characteristic and catalase test) (Table 1) as described by Ricciardi *et al*. (2005) was used to identify and classify the isolates. Strains were tested in duplicate to determine the test reproducibility. Identification of the strains were carried out according to Bergey's manual, Kandler and Weiss (1986); Hammes *et al*. (1992) integrated with supplementary information for strains isolated from natural populations obtained from Boukhemis *et al*. (2009); Khedid *et al*. (2009); Huidrom *et al*. (2012) and Yu *et al*. (2012).

Statistical Analysis

For effective representation of composition and relative position of LAB, Hierarchical cluster statistical analysis was carried out to group the isolates based on the phenotypic characters (Ricciardi *et al*. 2005; Di Cagno *et al*., 2010). To obtain an objective basis for grouping of the isolates, binary 0/1 matrices were created based on negative and positive results of phenotypic tests (the results were coded as 0 for tests showing negative results and 1 for tests with positive results). For morphology, two variables were used; 0 for coccus and 1 for rods/bacillus. Similarities were calculated using Jaccard coefficient and clustering was performed using Un-weighted Pair-Group Average Linkage Analysis in PAST software (version 2.17). To obtain a quantitative measurement of the phenotypic diversity, a Functional Evenness index (E) was calculated for samples for which at least 10 isolates were available using the procedure

Table 1. Phenotypic characteristics of the clusters of the LAB isolates from freshwater fish intestines

Cluster No.	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
No. of isolates	1	1	3	1	5	1	1	5	2	2	1	7	2	3	3	1	2	35
Morphology	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	C	B
CO_2 from glucose	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+
Growth at 15 °C	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+
Growth at 45 °C	-	+	+	+	-	-	-	+	+	+	+	+	+	+*	-	+	-	-
Arginine dihydrolysis	-	-	+	+	+	+	+	-	+	+*	+	+	+	+	+	-	+	-*
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	+	-	+	+	-	+	-	+	+	+	+	+*	+	-	+	w	+	+
Galactose	+	+	+	+	-*	+	+	+	-	+	+	+	+	+	+*	+	+	+*
Sucrose	-	-	-	-	-	+	-	+*	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	-	-	+	-	-	+	+	w	-	w	w	+	+	w*
Maltose	+	-	-	-	+	+	+	+	+	+	+	+	+	+	w	+	+	+
Mannitol	-	-	-	-*	-	-	-	-	-	-	-	-	-	-	w	+	+	+
Mannose	-	+	+	+	-	-	-	+	+	+*	-	+	+	+	+	+	+	+
Melezitose	-	-	-	+	-	-	-	-	-	-	-	+*	-	-	-	+	w*	+
Melebiose	+	+	-	-	+*	+	+	+	+	+*	+	-*	-	+	+*	+	+	w*
Raffinose	w	-	-	w	-	+	+	-*	-	+	-	+	+	+	+	+	w*	w*
Ribose	+	+	+	+	+*	+	-	-	-	+	+	+	+	+	+	w	+	+
Trehalose	+	+	+*	+	-	-	-	+	-	-	-	-*	-	-	+	w	+	+
Dextrose	+	+	+	+	+*	+	+	+	+	+	+	w	w	w	w*	+	+	+
Inositol	-	-	-	-	-	-	-	w	-	+*	+	-*	+	+	+*	-	-	w*
Sorbitol	-	-	-*	-	-	-	-	-	-	-*	-	-*	-	-	-	-	w	+
Salicin	+	+	+*	+	-	-	+	-	+	-	-	-*	w	w	-	-	+	+
Bile esculin	+	+	+	+	+	+	+	-*	+	+*	-	-*	-*	-*	+*	-	+	+
Growth in 2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 6% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

C= coccus; B= rods; -= negative; += positive; *= properties differing among strains of same type; w= weakly positive

described by Troussellier and Legendre (1981).

DNA Isolation, 16S rDNA Gene Amplification and Sequencing

Genomic DNA was extracted as described previously by Abed (2013). 16S rDNA gene amplification was carried out using universal bacterial 16S rDNA primers; forward primer (5'-AAGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGTTACCTTGTTACGACTT-3') which amplified 1500 bp amplicons (Stanley *et al*., 1995). The PCR mixture contained 100 ng of DNA template, 20 pmol of each primer, 2.5 Mm of dNTPs, 1×PCR buffer and 0.75 units of Taq polymerase. PCR was performed under the following conditions: initial de naturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. The PCR products were purified with purification kit (Fermentas, Europe) and subjected to partial DNA sequencing (Xcelris, India) of 900 bp containing V1, V2, V3, V4 and V5 variable regions.

The sequences were deposited in GenBank database. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program. The partial 16S rDNA sequences determined in the present study and those available in GenBank database were analyzed. Isolates were identified with ≥97% identity.

Probiotic Characteristics

Acid tolerance and Bile Tolerance Test

The acid and bile tolerances of the LAB isolates were determined as described by Ren *et al*. (2014). For acid tolerance test, *Lactobacillus* MRS broth was adjusted to pH 3 by using 1N HCL and for determining the bile tolerance capacity of the isolates, *Lactobacillus* MRS broth with 0.3 % Ox bile (Himedia) was used. Both the test broths were inoculated with active cultures of LAB strains (1 %, v/v) with initial bacterial concentration of 10^9 CFU mL^{-1} and incubated at 37 ^{0}C for 24 h. The viable number of LAB at pH 3 and 0.3% Ox bile were enumerated by pour plate count on MRS agar plate incubated at 37 ^{0}C for 48 h and compared to initial bacterial concentration.

Antagonistic Activity of the LAB Strains against *Aeromonas hydrophila*

The antagonistic activity of the isolates was determined using the well diffusion method (Srinu *et al*., 2013). The fish pathogen *Aeromonas hydrophila* isolated during outbreak period from experimental ponds of CIFA, Bhubaneswar, was used as the indicator strain and was lawn cultured in sterilie

Tryptic Soya Agar (TSA) (Himedia, India) plates with 6 mm diameter wells. 50 µL of filter sterilized supernatant obtained from each isolates of cell density 10^9 CFU mL^{-1} was placed in the wells in TSA plates. After 24 h of incubation at 37 ºC, the diameter of the zone of inhibition surrounding each agar well was measured. To test the proteinaceous nature of the inhibitory substances, 10 µl of a proteolytic enzyme, trypsin (Sigma, Madrid) solution (10 mg/ml in distilled water) was added in the cell free supernatant of the isolates and subjected to antibacterial assay (Ben Omar *et al*., 2008). The absence of zone of inhibition indicated the trypsin sensisitivity or proteinaceous nature of the inhibitory substances.

PCR Amplification of Known Bacteriocin Genes

PCR amplifications of genes involved in different bacteriocin production such as plantaricin (plnA, plnB, plnC, plnD, plnEF, plnI, plnJ, plnK, plnG, plnN genes) and plantaricin NC8 (tested for *Lactobacillus plantarum*, *L. pentosus*, *L. fermentum*, *L. brevis*), reuterin (for *L. reuteri*), salivaricin P (for *L. salivarius*), enterocin (for *Enterococcus faecium* and *E. durans*), pediocin PA-1 (for *Pediococcus pentosaceus* and *P. acidilactici*) and leucocin A (for *Weissella paramesenteroides* and *W. cibaria*) were carried out using the primers and conditions as described previously (du Toit *et al*., 2000; Barrett *et al*., 2007; Xiraphi *et al*., 2007; Ben Omar *et al*., 2008; Cadieux *et al*., 2008; Mlalazi *et al*. 2011). PCR products were separated by electrophoresis using a 2% agarose gel and 1×trisborate-EDTA buffer as described by Sambrook *et al*. (1989).

Results

Preliminary Identification

A total 76 number of strains were isolated and were considered to be LAB based on their positive Grams reaction, absence of motility, absence of spore formation and absence of catalase activity. The microorganisms were eventually grouped according to cell shape, as cocci and rods. 61 of 76 isolates found to be the rod-shaped strains were presumptively determined as derivatives of the genus *Lactobacillus.* Out of 8 coccus shaped isolates, 6 showed typical tetrad cell arrangement; therefore, they were tentatively referred to as *Pediococcus.* Moreover, 7 isolates showed typical lenticular or spherical shapes.

Phenotypic Characterization and Statistical Analysis

The isolates were characterized and identified by using 26 phenotypic tests. Based on phenotypic characters the dendrogram was constructed (Figure 1). The phenotypic characteristics of each cluster are shown in Table 1. 18 different LAB clusters were identified at 78 % similarity level. There were 7 major clusters (containing three or more strains) and 11 minor clusters (4, two member cluster and 7 one member clusters) containing different strains of *L. plantarum*, *L. fermentum*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. salivarius*, *P. pentosaceus*, *P. acidilactici*, *W. paramesenteroides*, *W. cibaria* and *E. faecium.* SM66 belonging to the genus *Pediococcus* could not be identified to species level. Within the clusters, the profiles were not identical but very similar. All the strains fermented glucose and grew at 2%, 4% and 6.5% NaCl. Most of the samples showed high phenotypic diversity with value of E, 0.772, 0.768 and 0.750 for Rohu, Mrigal and Catla respectively. (E can assume values between 0, for samples with no diversity, and 1, for samples with maximum diversity).

16S rDNA Gene Sequencing and Identification

The BLASTN search with partial sequence of 16S rDNA of all the isolates resulted in several hits having significant similarity with different LAB species. The percentage of similarity varied within the range of 97% to 100%. The identification of all the isolates along with accession number is shown in Table 2. *L. pentosus*, *L. reuteri* and *E. durans* were the species identified in addition to 10 species characterized and identified phenotypically. The frequency (%) of different isolates is shown in Figure 2.

Probiotic Properties

Acid Tolerance and Bile Tolerance Test

Acid and bile tolerance of the isolates is shown in Table 2. Most of the isolates, after 3 h of exposure to pH 3, showed a good survival with viability rate varying within the range of 10^5 to 10^8 CFU mL^{-1}. The strains could also quite effectively survive and tolerate bile concentration of 0.3% even after 24 h of exposure.

Antagonistic Activity of the LAB Strains against *A. hydrophila*

All the isolates of LAB except *L. brevis* SM54, *E. faecium* SM67, *E. durans* SM68 and *W. cibaria* SM74 showed antimicrobial activity against *A. hydrophila* with zone of inhibition varying within range of 11 mm to 20 mm in diameter (Table 2). In all the strains of *L. plantarum* along with *L. pentosus* SM35, *L. fermentum* SM51, *L. brevis* SM56, *L. salivarius* SM61, *P. pentosaceus* SM62, *P. acidilactici* SM64, *P. acidilactici* SM66 and all the strains of *W. paramesenteroides*, antimicrobial activity disappeared after addition of trypsin indicating the proteinaceous nature of the inhibitor.

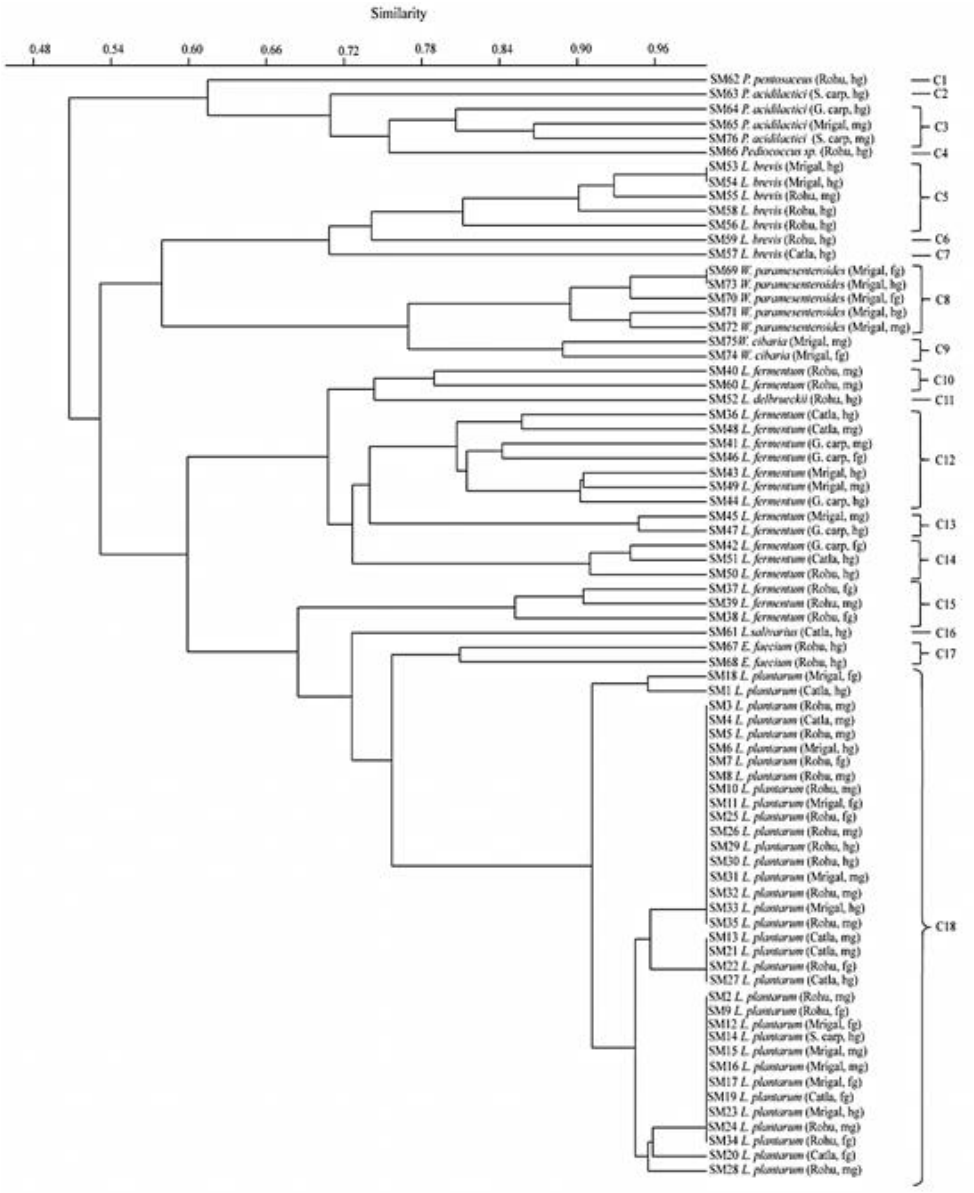

Figure 1. Dendrogram showing similarities and identifications based on phenotypic tests of 76 isolates of Lactic acid bacteria. Similarities were calculated using Jaccard coefficients and grouping was done by Hierarchical cluster analysis using Un-weighted Pair-Group Average Linkage Analysis. Clusters (C1 to C18) were established at 78% similarity level. Source of isolation is indicated within brackets. fg: foregut; mg: midgut; hg: hindgut.

Detection of Bacteriocin Gene

All the genes of the plantaricin cluster (plnA, plnB, plnC, plnD, plnEF, plnI, plnJ, plnK, plnG, plnN genes) were detected in all the 34 isolates of *L. plantarum* including *L. pentosus* SM35 (Figure 3A). Besides carrying all the genes of the plantaricin cluster, isolate *L. plantarum* SM33 was also found to be positive for plantaricin NC8 structural gene (Figure 3B). Similarly, *L. fermentum* isolate SM51 showed all the genes of the plantaricin cluster and in *L. brevis* isolate SM56 all the genes of plantaricin cluster were present except plnC. Bacteriocin, salivaricin P structural gene was detected in *L. salivarius* isolate SM61 (Figure 3C). Isolates SM62, SM64 and SM66 belonging to the genus *Pediococcus*, tested positive for pediocin PA-1. All the strains of *W. paramesenteroides viz.* isolates SM69, SM71, SM72, SM73 and *W. cibaria* isolate SM74 tested positive for presence of bacteriocin, leucocin A gene. However, isolate *L. reuteri* SM60 did not test positive for presence of reuterin gene and isolates *E. faecium* and *E. durans* did not show the presence of enterocin genes.

Discussion

It is evident from the present study that the occurrence of LAB is more frequent in the hindgut (40%) followed by midgut (37%) and less frequent in

Table 2. Identification based on 16S rDNA gene sequence (with accession numbers), acid and bile tolerance capacity in form of viable count in CFU mL^{-1} and antagonistic effect against *A. hydrophila* in form of zone of inhibition of LAB isolates

Isolates with accession numbers	Initial count (CFU mL^{-1})	Viable count at pH3 after 3h (CFUmL^{-1})	Viable count at pH3 after 24h (CFUmL^{-1})	Viable count at 0.3% bile after 24h (CFUmL^{-1})	Zone of inhibition (in mm) ± SEM against *A. hydrophila*
L. plantarum subsp. *plantarum* SM1 (KJ690717)	10^9	10^7	10^1	10^5	16.16±0.16
L. plantarum SM2 (KJ690718)	10^9	10^7	10^3	10^6	15.00±0.28
L. plantarum SM3 (KJ690719)	10^9	10^8	10^4	10^6	14.93±0.23
L. plantarum SM4 (KJ729071)	10^9	10^8	10^3	10^7	17.10±0.20
L. plantarum SM5 (KJ690720)	10^9	10^8	10^3	10^7	14.43±0.23
L. plantarum SM6 (KJ690721)	10^9	10^8	10^3	10^7	15.36±0.18
L. plantarum SM7 (KJ690722)	10^9	10^8	10^3	10^7	14.16±0.16
L. plantarum SM8 (KJ690723)	10^9	10^7	10^3	10^8	19.10±0.26
L. plantarum SM9 (KJ690724)	10^9	10^8	10^6	10^8	17.23±0.28
L. plantarum SM10 (KJ690725)	10^9	10^7	10^4	10^8	16.00±0.17
L. plantarum SM11 (KJ690726)	10^9	10^3	no growth	10^5	14.96±0.20
L. plantarum SM12 (KJ690727)	10^9	10^8	10^6	10^7	18.20±0.32
L. plantarum SM13 (KJ690728)	10^9	10^8	10^3	10^8	15.03±0.29
L. plantarum SM14 (KJ690729)	10^9	10^8	10^3	10^5	15.73±0.21
L. plantarum SM15 (KJ690730)	10^9	10^8	10^3	10^7	16.00±0.35
L. plantarum SM16 (KJ690731)	10^9	10^8	10^6	10^8	20.03±0.20
L. plantarum SM17 (KJ690732)	10^9	10^8	10^5	10^7	17.06±0.38
L. plantarum SM18 (KJ690733)	10^9	10^8	10^5	10^7	15.03±0.31
L. plantarum SM19 (KJ690734)	10^9	10^8	10^4	10^5	14.90±0.34
L. plantarum SM20 (KJ690735)	10^9	10^8	10^3	10^3	20.06±0.31
L. plantarum SM21 (KJ690736)	10^9	10^8	10^5	10^4	17.20±0.20
L. plantarum SM22 (KJ690737)	10^9	10^8	10^4	10^4	13.23±0.14
L. plantarum SM23 (KJ690738)	10^9	10^7	10^6	10^7	17.10±0.23
L. plantarum SM24 (KJ690739)	10^9	10^8	10^3	10^3	16.70±0.20
L. plantarum SM25 (KJ690740)	10^9	10^3	no growth	10^6	14.10±0.27
L. plantarum SM26 (KJ690741)	10^9	10^3	no growth	10^6	13.00±0.11
L. plantarum SM27 (KJ690742)	10^9	10^8	10^4	10^4	19.10±0.20
L. plantarum SM28 (KJ690743)	10^9	10^7	10^3	10^6	16.16±0.17
L. plantarum SM29 (KJ690744)	10^9	10^3	no growth	10^7	15.20±0.25
L. plantarum SM30 (KJ690745)	10^9	10^7	10^4	10^7	16.16±0.28
L. plantarum SM31 (KJ690746)	10^9	10^8	10^6	10^7	17.03±0.26
L. plantarum SM32 (KJ690747)	10^9	10^3	no growth	10^4	16.10±0.20

Table 2. Continued.

Isolates with accession numbers	Initial count (CFU mL^{-1})	Viable count at pH3 after 3h ($CFUmL^{-1}$)	Viable count at pH3 after 24h ($CFUmL^{-1}$)	Viable count at 0.3% bile after 24h ($CFUmL^{-1}$)	Zone of inhibition (in mm) ± SEM against *A. hydrophila*
L. plantarum SM33 (KJ690748)	10^9	10^8	10^7	10^8	18.33±0.24
L. plantarum SM34 (KJ690749)	10^9	10^3	no growth	10^6	16.90±0.30
L. pentosus SM35 (KJ690750)	10^9	10^8	10^5	10^6	16.30±0.15
L. fermentum SM36 (KJ690751)	10^9	10^5	10^2	10^2	15.06±0.23
L. fermentum SM37 (KJ690752)	10^9	10^5	no growth	10^2	11.03±0.21
L. fermentum SM38 (KJ690753)	10^9	10^7	no growth	no growth	13.10±0.29
L. fermentum SM39 (KJ690754)	10^9	10^5	10^3	10^4	13.26±0.33
L. fermentum SM40 (KJ690755)	10^9	10^4	no growth	10^5	12.40±0.20
L. fermentum SM41 (KJ690756)	10^9	10^7	10^5	10^4	13.03±0.31
L. fermentum SM42 (KJ690757)	10^9	10^7	10^6	10^5	11.13±0.24
L. fermentum SM43 (KJ690758)	10^8	10^6	10^5	no growth	15.10±0.20
L. fermentum SM44 (KJ690759)	10^8	10^6	10^5	no growth	15.26±0.26
L. fermentum SM45 (KJ690760)	10^9	10^7	10^5	10^5	13.00±0.28
L. fermentum SM46 (KJ690761)	10^9	10^7	10^6	10^4	14.13±0.24
L. fermentum SM47 (KJ690762)	10^9	10^7	10^5	10^4	12.10±0.37
L. fermentum SM48 (KJ690763)	10^7	10^5	no growth	10^5	13.03±0.31
L. fermentum SM49 (KJ690764)	10^8	10^5	no growth	10^5	14.83±0.16
L. fermentum SM50 (KJ690765)	10^9	10^5	10^2	no growth	12.00±0.23
L. fermentum SM51 (KJ729045)	10^9	10^7	10^6	10^6	16.13±0.18
L. delbrueckii subsp. *bulgaricus* SM52 (KJ729046)	10^9	10^6	10^4	10^4	13.23±0.23
L. brevis SM53 (KJ729047)	10^9	10^5	10^3	10^7	13.00±0.28
L. brevis SM54 (KJ729048)	10^9	10^3	no growth	10^6	no inhibition zone
L. brevis SM55 (KJ729049)	10^9	10^3	no growth	10^7	10.90±0.27
L. brevis SM56 (KJ729050)	10^9	10^3	no growth	10^7	16.10±0.20
L. brevis SM57 (KJ729051)	10^9	10^3	no growth	10^7	14.10±0.32
L. brevis SM58 (KJ729052)	10^9	10^3	no growth	10^6	13.06±0.17
L. brevis SM59 (KJ729053)	10^9	10^3	no growth	10^6	15.13±0.24
L. reuteri SM60 (KJ729054)	10^9	10^5	no growth	10^4	13.00±0.28
L. salivarius SM61(KJ729055)	10^9	10^7	10^5	no growth	16.13±0.24
P. pentosaceus SM62 (KJ729056)	10^9	10^1	no growth	10^2	12.06±0.29
P. pentosaceus SM63 (KJ729057)	10^9	10^6	10^2	10^7	14.20±0.20
P. pentosaceus SM64 (KJ729058)	10^9	10^7	10^4	10^7	15.10±0.20
P. acidilactici SM65 (KJ729059)	10^9	10^6	10^2	10^7	15.03±0.31
P. acidilactici SM66 (KJ729060)	10^9	10^6	no growth	10^6	13.10±0.37
E. faecium SM67 (KJ729061)	10^9	10^3	no growth	10^2	no inhibition zone
E. durans SM68 (KJ729062)	10^9	10	no growth	no growth	no inhibition zone
W. paramesenteroides SM69 (KJ729063)	10^8	10^2	no growth	no growth	13.10±0.30
W. paramesenteroides SM70 (KJ729064)	10^8	10^5	no growth	10^2	12.10±0.20

Table 2. Continued.

Isolates with accession numbers	Initial count (CFU mL^{-1})	Viable count at pH3 after 3h ($CFUmL^{-1}$)	Viable count at pH3 after 24h ($CFUmL^{-1}$)	Viable count at 0.3% bile after 24h ($CFUmL^{-1}$)	Zone of inhibition (in mm) ± SEM against *A. hydrophila*
W. paramesenteroides SM71 (KJ729065)	10^8	10^5	no growth	no growth	13.13±0.24
W. paramesenteroides SM72 (KJ729066)	10^8	10^2	no growth	no growth	13.36±0.18
W. paramesenteroides SM73 (KJ729067)	10^8	10^5	no growth	10^2	11.00±0.28
W. cibaria SM74 (KJ729068)	10^8	10^6	no growth	10^2	no inhibition zone
W. cibaria SM75 (KJ729069)	10^8	10^6	no growth	10^2	12.33±0.24
P. pentosaceus SM76 (KJ729070)	10^9	10^5	10^3	10^7	13.13±0.29

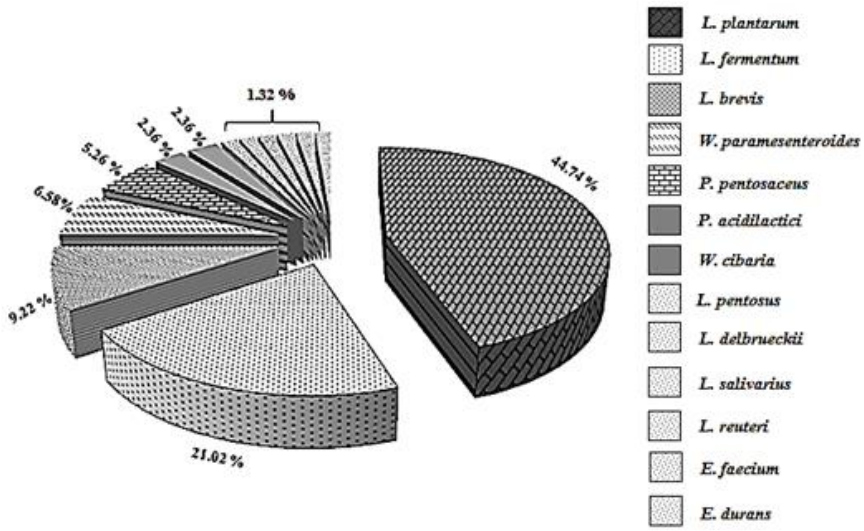

Figure 2. Frequency (%) of Lactic acid bacteria isolated from freshwater fish intestines.

the foregut (23%) of the intestine of freshwater fish. Similar results have been shown by Ayo Olalusi *et al*. (2012) and Jankauskiene (2002) who reported frequency of LAB higher in the midgut and hindgut region than observed in the foregut region of *Clarias gariepinus* and *Cyprinus carpio* respectively. It is known that LAB are associated with habitats rich in nutrients. Carbohydrates remain accumulated in the mid and hind segments of fish digestive tract (Jankauskiene, 2002). Moreover, earlier findings have shown that in general, the pH in the intestinal bulb and foregut region of Rohu, Catla and Mrigal varied within the range of 6.8 to 7.1 whereas pH in the hindgut region of these fish varied within the range of 6.2 to 6.5 which is favorable for the growth of LAB (Singh, 1985). A rich nutrient and low pH environment could be an explanation for a more frequent occurrence of LAB in the hindgut of these freshwater fish. Similarly, studies have shown that in general, pH of different segments of Silver carp and Grass carp intestines varies from 7.1 to 7.6 and 7.0 to 8.5 respectively (Hickling, 1966; Manadhar, 1977). A slight alkaline environment may not have favored the growth of LAB. Consequently, a low frequency (n=10) of occurrence of these bacteria was found in Silver carp and Grass carp.

The phenotypic diversity as measured by E value was found to be quite high in most of the samples. 76 isolates were phenotypically identified as different strains of *L. plantarum*, *L. fermentum*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. salivarius*, *P. pentosaceus*, *P. acidilactici*, *W. paramesenteroides*, *W. cibaria* and *E. faecium*. Hierarchical cluster analysis grouped all the isolates into 18 different clusters at 78% similarity level. According to the fermentation profile, it seems that the isolates assimilated variously a panel of carbohydrates that reflected their enzymatic and genetic potentials (Belhadj *et al*., 2014). The LAB genomes are predicted to carry a large number of carbohydrate transport and utilization genes that display substantial variations among strains (Ceapa *et al*., 2015). Many strains among the same clusters varied in the pattern of utilization of sugars indicating phenotypic

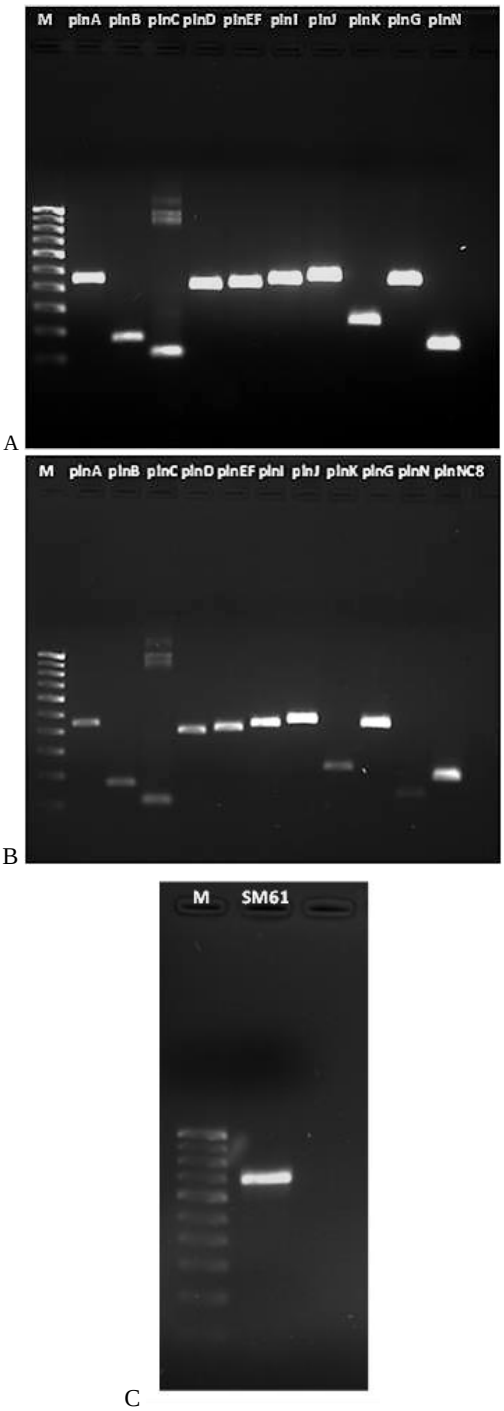

Figure 3. Agarose gel electrophoresis of PCR amplification products of bacteriocin **(A)** Plantaricin genes (plnA, plnB, plnC, plnD, plnEF, plnI, plnJ, plnK, plnG, plnN genes) in *L. plantarum* **(B)** Plantaricin genes along with plantaricin NC8 (plnNC8) in *L. plantarum* SM33 **(C)** Salivaricin P in *L. salivarius* SM61.

heterogeneity and diversity. Moreover, differences in carbohydrate utilization pattern were accounted among the present isolates and those referred from other sources such as milk (Khedid *et al*., 2009), fermented products (Boukhemis *et al*., 2009; Yu *et al*., 2012), faecal samples (Huidrom *et al*., 2012). As some niches display unique carbohydrate compositions, variability in carbohydrate utilization capacity is likely to reflect an important aspect of niche specific adaptation (Ceapa *et al*., 2015). Hence, to better understand the phenotypic diversity and niche adaptation of different strains of species of LAB analysis of carbohydrate fermentation profiling of different isolates is important (Salminen and Wright, 2004; Boukhemis *et al*., 2009).

In the current study, in an effort to corroborate phenotypic characterization, the identification of the isolates was confirmed by analysis of 16S rDNA

gene. In some of the isolates, discrepancies in identification at species level were noticed by both the methods. In 16S rDNA gene sequencing, isolates, SM35, SM60 and SM68 were identified as *L. pentosus*, *L. reuteri* and *E. durans* respectively whereas by phenotypic traits they were differently identified as *L. plantarum*, *L. fermentum* and *E. faecium* respectively. Similarly, strains SM63, SM64 and SM76 identified as *P. pentosaceus* by 16S rDNA sequencing were differently identified as *P. acidilactici* by phenotypic tests. Strain SM66 which could not be identified biochemically to the species level was identified as *P. acidilactici* by 16S rDNA sequencing. This may probably be due to the selected limited characters used for phenotypic study, or by the similarity of the metabolic patterns expressed by the isolates, due to similar nutritional requirements and growth under similar environmental conditions or by due to possible loss or acquisition of plasmids that encode many carbohydrate fermentation traits. As reported elsewhere (Belhadj *et al*., 2014), simple phenotypic tests used for a clear identification of LAB strain to species level are thus, often ambiguous. Therefore, 16S rDNA gene sequencing is used as molecular based characterization approach for discrimination and assignment of a given LAB strain to its taxonomic status (Soto *et al*., 2010).

Among the samples, a high diversity in terms of bacterial species was found (Figure 2). Genus *Lactobacillus* dominated (80.26%) among the total isolates and *L. plantarum* was the species most often isolated (44.74%) followed by *L. fermentum* (21.02%). *L. plantarum* is known as the most adaptable *Lactobacillus* species due to its large genome, capability in metabolizing different carbon sources and growth ability. By having such characteristics, it enables such a species to colonize different environments (Mirlohi *et al*., 2009) and could be the reason for *L. plantarum* to be the species most often isolated in the present study.

There have been several reports of different strains of LAB isolated from fish intestine showing relatively high resistance to low pH and high fish bile concentration (Balcazar *et al*., 2008; Buntin *et al*., 2008). Most of the isolates particularly strains of *L. plantarum* survived after 3 h of exposure to pH 3 and tolerated bile concentration of 0.3% even after 24 h of exposure. However, the isolates belonging to *L. brevis* and *Pediococcus* could not tolerate pH 3 but showed a longer survival in the presence of bile indicating the strain specific nature for acid and bile tolerance and are in consistent with earlier findings (Duangjitcharoen *et al*., 2014).

All the isolates of LAB except *L. brevis* SM54, *E. faecium* SM67, *E. durans* SM68 and *W. cibaria* SM74 showed antimicrobial activity against fish pathogen *A. hydrophila*. Most of the strains of *L. plantarum* showed the strongest antimicrobial effect followed by strains of *L. fermentum* (SM36, SM43, SM44, SM51), *L. salivarius* (SM61), *L. brevis* (SM56, SM59) and *Pediococcus* (SM64, SM65). These results are in agreement with earlier studies where similar *in vitro* antagonisms against fish pathogens have been reported by using *Lactobacillus* spp. (Allameh *et al*., 2013; Butprom *et al*., 2013; Dash *et al*., 2014). Subsequent use of these isolates in *in vivo* study as dietary probiotic gave successful results in challenge experiments against potential fish pathogens. The antimicrobial activity of the LAB isolates is thought to be multi factorial and to be due to the synergy effect of production of organic acids (acetic acid or lactic acid) and strain specific metabolites or non-lactic acid molecules, bacteriocins etc which serve as antibacterial agents to eliminate growth of competing microbes (Hagi and Hoshino, 2009). During preliminary screening for bacteriocin production, all the strains of *L. plantarum* along with *L. pentosus* SM35, *L. fermentum* SM51, *L. brevis* SM56, *L. salivarius* SM61, *P. pentosaceus* SM62, *P. acidilactici* SM64, *P. acidilactici* SM66 and all the strains of *W. paramesenteroides*, showed no zone of inhibition after addition of trypsin indicating the proteinaceous nature of the inhibitor. All these strains also tested positive for presence of different bacteriocin genes indicating the correlation between the bacteriocin and antagonistic activity of the LAB and is in consistent with earlier findings (Ben Omar *et al*., 2008; Hurtado *et al*., 2011; Ruiz Rodriguez *et al*., 2013). Bacteriocins permeabilise the target cells by forming pores in the cytoplasmic membrane that subsequently leads to the depletion of the intracellular ATP pool, disturbance of the proton motif force and eventually the cell death (Suzuki *et al*., 2005).

All the strains of *L. plantarum* carried all the genes of plantaricin cluster described previously in *L. plantarum* strains C11 (Diep *et al*., 1996) and WCFS1 (Kleerebezem *et al*., 2003) suggesting similarity in gene organizations and /target sequence. Besides carrying all the genes of plantaricin cluster, isolate *L. plantarum* SM33 tested positive for presence of plantaricin NC8 structural gene. Hurtado *et al*. (2011) also reported the presence of NC8 structural gene from six strains isolated from fermented olive. In *L. pentosus* SM35 and *L. fermentum* SM51 all the genes of plantaricin cluster were found whereas in *L. brevis* SM56 all the plantaricin genes except plnC were detected. Bacteriocin production is frequently associated with mobile genetic elements that may facilitate the transfer of genes between species and strain sharing the same niche. Also, it seems reasonable that closely related species must present similar characteristics to survive in the same conditions. This is in agreement with Ben Omar *et al*. (2008) who first reported the detection of plantaricin gene cluster in *L. fermentum* and *L. plantarum* isolated from the same niche and Hurtado *et al*. (2011) who reported the presence of plantaricin genes in closely related species *L. plantarum* and *L. pentosus*. Also, the strains of *L. plantarum* along with *L. pentosus* SM35, *L. fermentum* SM51 and *L. brevis*

SM56 with similar gene of plantaricin cluster, exhibited almost similar zone of inhibition diameter (16mm approximately) against *A. hydrophila.* The isolate *L. salivarius* SM61 was positive for bacteriocin, salivaricin P structural gene. Studies have shown salivaricin P and other closely related variants of abp118β (a two-component class IIb bacteriocin) occur frequently in intestinally derived *L. salivarius* isolates from different hosts (Barrett *et al*., 2007; Li *et al*., 2007). This feature may be important for the successful establishment of *L. salivarius* within the GIT (O Shea *et al*., 2011). A 1044 bp DNA fragment, corresponding in size to the structural gene of pediocin PA-1 was amplified in isolates SM62, SM64 and SM66 belonging to the genus *Pediococcus.* Pediocin PA-1 originally isolated from *P. acidilactici* PAC1, is a class IIa bacteriocin that has been well studied (Marugg *et al*., 1992; Mlalazi *et al*., 2011) and shows a wide range of inhibitory spectrum. Also in the present study, all the strains of *W. paramesenteroides* (SM69, SM71, SM72, SM73) and *W. cibaria* isolate SM74 were positive for presence of bacteriocin, leucocin A gene. A survey on the strains of *Leuconostoc* and *Weissella* has also revealed the production of leucocin A, B and C type bacteriocins (Papathanosopoulos *et al*., 1997). As discussed above, the presence of bacteriocin genes correlated with the antimicrobial acitivity of the isolates. Also, antimicrobial activity varied among genus *Lactobacillus* (average zone of inhibition 16.3 mm), *Pediococcus* (average zone of inhibition 13.3 mm), *Weissella* (average zone of inhibition 12.5 mm) indicating different bacteria usually differ in the incidence and expression of bacteriocin genes, as well as in their antimicrobial activity (Ruiz Rodriguez *et al*., 2013). However, detection of various bacteriocin producing genes and antagonistic activity, the highly desirable probiotic traits, from different isolates in the present study, suggests that this may help these strains predominate in the LAB population in the gut microflora and establish as potential alternatives to traditional antibiotics with respect to controlling pathogens within the gut and overcoming complications such as the proteolytic degradation of orally delivered antimicrobial peptides during gastric transit (O Shea *et al*., 2011).

The study revealed a diversified LAB population in the intestines of freshwater fish. Selective putative strains with good acid and bile tolerance capability, ability to produce bacteriocins and suppress pathogen growth under *in vitro* conditions, could well be potential *in vivo* probiotic candidates for sustainable and environmental friendly aquaculture.

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