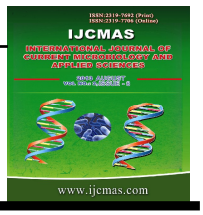
Int.J.Curr.Microbiol.App.Sci (2013) 2(8): 234-244

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 8 (2013) pp. 234-244**
http://www.ijcmas.com



Original Research Article

Screening and characterization of potential probiotic lactic acid bacteria isolated from vegetable waste and fish intestine

Pradipta Ranjan Rauta[1], Madhusmita Dhupal[2], Bismita Nayak[1]*

[1]Department of Life Science, National Institute of Technology, Rourkela, Odisha-769008, India
[2]Department of Botany, Gangadhar Meher Autonomous College Sambalpur, Odisha - 768004, India
***Corresponding author e-mail:** bismita.nayak@gmail.com, nayakb@nitrkl.ac.in*

Keywords

Probiotics; Lactic acid bacteria; Fish intestine; vegetable waste; Bacteriocin; Molecular analysis.

A B S T R A C T

Generally the microorganisms isolated from kitchen waste are poorly correlated with potential probiotics. For the first time we have correlated probiotic bacterial strains with their high bacteriocin producing properties to the environmental niches they have been isolated. In order to identify the predominating Lactic acid bacteria (LAB) organisms from kitchen waste and fish intestine, a total number of 16 strains of LAB were isolated in *Lactobacillus* selective media (MRS broth). Among all the isolated strains, six strains were selected on the basis of LAB specific morphological and biochemical properties. The phenotypic characteristics (morphological and biochemical) of these strains were determined by molecular characterization method using species-specific PCR techniques, 16 S rDNA sequencing. The five strains (KTIT, KT2W, KT1B, KA2, FS) have been identified as *Lactobacillus casei* and sixth strain KT1 has been identified as *Lactobacillus delbrueckii*. Out of these six strains KT1 (*Lactobacillus delbrueckii*) has proved its probiotic credibility more than other strains by producing antimicrobial bacteriocin-like compound, and inhibitory property against all tested potential pathogenic strains (*Protius vulgaris, Bacillus subtilis, Pseudomonas aeruginosa, E. coli, Kleibsella pneumoniae*). The study is extremely promising, that underscores the important role of *Lactobacillus* strains, having probiotic effects, which can be used to improve quality and safety of preserved food and beverages.

Introduction

Lactic acid bacteria (LAB) are the most common types of microbes used as probiotics (Herich and Levkut, 2002) , which are safely applied in medical and veterinary functions (Divakara *et al*., 2010). Lactic acid bacteria are present in foods (dairy products, sour dough, fermented meat, fermented vegetables, silage, beverages), in sewage, on plants, as well as within the genital, intestinal and respiratory tracts of humans and animals (Schleifer and Ludwig, 1995; Hammes *et al*., 1991). Bacteriocins of LAB are considered as safe natural preservatives or

bio-preservatives, because they are degraded by the proteases in gastrointestinal tract unlike traditional antibiotics and they also reduce the use of chemical preservatives in foods (Cleveland *et al*., 2001). In recent years, much attention is being given to a large variety of bacteriocinogenic Lactic acid bacteria from different sources (dairy, meat, plant products, and traditional fermented products) (Meera and Charitha Devi, 2012).

Current methodologies used for classification of LAB mainly rely on 16S ribosomal ribonucleic acid (rRNA) analysis and sequencing (Morgan *et al*., 2009). Antimicrobials have been used progressively as a primary intervention for inhibition or inactivation of pathogenic microorganisms in foods (Davidson and Zivanovic, 2003). Finally, the optimization of the use of probiotic lactobacilli for the gastrointestinal disorders requires the knowledge of their antibiotic resistance to reinforce the concomitant antibiotic therapy (Salminen *et al*., 1998). The present study was undertaken to screen out the potential LAB isolates producing bacteriocins from fermented vegetables and fish intestines.

Materials and Methods

Isolation of Lactic Acid Bacteria

Vegetable peels based fermented kitchen wastes were collected from dump yard of a local market at Rourkela, Odisha, India. Fresh water fishes such as Kau (*Anabas scandens)*, Singhi (*Heteroneuster fossilis)* were obtained from Pahad kata fish market, Rourkela, Odisha in living condition. The LAB were isolated from kitchen waste as well as fish intestine by selective enrichment procedure on MRS agar plate and selected by performing LAB specific morphological (gram's staining) and biochemical tests (Oxidase and catalase tests).

Selection of *Lactobacillus* sp. by specific biochemical tests and lactobacillus specific gene amplification

The isolated strains on *Lactobacillus* selective media (MRS agar) were subjected to some lactobacillus specific biochemical tests (gram staining, catalase activity and oxidase test) and *Lactoacillus* group specific gene amplification (~327 bp products) by using specific primers (Forward primer: 5'AGCAGTA GGGAATCTTCCA3' and Reverse primer: 'ATTYCACCGCTACACATG3') (Vanhoutte *et al*., 2004). Each PCR reaction consisted of 40.70µl dH_2O, 5 µl 10X buffer (HIMEDIA), 0.5µl 10 mM dNTPs (Chromus Biotech), 1 µl (10 µ mol) of each forward and reverse primer, followed by 1µl (1 U) Taq DNA polymerase (Himedia) and 2.5 µl of bacterial cell lysate (prepared by boiling lysis method). All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 52°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min. The generated PCR products (8µl) were then analyzed by electrophoresis on 1% agarose gel. The PCR positive isolates (confirmed as *Lactobacillus* sp.) were used for further analysis.

Bacteriocin preparation and bacteriocin assay

The bacteriocins from selected *Lactobacillus* sp. were purified partially according to Coventry *et al*., (1996).

Briefly, Cells were harvested from 48 hrs culture by centrifugation (12,000 × g for 30 min at 4^0C) and the supernatant was filtered (by syringe filter pore size, 0.45 µm). Crude concentrated preparations of bacteriocins were prepared by precipitating the filtered culture supernatants (2 litres) with 516 g of ammonium sulfate per liter at 4 ^{0}C overnight and resuspending the collected pellet (by centrifugation at 12,000 × g for 30 min at 4^0 C) in a minimum volume of sterile distilled water.

The antibacterial spectrum of the bacteriocin from above isolated cultures was determined using the well diffusion method (Takahiro *et al*., 1991). Aliquots (50 µl) of the sterile supernatant (filtered through a 0.45 µm pore size membrane filter) were placed in 4-mm-diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with the indicator bacteria (*P. vulgaris, B. subtilis, P. aeruginosa, E. coli, K. pneumoniae*). After 12-18 h of incubation, the diameters of the zones of growth inhibition were measured (Rammelsberg and Radler, 1990).

Identification of Lactic Acid Bacteria

Pure culture colonies of the selected isolates (six isolates) were characterized using morphology, cultural and biochemical characteristics (Table 2) (Rauta *et al*., 2011). The 16S rDNA sequence was amplified using 16S universal primers (B27F: 5' AGAGDDDGATCCPGGCTCAG 3' and U1492R: 5' GGTTACATTGTTACGAC TT 3') of 1.53 kb size. Every PCR reaction mixture was prepared according to same protocol as followed in amplifying *Lactoacillus* group specific gene. All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 49°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min.

PCR products were purified with a Gene Elute PCR DNA purification kit (Sigma Aldrich) and analyzed by agarose gel electrophoresis. Then, the purified PCR products were sent to Accelrys, Bangalore, India for sequencing.

The obtained sequences were then compared to sequences available in GenBank using the NCBI–BLAST program. Phylogenetic analysis was performed using the neighbour-joining algorithm with MEGA software (version 4.1) and the resulting tree was displayed with Tree View software (version 1.6.6) (Rauta *et al*., 2011).

All published *Lactobacillus* genomic sequences, obtained from GenBank were used to confirm the different relationships between the present isolates used in this study and others. Bootstrapping was performed to assess the confidence values of the clusters formed. Identification to the genomic species level was defined as a 16S rDNA sequence similarity above 99% with the query sequence (Drancourt *et al*., 2000).

Antibiotic sensitivity Test

Drug sensitivity of above selected isolates to various antibiotics (HiMedia, Mumbai) was assayed by Kirby–Bauer disc diffusion method on Mueller–Hinton agar (Himedia, Mumbai) plates as described by Bauer *et al*., (1966). The concentrations of tested antibiotics are given in Table 3.

Results and Discussion

Isolation of *Lactobacillus* strains has been accomplished by using *Lactobacillus* selective medium MRS agar (HIMEDIA). Sixteen strains has been isolated from kitchen waste and fish intestine and out of which, six Strains (KT1, KT2W, KT1T, KT1B, KA2 from kitchen waste and FS from fish intestine) were selected on the basis of LAB specific morphological and biochemical properties such as gram positive, oxidase negative, catalase positive bacilli (Bukola *et al*., 2008). Above six strains were also confirmed as belong to LAB by amplifying ~327 bp products (figure 1 and 2) from bacterial cell lysate using genus specific primer designed from regions of identity within the 16S ribosomal DNA (rDNA) sequence (Byun *et al*., 2004). All the strains were found to be facultative anaerobes.

As shown in Table 1, the antimicrobial spectra of LAB strains were assessed against *E. coli, P. aeruginosa, B. subtilis, P. vulgaris* and *K. pneumoniae.* All srains showed maximum inhibition against *E. coli* and *P. vulgaris*. All strains except KT2W (*L. casei)* showed inhibition zone against *P. aeruginosa.* Strain KT1 showed maximum inhibition zone against all tested microorganisms. Similarly, LAB were found to be effective against many bacterial species (Tripathy and Saini, 2012).

The stages of the pre-identification based on morphological aspects showed that LAB strains were Gram positive rods found in chain, singly and in pairs. Their colonies were circular (KTI, KT2W, KT1B, KT1T, KA2), flat (FS), low convex with entire margin, as also reported previously (Bukola *et al*., 2008; Byun *et al*., 2004; Tripathy and Saini, 2012). All the 6 isolates were subjected to different biochemical tests results (table 2). Most of the phenotypical and biochemical characteristics of strains, KT2W, KT1B, KT1T, KA2, FS were in accordance with published characters of *Lactobacillus casei* (Erdorul and Erbilir, 2006; Cai *et al*., 2007), whereas strain KT1 showed similarity with *Lactobacillus delbrueckii* (Divakara *et al*., 2010; Djilali *et al*., 2012).

Now a days, bacterial species identification using the 16S rDNA-based method is the most widely accepted, as large public-domain sequence databases are available in GenBank for comparison and also this method has substantially higher percentage accuracy as compared to the other conventional methods (Morgan *et al*., 2009). In the current study, the obtained 1318 bp of DNA fragments of strains KT1T, KT2W, KT1B, KA2, FS, coding the 16S rRNA (GB accession no. KC404970: KT1T, KC404971: KT2W, KC404972: KT1B, KC404973: KA2, KC404975: FS) after comparison with the sequences of 16S rRNA available in GenBank showed $\geq$ 99% homology with that of *L. casei* (GenBank accession no. D16552.1) (Figure 1, 2). Similarly, the obtained 16S rDNA sequence of strain KT1 showed $\geq$ 99% homology with that of *L. delbrueckii* (GenBank accession no. M58814.1). The phylogenetic tree was constructed using the above six isolated strains used in the current study and 18 *Lactobacillus* strains of different species, *L. delbrueckii* (M58814.1), *L. casei* (D16552.1), *L. acidophilus* (M58802.1), *L. amylolyticus* (Y17361.1), *L. amylovorus* (M58805.1), *L. brevis* (M58810.1), *L. fermentum* (AJ575812.1), *L. gastricus* (AY253658.1), *L. helveticus* (AM113 779.1), *L. intestinalis* (AJ306299.1), *L. jensenii* (AF243176.1), *L. johnsonii* (AJ002515.1),

Table.1 Bacteriocin Assay by Agar well diffusion method

Sl No.	Pathogen	KT2W	KT1	KT1T	KT1B	KA2	FS
1	*E. coli*	+++	+++	+++	++	++	++
2	*Pseudomonas aeruginosa*	+	+++	+	++	++	++
3	*Bacillus subtilis*	+++	+++	+++	+++	++	++
4	*Proteus vulgaris*	+++	+++	++	++	++	+++
5	*Klebsiella pneumoniae.*	++	+++	++	++	++	+++

Degree of inhibition: + = Moderate inhibition zone (6-9 mm); ++ = Strong inhibition zone (10-14mm); +++ = Very strong inhibition zone (15-18mm); - = No inhibition zone

Table.2 Biochemical characteristics of isolated LAB strains

Strain	KT2W	KT1	KT1T	KT1B	KA2	FS
Gram Staining	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Endospore	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Gas Prduction	-	-	-	-	-	-
Malonate	-	+	-	-	-	-
V.P.	-	-	-	-	-	-
Citrate	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+
Arginine	-	-	-	-	+	+
O.N.P.G.	-	-	-	-	-	-
Esculin Hydrolysis	+	+	-	+	+	-

Positive reaction (+), negative reaction (-)

Figure.1 Lactobacillus genus specific gene amplification of KT2W, KT1, KT1B

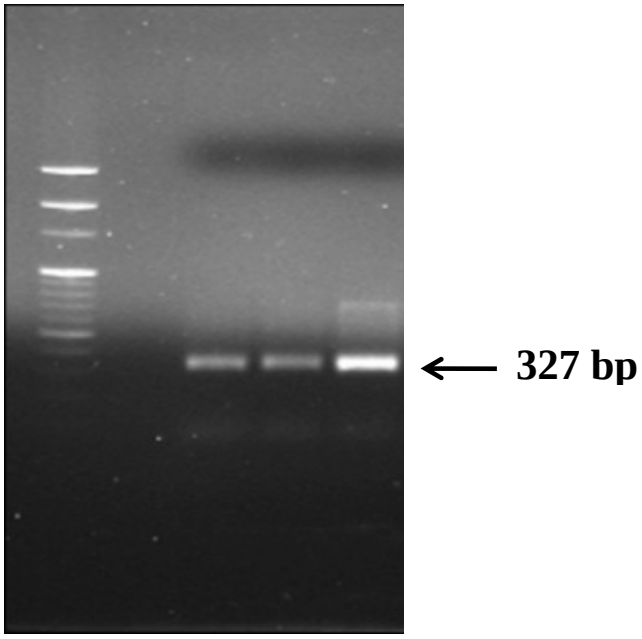


Figure.2 Lactobacillus genus specific gene amplification KT1T, KA2, FS

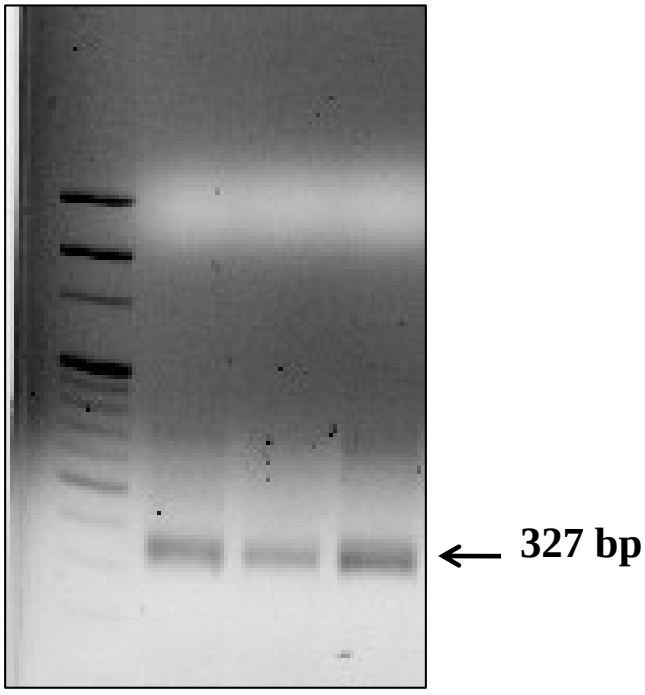


Table.3 Carbohydrate utilization study of isolated LAB strains.

Sl.No.	Tests	KT2W	KT1	KT1T	KTIB	KA2	FS
1	Lactose	+	+	+	+	+	+
2	Xylose	-	-	-	-	-	+
3	Maltose	+	+	+	+	+	+
4	Frucose	+	+	+	+	+	+
5	Dextrose	+	+	+	+	+	+
6	Galactose	+	+	+	+	+	+
7	Raffinose	-	-	-	-	-	-
8	Trehalose	+	-	+	+	+	+
9	Melibiose	+	-	+	+	+	+
10	Sucrose	+	_	+	+	+	+
11	L-Arabinose	+	_	+	+	+	+
12	Mannose	+	-	+	+	+	+
13	Inuline	+	-	+	+	+	+
14	Sodium gluconate	+	-	+	+	+	+
15	Glycerol	+	+	+	+	+	+
16	Salicin	+	+	+	+	+	+
17	Dulcitol	+	_	+	+	+	+
18	Inositol	+	_	+	+	+	+
19	Sorbitol	+	+	+	+	+	+
20	Mannitol	+	_	+	+	+	+
21	Adonitol	+	_	+	+	+	+
22	Arabitol	+	_	+	+	+	+
23	Erythritol	+	_	+	+	+	+
24	α-methyl D-glucoside	+	+	+	+	+	+
25	Rhamnose	-	_	-	-	-	-
26	Cellobiose	+	+	+	+	+	+
27	Melizitose	+	+	+	+	+	+
28	α-methyl D-Glucose	+	+	+	+	+	+
29	Xylitol	+	+	+	+	+	+
30	D-Arabinose	+	_	+	+	+	+
31	Sorbose	+	+	+	+	+	+

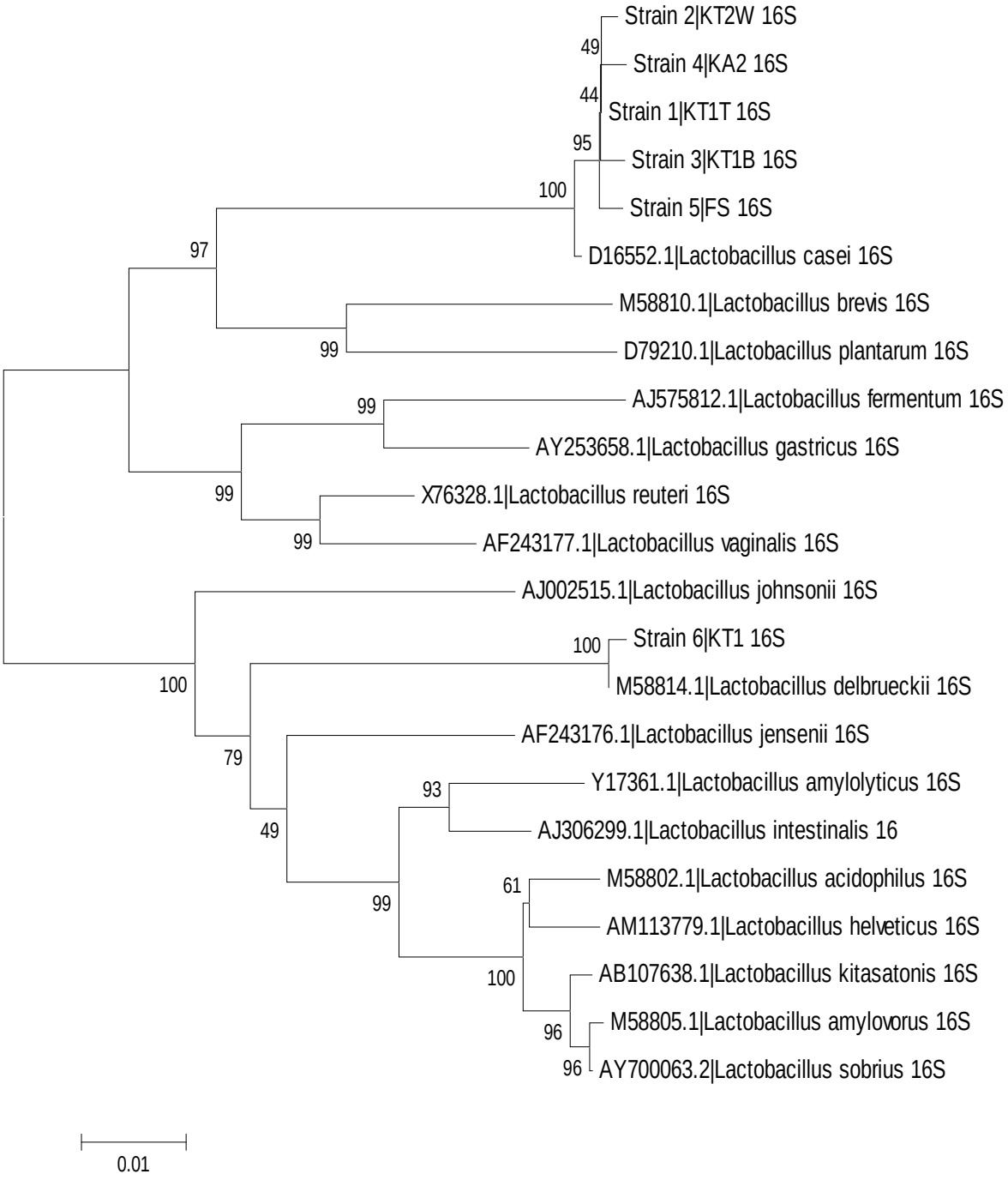
Positive reaction (+), negative reaction (-)

Table.4 AntiBiogram study of isolated LAB strains 19 different types of antibiotics

Sl.No.	Antibiotics	KT2W	KT1	KT1T	KTIB	KA2	FS
	ß-lactam						
1	Ampicillin	+	+++	+	+	+	+
2	Amoxycillin	+++	+++	+	+	+	+
3	Piperacillin	++	+++	+	+	+	+
4	Methicillin	+++	+++	+++	+++	+++	+++
	Peptides Glycopeptide						
5	Vancomycin	+++	+++	+	+++	+++	+
	Polypeptides						
6	bacitracin	++	+++	++	+++	+++	+
	Macrolides						
7	Erythromycin	+	+	+	+++	+	++
	Fenicols						
8	Chloramphenicol	+	+	+	+	+	+
	Nitrofurantoin						
9	Nitrofurantoin	+++	+++	+	+	+++	+++
	Aminoglycosides						
10	Amikacin	+++	+++	+++	+++	+++	++
11	Kanamycin	+	+++	+++	+++	++	+++
12	Streptomycin	++	+++	+++	+++	+++	+++
13	Gentamycin	+	+++	+++	++	+++	++
	Tetracyclines						
14	Tetracycline	+	+++	+	+	+	+
	Quinolones						
15	Norfloxacine	+++	+++	+	++	+++	+++
16	Lomefloxacin	+++	+++	+++	+++	+++	+++
17	Gatifloxacine	+	+++	+++	++	+++	+++
	Others						
18	Cephataxime	+	+++	+	+	+	+
19	Clindamycine	+	+++	+	+++	+	+++

(Resistant: +++, intermediate: ++, susceptible: +)

Figure. 3 Phylogenetic analysis of LAB isolates using 16S rDNA sequence.. The 16S rDNA sequences were aligned and used to construct the neighbour-joining phylogenetic tree. Scale bar indicates the genetic distance and the numbers shown next to each node indicate the bootstrap values from 1000 replicons**.**



L. kitasatonis (AB107638.1), *L. plantarum* (D79210.1), *L. reuteri* (X76328.1), *L. sobrius* (AY700063.2), *L. vaginalis* (AF243177.1) (figure 3). The constructed tree showed that five strains (KT1T, KT2W, KT1B, KA2, FS) are in the same cluster along with *L. casei* (D16552.1), where one strain (KT1) was in the same cluster with *L. delbrueckii* (M58814.1) (figure 3). This confirms the close relationship between the query sequences i.e. KT1T, KT2W, KT1B, KA2, FS with *L. casei* and KT1 with *L. delbrueckii*. From the above phenotypical, biochemical and molecular results, it may be concluded that the isolates i.e. KT2W, KT1B, KT1T, KA2, FS can be identified as is *L. casei* and isolate KT1 as *L. delbrueckii.*

The susceptibility of these LAB isolates tested against 19 different types of antimicrobial agents in the present study (table 3) showed that strain KT1 (*L. delbrueckii*) is resistant to the ß-lactam group of antibiotics (Ampicillin, Amoxycillin, Piperacillin) like similar results of Halami *et al*. 2000. But all other strains, KT2W, KT1B, KT1T, KA2, FS (*L. casei*) are susceptible to above mentioned antibiotics like Liasi *et al*. 2009. Methicillin is the only one the ß-lactam group of antibiotic, to which all six isolates are resistant. But, in the current study all the isolates are susceptible to erythromycin, chloramphenicol, nitrofurantion. In case of tetracycline, all isolates are susceptible except strain KT1.

All the six LAB isolates were mostly resistant to the aminoglycosides, sulfanamides and quinolones groups of antimicrobial agents (table 3). All LAB isolates in this study showed that they are resistant to gram-negative spectrum antibiotic (nalidixic acid), broad spectrum antibiotic (norfloxacine) and aminoglycoside antibiotics (amikacin, kanamycin, streptomycin and gentamycin). These findings matched with previous results that explain the resistance nature of most Lactobacillus strains used as probiotic to gram-negative spectrum and aminoglycoside antibiotics (Halami *et al*., 2000; Liasi *et al*., 2009).

The inhibitory effect demonstrated by *L. casei* and *L. delbrueckii* against the above mentioned bacteria is an indication of possession of probiotic properties to replace chemical antibiotics in animal and in fish feed industry. The study is extremely promising, that underscores the important role of *Lactobacillus* strains, having probiotic effects, which may play an important role in food industry as starter-culture, co-culture and bio protective cultures to improve quality and safety of preserved food and beverages.

References

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and Twick, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493–496.

Bukola, C.A. and Onilude, A.A. 2008. Screening of Lactic Acid Bacteria Strains Isolated from Some Nigerian Fermented Foods for EPS Production. World Applied Sciences Journal 4 (5): 741-747.

Byun, R., M.A. Nadkarni, K.L. Chhour, F.E. Martin, N.A. Jacques and Hunter, N. 2004. Quantitative Analysis of Diverse *Lactobacillus* Species Present in Advanced Dental Caries. J. Clin. Microbiol. 42(7): 3128–3136.

Cai, H., B.T. Rodrı´guez, W. Zhang, J.R. Broadbent and Steele, J.L. 2007. Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. Microbiol. 153: 2655–2665.

Cleveland, J., T.J. Montville, I.F. Nes and Chikindas, M.L. 2001. Bacteriocins: safe,

natural antimicrobials for food preservation. Int. J. Food Microbiol. 71: 1-20.

Coventry, M.J., J.B. Gordon, M. Alexander, M.W. Hickey and Wan, J. 1996. A food-grade process for isolation and partial purification of bacteriocins of Lactic acid bacteria that uses diatomite calcium silicate. Appl. Environ. Microbiol. 62(5): 1764–1769.

Davidson, P.M. and Zivanovic, S. 2003. Food antimicrobials. In: Davidson, P. M., Sofos, J. N. And Branen. A. L. Antimicrobials in foods.: CRC press, USA.

Divakara, R., B.K. Manjunatha and Paul, K.2010. Lactic acid bacteria as Probiotics: Role in human health. Res. Rev. Biomed. Biotechnol. 1(1): 1-5.

Djilali, B., A. Bouziane, H. Ahmed, I. kada and Nawal, O. 2012. Study of the behavior of *Lactobacillus delbrueckii* Subsp. *bulgaricus* in Date Syrup in batch fermentation with controlled pH. Biotechnology and Biomaterials. 2 (2): 1-5.

Drancourt, M., C. Bollet and Carlioz, A. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38: 3623–3630.

Erdorul, Ö. and Erbilir, F. 2006. Isolation and characterization of *Lactobacillus bulgaricus* and *Lactobacillus casei* from various foods. Turk. J. Biol. 30: 39-44.

Halami, P.M., A. Chandrashekar and Nand, K. 2000. *Lactobacillus farciminis* MD, a newer strain with potential for bacteriocin and antibiotic assay. Lett. Appl. Microbiol. 30: 197–202.

Hammes, W.P., N. Weis and Holzapfel, W.P. 1991. The genera *Lactobacillus* and *Carnobacterium*. In: *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, A. Balows, H. G. Truper, M. Dworkin, W. Harder, K. H. Schleifer (Eds.), Springer, New York. (1991). pp. 1535-1594.

Herich, R. and Levkut, M. 2002. Lactic acid bacteria, probiotics and immune system. Vet. Med. Czech. 47(6): 169–180.

Liasi, S.A., T.I. Azmi, M.D. Hassan, M. Shuhaimi, M. Rosfarizan and Ariff, A.B. 2009. Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. Malays J. Microbiol. 5(1): 33-37.

Meera, N.S. and Charitha Devi, M. 2012. Partial characterization and optimization of parameters for bacteriocin production by probiotic Lactic acid bacteria. J. Microbiol. Biotech. Res. 2 (2):357-365.

Morgan, M.C., M. Boyette, C. Goforth, V.S. Katharine and Shermalyn, R. 2009. Comparison of the Biolog OmniLog Identification System and 16S ribosomal RNA gene sequencing for accuracy in identification of atypical bacteria of clinical origin. Greece J. Microb. Meth. 79: 336–343.

Rammelsberg, M. and Radler, F. 1990. Antibacterial polypeptides of Lactobacillus species. J. Appl. Bacteriol. 69:177-184.

Rauta, P.R., K. Kumar and Sahoo, P.K. 2011. Emerging new multi-drug resistant bacterial pathogen, *Acinetobacter baumannii* associated with snakehead *Channa striatus* eye infection. Curr. Sci. 101(4):548-553.

Salminen, S., A. Von Wright, L. Morelli, P. Marteau, D. Brassart, W.M. De Vos, R. Fonden, M. Saxelin, K. Collins, G. Mogensen, S.E. Birkeland and Mattila-Sandholm, T. 1998. Demonstration of safety of probiotics—a review. Int. J. Food Microbiol. 44:93–106.

Schleifer, K.H. and Ludwig, W. 1995. Phylogenetic relationships of lactic acid bacteria. In: The Genera of Lactic Acid Bacteria, B. J. B. Wood, W. H. Holzapfel (Eds.), Blackie Academic & Professional, Glasgow. pp. 1–18.

Takahiro, T., Y. Emiko and Takatoshi, I. 1991. Lacticin, a bacteriocin produced by *Lactobacillus delbrueckii* sub sp. *Lactis*. Lett. Appl. Microbiol. 12: 43-45.

Tripathy, P.P. and Saini, M.R. 2012. Spectrum of antimicrobial activity of lactic acid bacteria (*Lactobacillus* KSBT 56) isolated from indigenous fermented products of Odisha. Afr. J. Food Sci. 6(24): 560-566.

Vanhoutte, T., G. Huys, E. Brandt and Swings, J. 2004. Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and groupspecific 16S rRNA gene primers. FEMS Microbiol. Ecol. 48: 437–446.