

Isolation and screening of *Lactobacillus* species from dogs for probiotic action

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ABSTRACT

The present study aimed at the detection of suitable *Lactobacillus* species from dogs for usage as probiotic. A total of 67 rectal swabs from healthy pups were analyzed and 49 (73.1%) *Lactobacillus* isolates were identified based on morphological, biochemical characteristics and confirmed by genus specific PCR targeting 16S rRNA gene. A total of 20 isolates that showed strong aggregation, high cell surface hydrophobicity, acid and bile tolerance were screened for *in-vitro* antibacterial activity by agar well diffusion assay, where prominent inhibitory zones were observed against majority of the test pathogens. Reduction in antibacterial activity was noticed after neutralization, proteinase K and heat treatment of supernatants. Partial 16S rRNA nucleotide sequence analysis of seven *Lactobacillus* isolates that showed prominent *in-vitro* antibacterial activity revealed maximum sequence homology with *Lactobacillus fermentum* (for six isolates) and *Lactobacillus agilis* (for one isolate). The *in-vitro* antibacterial activity results highlighted the need for *in-vivo* studies in India to establish probiotic potential of canine faecal *Lactobacillus* species in the near future.

Key words: Dogs, *In-vitro* antibacterial activity, *Lactobacillus*, Probiotic.

INTRODUCTION

Probiotics are live micro-organisms, which when consumed in adequate amounts confer a health benefit on the host (Sanders, 2008). Probiotic agents exert beneficial effects through a wide array of actions *viz.*, receptor competition, production of antimicrobial substances (such as lactic acid, free radicals, acetaldehyde, antioxidants, hydrogen peroxide and antibacterial peptides like bacteriocins), immunomodulation, degradation of toxins (biodegradation), inhibition of pathogen adhesion, stimulation of brush border enzyme activity and maintenance of intercellular integrity of tight junctions (Oyetayo and Oyetayo, 2005). Despite the increasing interest on probiotics as a therapeutic tool, there are only a few studies focused on their use in companion animals (Baillon *et al.*, 2004 and McCoy and Gilliland, 2007).

Lactic acid bacteria (LAB) of the genus *Lactobacillus* constitute the major group of microbes to be used as probiotics (Baillon *et al.*, 2004). Choosing correct LAB strain is vital as not all strains have the same beneficial effects. For successful use as a probiotic in canines, the *Lactobacillus* species should be of canine intestinal origin as these species exhibit host specificity (McCoy and Gilliland, 2007). Perusal of the available literature revealed the need for a novel probiotic strain of canine origin in India. Keeping this in view, the present study was carried out with an objective of detection of suitable *Lactobacillus* spp. from dog faeces for usage as probiotic.

MATERIALS AND METHODS

Reference strains: *Lactobacillus acidophilus* (*L. acidophilus*) (MTCC 10307), *L. delbrueckii* sub-species *lactis* (MTCC 911), *L. plantarum* (MTCC 9496), *L. rhamnosus* (MTCC 1408) and *Salmonella enterica* serovar Typhimurium (MTCC 3231) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh (India). Culture of *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603) and *Salmonella enterica* serovar Enteritidis (ATCC 13076) were procured from M/s. Hi-Media Laboratories (Mumbai).

Collection of samples: Rectal swabs were collected from a total of 67 healthy pups (less than six months of age) of different breeds *viz.*, mongrel dogs (27), labrador (20), pomeranian (20) and transferred to pre-reduced de Man, Rogosa and Sharpe (MRS) broth (with 0.05% L-cysteine) immediately after collection. The duration of study lasted from October 2015 to July 2016.

Isolation and biochemical characterization of *Lactobacillus* species:

Rectal swabs in pre-reduced MRS broth were incubated under micro-aerophilic conditions in candle jar at 37°C for 48 hours. Loopful enriched broth culture was streaked onto MRS agar plates. Presumptive *Lactobacillus* colonies (2-3 mm, pale white/yellow, transparent) were examined for Gram's reaction (Gram positive rods), spore staining (non-spore formers), motility (non-motile), haemolysis (non-haemolytic) and biochemical tests like catalase (negative), oxidase (negative), indole

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(negative), methyl red (variable), Voges-Proskauer (variable), citrate utilization (negative), nitrate reduction (negative), sugar fermentation (positive for glucose, maltose, sucrose, lactose and negative for mannitol) and milk coagulation (positive) (Sneath and Holt, 2001).

Confirmation of *Lactobacillus* spp. by genus specific PCR:

Whole cell DNA of all *Lactobacillus* isolates was extracted by boiling and snap chilling method (Wani *et al.*, 2006) and was subjected to genus specific PCR using genus specific primers (Lac 1F, 5'-AGCAGTAGGGAATCTTCCA-3' and Lab-0677R, 5'-CACCGCTACACATGGAG-3') targeting 16S rRNA gene (Garcia-Mazcorro *et al.*, 2011) with optimized 25 μ l reaction mixture containing 1.5 μ l of DNA template prepared from each isolate; 2.5 μ l 10x *Taq* buffer; 0.5 μ l 10mM dNTP mix; 1.5 μ l 25mM MgCl₂; 10 pmol forward primer; 10 pmol reverse primer; 1 U *Taq* DNA polymerase and 16.0 μ l nuclease free water to make up the volume to 25 μ l, under the following standardized thermal cycling conditions: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, elongation at 72°C for 1 min and final elongation at 72°C for 7 min, with an expected amplicon size of 341 bp. The DNA of *L. acidophilus* (MTCC 10307) was used as positive control. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and the bands were visualized under UV transilluminator (Alpha Innotech Gel Documentation system, USA).

Aggregation test: Overnight grown *Lactobacillus* cultures were kept undisturbed in Eppendorf tubes and examined after 15 min, in order to determine the degree of aggregation to the bottom of the tubes (Vineetha *et al.*, 2016).

Cell surface hydrophobicity: Cell surface hydrophobicity was determined by microbial adherence to hydrocarbons (MATH) method using xylene as solvent as described by Vineetha *et al.* (2016). Overnight grown *Lactobacillus* cultures were centrifuged (7500 rpm for 5 min at 4°C), washed twice with PBS buffer and finally resuspended in the same buffer. The initial absorbance (A_0) at 600 nm was measured. Two ml of bacterial suspension was then transferred into round bottom test tube and 0.4 ml of xylene was added. Tubes were vortexed for 2 min and then left undisturbed for 1 hour to allow the phase separation. The lower aqueous was separated and absorbance (A_1) was measured at 600 nm. Hydrophobicity was calculated as the percentage (H%) decrease in the absorbance of the bacterial suspension using the following formula, $H\% = (1 - A_1/A_0) \times 100$.

Acid tolerance test: Overnight *Lactobacillus* cultures were inoculated into MRS broth previously adjusted to pH values 2, 3, and 6.5 (control) with 1N HCl or NaOH, and incubated at 37°C overnight under micro-aerophilic conditions. The viability was checked by plating onto MRS agar (Khalil *et al.*, 2007).

Bile tolerance test: Overnight cultures were inoculated in MRS broth containing 0.15, 0.3 and 1.0% bile salt, and incubated at 37°C overnight under micro-aerophilic conditions. Absorbance was measured at 600 nm. MRS broth without bile was kept as control. (Khalil *et al.*, 2007).

Detection of *in-vitro* antibacterial activity of *Lactobacillus* species:

Antibacterial activity of the dog faecal *Lactobacillus* isolates towards specific serotypes of *E. coli* (Rough, O141, O20, O49, O128), *Klebsiella* and *Enterobacter* species isolated from diarrhoeic dogs as well as towards reference strains *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), *S. typhimurium* (MTCC 3231) and *S. enteritidis* (ATCC 13076) was studied by agar well diffusion assay using Mueller Hinton (MH) agar. Cell free supernatant of *Lactobacillus* isolates was prepared by centrifugation of 48 h old *Lactobacillus* MRS broth culture at 10,000 rpm at 4°C for 20 minutes and then filter-sterilized using 0.22 μ m syringe filters. The inoculums of test pathogens were spread on MH agar to get lawn culture. Cell free-supernatant (100 μ l) of *Lactobacillus* isolates was then added into pre-sealed wells (7 mm) made in the MH agar and incubated aerobically at 37°C for 18-24 hours. The degree of inhibition of test pathogens was interpreted as high (> 15mm diameter of zone of inhibition, +++), medium (10-15 mm diameter of zone of inhibition, ++), low (< 10 mm diameter of zone of inhibition, +) and absent (-) (Nair, 2000).

Factors influencing probiotic potential of *Lactobacillus* species:

To determine the nature of inhibitory substances, cell free supernatants of *Lactobacillus* isolates were neutralized with 1N NaOH, boiled at 100°C for 15 min, treated with proteinase K @ 1 mg/ml, 37°C for 1h in triplicate and inhibitory action was re-checked (Bilkova *et al.*, 2011).

Sequencing: Genus specific PCR products of *Lactobacillus* isolates that showed prominent *in-vitro* antibacterial effect were sequenced by Sanger sequencing, submitted to the Genbank and compared with known 16S rRNA gene sequences by NCBI – BLAST.

RESULTS AND DISCUSSION

A total of 49 (73.1%) isolates showed morphological and biochemical features in accordance to genus *Lactobacillus* (Fig. 1) and found to be positive for 16S rRNA gene in genus specific PCR (Fig. 2). Out of 49, about 28 isolates showed a high degree of aggregation by settling down within 15 min and high hydrophobicity above 70%. Taheri *et al.* (2009) showed that the strains with high aggregation (short aggregation times) had better attachment to epithelial cells and high adhesion ability to mucus. In the present study, all these 28 isolates with short aggregation time and high hydrophobicity were selected for further screening as these properties indicated the adhesion ability to gastrointestinal tract (GIT) epithelium. Our results were in agreement with Vineetha *et al.* (2016) from India who reported higher cell



Fig. 1: A) MRS agar plate showing pure white transparent colonies of *Lactobacillus* species
B) MRS blood agar plate showing non-haemolytic colonies of *Lactobacillus* species

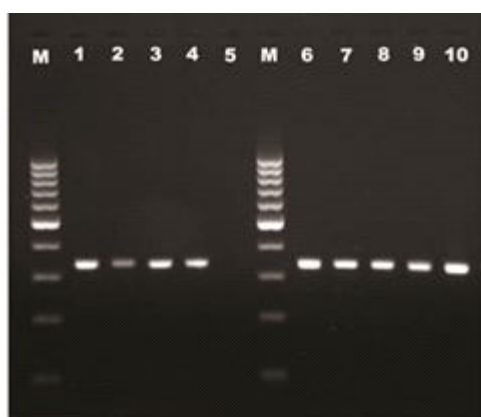


Fig. 2: Confirmation of dog faecal *Lactobacillus* spp. by genus specific PCR targeting 16S rRNA gene (341 bp). Lane M: Molecular weight marker (100 bp), Lane 1: DNA standard of *L. delbrueckii* subsps *lactis* (MTCC 911), Lane 2: DNA standard of *L. acidophilus* (MTCC 10307), Lane 3: DNA standard of *L. rhamnosus* (MTCC 1408), Lane 4: DNA standard of *L. plantarum* (MTCC 9496), Lane 5: Negative control, Lane 6-10: Dog faecal *Lactobacillus* isolates positive for 16S rRNA gene (341 bp).

surface hydrophobicity (above 76%) for *Lactobacillus* isolates. Ehrmann *et al.* (2002) reported that values higher than 66% were regarded as strong hydrophobicity. Resistance to low pH and bile salts is a characteristic feature of probiotic strains for survival in the GIT (Vineetha *et al.*, 2016). A total of 20 (L2, L6, L8, L11, L12, L13, L14, L15, L16, L17, L18, L20, L26, L31, L33, L35, L36, L38, L41 and L42) out of 28 isolates showed satisfactory growth at pH 2 and 3, while other isolates showed very meager growth. All these 20 isolates showed good turbidity at 0.15% bile salt concentration, while growth was inhibited at 0.3 and 1% bile salt concentration. The present results corroborate with the findings of Taheri *et al.* (2009) and Vineetha *et al.* (2016).

The supernatants of the selected 20 isolates showed inhibition against majority of the test pathogens examined (Fig. 3.A). However, the degree of inhibition as measured by the diameter of zone of inhibition varied with each test pathogen and *Lactobacillus* isolate studied (Table 1). Prominent inhibition zones i.e. above 15 mm (+++) were observed in a total of 6 isolates (L8, L12, L15, L16, L18 and L38) against 9 out of 11 test pathogens examined, 4 isolates (L13, L26, L33 and L42) against 5 out of 11 test pathogens examined, one isolate (L6) against 6 out of 11 test pathogens examined, one isolate (L2) against 5 out of 11 test pathogens examined, 5 isolates (L11, L14, L17, L35 and L41) against 1 out of 11 test pathogens examined, whereas in 3 isolates (L20, L31 and L36) inhibition zones above 15 mm (+++) were not observed (Table 1). The differences among antimicrobial activities displayed by different strains tested were supposed to be due to differences in production of organic acids like lactic acid or proteinaceous inhibitory substances (Martin *et al.*, 2010). Antibacterial activity was found to be prominent (with inhibition zones above 15 mm) against O49, O128 and rough (R) serotypes of *E. coli* compared to O20 and O141 *E. coli* serotypes (Table 1). The inhibition zones were large and clear against the reference strains of *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), *S. typhimurium* (MTCC 3231) and *S. enteritidis* (ATCC 13076) but limited and hazy zones were observed against *Enterobacter* species. Silva *et al.* (2013) from Brazil reported promising *in-vitro* antibacterial activity in *Lactobacillus* isolates of dog faecal origin against *E. coli* and other pathogenic bacteria. Vineetha *et al.* (2016) from India reported promising *in-vitro* antibacterial activity in *Lactobacillus* isolates of poultry faecal origin against *E. coli*, *S. typhimurium* and *S. enteritidis*.

When the pH of the *Lactobacillus* supernatants was adjusted to pH 7.0, reduction in antibacterial activity was noticed against all the test pathogens (Fig. 3.B), suggesting that

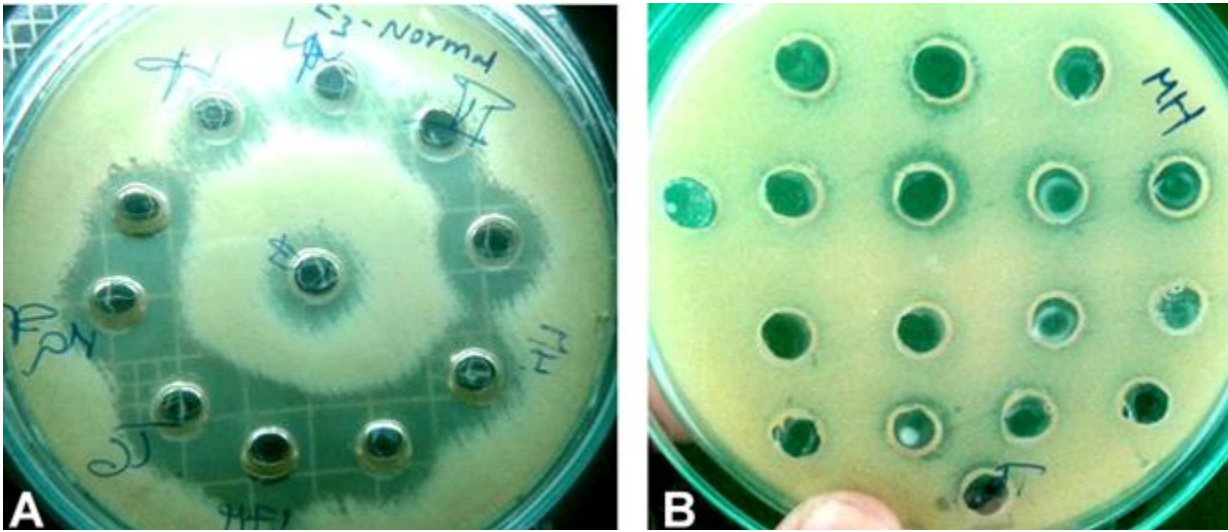


Fig. 3: A) *In vitro* inhibitory effect of canine faecal *Lactobacillus* cell free supernatants on *E. coli* (Rough serotype) B) Reduction in the *in vitro* inhibitory effect of canine faecal *Lactobacillus* cell free supernatants after neutralization.

Table 1: *In vitro* antibacterial activity of dog faecal *Lactobacillus* isolates on test pathogens.

<i>Lactobacillus</i> isolates (Dog breed)	Test pathogens										
	<i>Escherichia coli</i>						<i>K. pneumoniae</i> <i>Enterobacter</i>				
	ATCC 25922	Rough	O20	O49	O128	O141	ATCC 700603	Isolate K3	Isolate E25	<i>Salmonella</i> <i>typhimurium</i> (MTCC 3231)	<i>Salmonella</i> <i>enteritidis</i> (ATCC 13076)
L2 (M)	++	+++	++	+++	+++	++	++	++	+	+++	+++
L6 (L)	+++	+++	-	+++	+++	++	++	++	+	+++	+++
L8 (M)	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
L11 (M)	+++	++	+	++	++	-	++	++	+	++	++
L12 (M)	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
L13 (P)	+++	+++	++	+++	+++	++	++	+++	++	++	++
L14 (M)	++	++	++	++	++	++	++	+++	+	++	++
L15 (M)	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
L16 (M)	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
L17 (L)	+++	++	++	++	++	-	++	++	+	+	+
L18 (L)	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++
L20 (M)	++	++	++	++	++	++	++	++	+	+	+
L26 (M)	+++	+++	++	+++	+++	++	++	+++	++	++	++
L31 (P)	++	++	+	++	++	-	++	++	+	+	+
L33 (M)	+++	+++	++	+++	+++	++	++	+++	++	++	++
L35 (M)	+++	++	++	++	++	++	++	++	+	++	++
L36 (L)	++	++	-	++	++	+	++	++	+	+	+
L38 (L)	+++	+++	+++	+++	+++	+++	++	+++	+	+++	+++
L41 (M)	+++	++	++	++	++	++	++	++	+	+	+
L42 (P)	+++	+++	++	+++	+++	++	++	+++	++	++	++

+++ Represents inhibition zone diameter above 15 mm (upto 17 mm)

++ Represents inhibition zone diameter between 10-15 mm

+ Represents inhibition zone diameter less than 10 mm

- Represents absence of inhibition zone

(M) – Mongrel; (P) – Pomeranian; (L) – Labrador

inhibition effect might be in part related to the organic acids. In addition, reduction in the antibacterial activity was noticed after proteinase K and heat treatment of supernatants, indicating that inhibition effect might also be in part related to heat labile antimicrobial proteins. Prominent antibacterial effect in canine *Lactobacillus* isolates on pathogenic bacterial strains was reported from earlier studies in India (Kumar *et al.*, 2017) and other countries like Uruguay (Perelmuter *et al.*, 2008) and Slovakia (Strompfova and Laukova, 2014).

The partial 16S rRNA nucleotide sequences of seven isolates that showed promising antibacterial activity (inhibition zones > 15 mm), were submitted to the Genbank database. The NCBI accession numbers for isolates L12, L13, L15, L16, L18, L26 and L33 were KX929077, KX929079, KX929080, KX929081, KX929082, KX929083 and KX929084, respectively. A total of six isolates (L12, L13, L15, L16, L18 and L33) showed nucleotide sequence similarity of 99% with *L. fermentum* strain RCM 14 and one isolate (L26) with *L. agilis* strain 76CL. Beasley *et al.* (2006) from Finland reported LAB in 67% of dog faecal samples of which *Lactobacillus casei* (11.8%), *L. salivarius* (11.8%), *L. rhamnosus* (11.1%), *L. mucosae* (10.5%) and *L. fermentum* (9.2%) were frequently encountered. In a study from Finland, Tang *et al.* (2012) identified seventy-four lactobacilli isolates from beagle dogs by 16S rRNA gene sequencing, in which *L. acidophilus* was reported to be most dominant followed by *L. murinus*, *L. reuteri*, *L. johnsonii*. Vineetha *et al.* (2016) from India reported detection of *L. plantarum* and *L. reuteri*

from the GIT of guinea fowl by 16S rRNA gene sequencing. Of the seven *Lactobacillus* isolates (six *L. fermentum* and one *L. agilis* strains) that showed prominent *in-vitro* antibacterial activity in the present study, five strains were isolated from mongrel pup faeces, one from pomeranian and one from labrador pup faeces. Benno *et al.* (1992) and Greetham *et al.* (2002) reported higher *Lactobacillus* count in indigenous dogs of younger age with colonization resistance against pathogens. Kumar *et al.* (2016) from India reported the superiority of canine-origin probiotic over the dairy-origin in improving the metabolic response and overall health of dogs.

For further usage of lactobacilli in clinical trials for probiotic action, information regarding colonization patterns, safety and other properties of *Lactobacillus* strains in dogs is essential. As the present study demonstrated the prominent *in-vitro* antibacterial activity, it would seem logical to pursue further studies regarding this organism. Future studies should focus on increasing the number of samples and clinical trials, so that suitable *Lactobacillus* strain as probiotic could be selected for therapeutic purpose as well as for preserving the healthy gut microbiota of canines.

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