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

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Received: 01-05-2017 | Accepted: 21-06-2017

**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 exerts 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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overnight grown 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. Absorbance was measured at 600 nm. MRS broth without bile was kept as control. (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 µm syringe filters. The inoculums of test pathogens were spread on MH agar to get lawn culture. Cell free-supernatant (100µl) 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
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...
Table 1: *In-vitro* antibacterial activity of dog faecal *Lactobacillus* isolates on test pathogens.

<table>
<thead>
<tr>
<th>Lactobacillus isolates (Dog breed)</th>
<th>Escherichia coli</th>
<th>K. pneumoniae</th>
<th>Enterobacter</th>
<th>Salmonella typhimurium (MTCC 3231)</th>
<th>Salmonella enteritidis (ATCC 13076)</th>
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<tr>
<td>ATCC 25922</td>
<td>Rough O20 O49 O128 O141</td>
<td>ATCC 700603 Isolate K3</td>
<td>Isolate E25</td>
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<td>L2 (M)</td>
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<td>L42 (P)</td>
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+++ 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

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.
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 (Perelmutter 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.

ACKNOWLEDGEMENT

Financial support as postgraduate fellowship and other contingency support for the work was provided by Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh.

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