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ISOLATION AND CHARACTERIZATION OF A LIPOLYTIC AND PHYTASE PRODUCING PROBIOTIC FOR POTENTIAL APPLICATION IN POULTRY FEED

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ABSTRACT: *In the current study a total of 35 bacterial isolates from 17 food and fecal samples were examined. Five among those were earmarked as putative probiotic candidates. All the selected isolates survived the low pH conditions of 2.0, and resisted the presence of bile salts (0.02-0.25%) and NaCl (2-14 %), indicating their ability to survive in the gastrointestinal (GI) tract conditions and hence making them suitable candidates for probiotic applications. The selected probiotic isolates showed considerable levels of hydrophobicity indicating their potential adhering properties with the gut epithelium. In addition the five selected probiotic candidates depicted substantial antagonistic action against potent pathogens like Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas aeruginosa, P. alcaligenes, Staphylococcus aureus and Streptococcus sp. The isolates CM-4 and KD-7 were most remarkable as they inhibited all the pathogens tested including S. aureus and Streptococcus sp. Extracellular enzymatic studies showed that all the five strains produced phytase whereas isolate CM-4 and KD-7 were the only lipase producers found. However no amylase protease activity was detected. Isolate CM-4 was found to be the best among all five as it showed all desirable probiotic features viz. bile salt, NaCl and pH tolerance, maximum hydrophobicity, antagonistic action against pathogens, phytase and lipase activity, therefore was identified by using 16S rDNA sequencing and MEGA BLAST.*

ORIGINAL ARTICLE

Key words: *Probiotics, Phytase, Lipases, Hydrophobicity, Antibacterial Activity*

INTRODUCTION

Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance. The probiotic bacteria most commonly studied include members of the genera *Lactobacillus* and *Bifido bacterium*. *Saccharomyces boulardii, Escherichia coli* and *Enterococcus* strains are used as probiotics in non-food format (Holzapfel et al., 2001). Correspondingly, in feed regulation, probiotics are included in the group of feed additives for stabilising the microbial communities of the digestive tract in monogastric animals and ruminants. They are also known as digestive bioregulators or direct-fed microbials (DFMs). In a narrower sense, the term probiotics is confined to products which consist of one, or a few, well-defined strains of microorganisms. Besides imparting all these beneficial attributes probiotics are also known to produce various enzymes that help in the digestion of monogastric animals, phytases and lipases being the important ones (Khattak et al., 2006). Phytic acid (*myo*-inositol hexakis phosphate, phytate) is the major storage form of phosphorus in cereal, oil and legume (Khattak et al., 2006). Phytase, a specific group of phosphatase hydrolizes phytic acid to myo-inositol and phosphoric acid. In terms of animal nutrition, monogastric animals such as swine, poultry and human are not capable of metabolizing phytate phosphorus owing to the lack of digestive enzymes hydrolyzing the substrate, and therefore, inorganic phosphate is added to their diet to meet the phosphorus requirement, while undigested phytate phosphorus is excreted in manure and poses a serious phosphorus pollution problem, contributing to the eutrophication of surface water in areas of intensive livestock production (Bajaj and Wani, 2011). In addition, phytic acid also acts as an anti-nutritional agent forming complexes with proteins and various metal ions, thereby decreasing the dietary bioavailability of these nutrients. Recently, microbial plants and animal-derived phytases have been made available as feed supplements. They have become the most popular and widely used enzymes in animal farming systems. Due to the ever increasing incidences of bacterial antibiotic resistance, the European Union (EU) has decided to ban antibiotics as feed additives from 1st January 2006 onwards (Simon, 2005). Therefore, a massive hunt has been launched to establish other substances with beneficial effects on animals via modifications of the intestinal microbiota. Among these so called "alternatives to antibiotics" important ones are probiotics. Probiotics due to their ability to create a healthy equilibrium between beneficial and potentially harmful

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microorganisms in the gut by competitive exclusion, and by the production of organic acids etc. are found to be the leaders in feed supplements. The health benefitting attributes of probiotics show wide variation with respect to strain, species and genus therefore, the quest for novel strains with superior health benefitting features is never ending. In the current study a total of 35 lactic acid bacteria (LAB) isolated from food and fecal samples were screened for their probiotically important parameters. The isolate CM-4 which had the most of the desirable probiotic features was identified using 16S rDNA sequencing.

MATERIALS AND METHODS

Chemicals and bacterial pathogens

All chemicals and media components used in this study were purchased from Himedia, Sigma, Ranbaxy and SD fine chemicals, and were of analytical quality. The pathogenic cultures used in this study were: *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas aeruginosa, P. alcaligenes, Staphylococcus aureus* and *Streptococcus* sp., and were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Isolation of bacteria

Bacteria were isolated from samples collected from various sources viz. cow or goat milk, curd, cheese, or cattle excreta. The samples were collected in sterile vials and maintained at 4^{o}C till processed further. For isolation of LAB, the samples were enriched in MRS broth supplemented with acetic acid (1ml/l) and gentamycin (100mg/l), for 18 h and then spread plated on MRS agar and incubated in anaerobic chamber (HI-MEDIA) for 24-48 h. Pin head sized colonies were selected, pure cultured and maintained on skimmed milk broth (10 % skimmed milk and 5 % pH indicator). The cultures were revived after every two weeks. A total of 17 samples were used and 35 pin head colonies were picked up and subjected to morphological and biochemical tests. Five putative isolates CM-4 (from cow milk), KD-7 (from kudan, an indigenous milk product produced upon prolonged boiling of milk), HD (from horse dung), GD (from goat dung) and GM (from goat milk), were studied according to Bergey's Manual of Determinative Bacteriology (Garrity et al., 2004).

Study of probiotically important features of LAB isolates

For studying the ability of isolated LAB to grow over (and tolerate) wide range of pH the MRS broth was adjusted at different pH (2-12) using 1N HCl or 1N NaOH. The LAB cultures were activated by growing them in MRS broth for 18 h and then used this cell suspension for inoculation (@1%, v/v) into different MRS broth tubes (having varying pH). For assessing the ability of LAB to grow in presence of different concentrations of sodium chloride (2-14 %, w/v) and bile salts (0.02-0.25 %, w/v), the MRS broth was added with corresponding amounts of NaCl and bile salts. Incubation at 37˚C under static conditions was given for 24 h and growth was measured spectrophotometerically at 660nm using a UV-VIS spectrophotometer (λ 35, PerkinElmer, USA). Growth in MRS broth at pH 6.5 served as control while assaying growth for various parameters.

Assaying antibacterial activity of LAB

For determining antibacterial activity, the selected LAB was grown in MRS broth for 24 h at 37˚C under static conditions. Samples drawn at various time intervals were centrifuged at 8000g for 5 min (Sigma, 3K30) and the supernatant was filtered through a bacterial filter (Whatman, 0.22 µ). The filtrate obtained was used for assaying the antimicrobial activity after adjusting the pH to 6.5. The test organisms (*Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas aeruginosa, P. alcaligenes, Staphylococcus aureus* and *Streptococcus* sp.) were cultivated for 18 h at 37˚C (A_{660} 0.8) in Luria-Bertani (LB) broth and spread plated on Mueller-Hinton (MH) agar plates. Wells of 6mm were cut and 50µl supernatant of the test isolates was poured into each well and incubated for 24-48 h at 37 ˚C. Antimicrobial activity was analyzed by measuring the zone of inhibition around the well.

Qualitative assay for enzymes

The five selected strains were examined for their ability to produce extracellular enzyme (i.e., amylase, protease, lipase, and phytase) activities according to Kim et al. (2007) with minor modifications. To detect the amylase, lipase, and protease activities, the selected LAB were subcultured and then centrifuged and the supernatant was considered as the crude enzyme. Amylase activity was examined using starch agar medium (starch 0.25%, and agar 1.5%). For detecting the clear zones of amylase activity, iodine solution was poured over the plates. For lipase activity, the MRS broth was used to subculture the strains. Activity was detected by using a medium that consisted of nutrient agar and tributyrin oil (1%, v/v). For detection of protease activity, the strains were cultured in MRS broth and after anaerobic incubation for 24 h at 37°C, 50 µl of culture supernatant was transferred to wells in the medium consisting of skim milk (0.5%) and agar (1.5%). Clear zone surrounding each well was measured.

Phytase activity was measured using sodium phytate as substrate. The MRS broth that contained 0.25% sodium phytate (HIMEDIA) was used to subculture the strains and the medium consisted of (%, w/v): glucose 1.5, sodium phytate 0.5, NH_4NO_3 0.5, KCl 0.05, $MgSO_4 \cdot 7H_2O$, 0.05, $MnSO_4 \cdot 7H_2O$ 0.02, $FeSO_4 \cdot 7H_2O$ 0.001 and agar 1.5 (pH 7.0). The cultural supernatant was put in the wells cut on the medium of the same composition. Appearance of halos was suggestive of phytase production.

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Cell surface hydrophobicity test:

Cell surface hydrophobicity was determined by the method of Rosenberg et al. (1980). Cultures were grown in 5 ml of MRS broth, centrifuged at 7500 g for 5 min and the cell pellet was washed with 9 ml of Ringer solution (%, w/v): NaCl 6, KCl 0.0075, $CaCl_2$ 0.01 and $NaHCO_3$ 0.01, re-suspended in a cyclomixer and washed thrice. Then 1 ml of the suspension was taken and the absorbance at 580nm was measured. Then 1.5 ml of the cell suspension was mixed with 1.5 ml of n-hexadecane in triplicate and mixed thoroughly in a cyclomixer for 2 min. The two phases were allowed to separate for 30 min. Then 1 ml of the lower phase was taken and the absorbance at 580nm was measured. The percentage of hydrophobicity of strain adhering to hexadecane was calculated using the equation:

$$\text{Percentage of hydrophobicity} = \frac{A_{580}\,(\text{before mixing}) - A_{580}\,(\text{with hexadecane}) \times 100}{A_{580}\,(\text{before mixing})}$$

Identification of isolate CM-4 based on 16S rDNA sequencing

Total DNA was extracted using a HIMEDIA DNA extraction kit as per the manufacturer's instructions. Identification was carried out based on primers targeted against variable regions of the 16S rRNA genes. The universal primer pair used for amplification of 16S rRNA was lac1-27F 5'-AGAGTTTGATCCTGGCTCAG and lac 1 - 1492R 5'TACGGYTACCTTGTTACGACT (IDT/PROMEGA). DNA amplification of the (~1,500 bases) fragment was carried out in a 20µl reaction mixture. All PCR chemicals were obtained from Fermentas. Amplification was performed in a thermocycler (Eppendorf, Mastercycler gradient) with the following running program: initial denaturation 95ºC for 5 min, denaturation 92ºC 1min, annealing 55ºC for 30 s, extension 72ºC 1 min and a total number of 25 cycles were run. The amplicon obtained was sent to Department of Biochemistry, University of Delhi, and South campus for sequencing. Sequence homology and analysis were performed using the Blast program available online at the National Center for Biotechnology Information, NCBI (www. http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Isolation of bacteria

Of a total of 35 bacterial isolates from 17 samples, 5 fulfilled the set criteria for probiotics, based on morphological (Table 1) and biochemical tests (Table 2), and these were subjected to further studies with regard to probiotically important features like their ability to grow over (and tolerate) broad pH range, and at different concentrations of NaCl and bile salts.

Table 1 - Characterization of the selected lactic acid bacteria (LAB) isolates

Samples	Isolates	Gram- staining	Morphology	Catalase test	Spore- staining	Motility
Cow milk	CM-4	Gram- positive	Cocci	Negative	Negative	Negative
Kudan	KD-7	Gram- positive	Cocci	Negative	Negative	Negative
Horse dung	HD	Gram- positive	Bacilli	Negative	Negative	Negative
Goat dung	GD	Gram- positive	Bacilli	Negative	Negative	Negative
Goat milk	GM	Gram- positive	Bacilli	Negative	Negative	Negative

Table 2 – Carbohydrate utilization pattern of the isolates

Carbohydrates	CM-4	KD-7	HD	GD	GM
Lactose	+	+	+	+	-
Xylose	+	+	+	+	-
Maltose	+	+	+	+	+
Fructose	+	+	+	+	+
Dextrose	+	+	+	+	+
Galactose	+	+	+	+	+
Raffinose	+	+	+	+	+
Trehalose	+	+	+	+	+
Mellibiose	+	+	+	+	+
Sucrose	+	+	+	+	+
L-arabinose	+	+	+	+	+
Mannose	+	+	+	+	-
Inulin	+	+	-	+	-
Sodium gluconate	+	+	-	+	-
Glycerol	+	+	-	+	-
Salicin	-	+	+	+	-
Dulicitol	-	+	-	+	-
Inositol	-	+	-	+	-
sOrbitol	-	+	+	+	-
Mannitol	+	+	+	+	-
Adonitol	-	+	-	+	-
Arabitol	-	+	-	+	-
Erythritol	-	+	-	+	-
L-methy D	-	+	+	+	-
Rhamnose	-	+	-	+	-
Cellobiose	+	+	+	+	-
Melezitose	-	+	+	+	-
L-methyl –d –mannose	-	+	+	+	-
Xylitol	-	+	-	-	+
ONPG	-	-	-	-	-
Esculin	+	+	+	+	+
d- arabinose	+	+	-	+	+
Citrate	-	-	-	-	-
Malonate	-	-	+	-	-
Sorbose	+	+	+	+	-

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Study of probiotically important features

Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition", interest in this area was initiated by Elie Metschnikov about 100 years ago (Ljung and Wadstrom, 2006). Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al., 2001). Therefore, growth of the isolates was studied spectrophotometrically (A_{660}) in MRS at different pH (2 - 12) at 37 ˚C for 72 h. Growth in MRS at pH 6.5 served as control (Fig. 1). All the LAB isolates except HD showed growth at pH range of 2 to pH 12, (Fig. 2). However, isolates HD and GM displayed a considerable growth at pH of 3 - 4. All the isolates were able to tolerate a low pH of 2 - 4, though an appreciable growth was not reported. At pH 2, isolate CM-4 showed maximal growth which was followed by isolates KD-7 and GM, while at pH 3 highest growth of CM-4 was followed by GM and KD-7. Thus, the entire selected LAB grew over a broad pH range of 2-12 which is a desirable feature for the organism to be a good probiotic. Ability of probiotics to grow or tolerate low pH has been studied by many researchers. Roopashri and Varadaraj (2009) reported that *Lactobacillus plantarum* MTCC 5422 and *Bifidobacterium adolescentis* MTCC 5423 showed good growth at acidic pH of 3.5 - 5.5 in addition to other probiotically important attributes. Similarly in our study, isolate CM-4 was found to grow and survive at a pH of 2. *L. salivarius* MTC 106 survived in the gastric juice at pH 2 (Tinrat et al., 2011). However, among the 15 isolates of lactic acid bacteria examined only three showed resistance to pH 3 (Tatsadjieu et al., 2011).

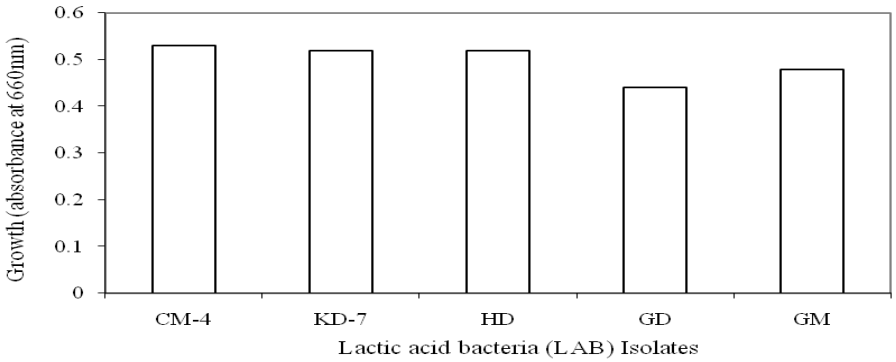

Figure 1 - Growth of lactic acid bacteria (LAB) isolates in MRS at pH 6.5 (Control)

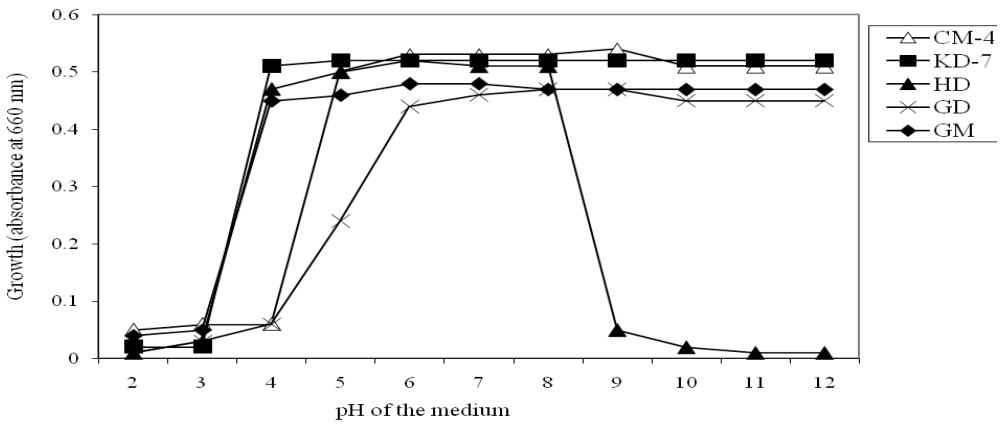

Figure 2 - Growth of the selected LAB isolates in MRS at varying pH

All the LAB isolates exhibited excellent growth in MRS containing 2 - 7% NaCl (Fig. 3). In this range maximum growth was shown by isolate KD-7 and was followed by CM-4, GD, GM and HD. With further increase in NaCl concentration, growth decreased. At 8% NaCl concentration highest growth was observed for the isolate KD-7 which was followed by HD, GD, CM-4 and GM. The isolate CM-4 showed constant good growth up to 13% NaCl concentration while KD-7 could not grow in MRS having above 11% NaCl. Eight to nine % NaCl was the limiting concentration for growth of GM, HD and GD (Fig. 3). Lactic acid bacteria generally tolerate high salt concentrations. It allows the bacteria to begin metabolism, which produce acid that further inhibit the growth of undesirable organisms. When bacterial cells are grown in medium with salt, they experience a loss in their turgor pressure, which in turn affects the metabolism and their enzyme and water activity. Cells overcome this situation by regulating the pressure inside and outside of the cell by inducing osmolytes, such as glycine, betaine, as an adaptive mechanism to withstand increased osmotic potential (Robert et al., 2000). Aswathy et al. (2008) evaluated probiotic characteristics of newly isolated LAB and found that one isolate was capable of growing at NaCl concentration of 12% while majority of others grew well at 8% NaCl concentration. In the present study, it was

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observed that the isolate CM-4 showed good growth up to 13% NaCl concentration while the other isolates were not able to grow beyond 11% of NaCl.

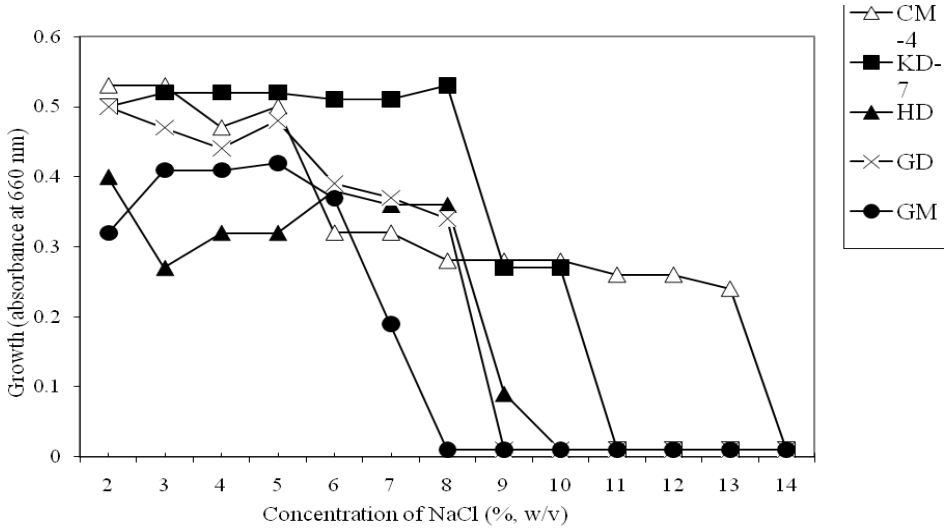

Figure 3 - Growth of LAB isolates at different concentrations of NaCl

LAB isolates were evaluated for their ability to grow in MRS broth containing varying concentrations of bile salts (0.025 - 0.25 %). All the LAB isolates showed good growth at different concentrations of bile salts. The isolates HD, KD-7 and CM-4 displayed maximum growth over all the bile salt concentrations used, while the isolates GM and GD showed a reasonable growth (Fig. 4). Barring isolate GD which realized a sharp decline in growth at concentration above 0.2 % of bile salts, all other isolates grew exceptionally well in MRS with 0.25 % bile salts. Bile tolerance has been described as an important factor for the survival and growth of LAB in the intestinal tract. Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyse conjugated bile, thus reducing its toxic effect. BSH activity has most often been found in organisms isolated from the intestines or faeces of animals (Mourad and Nour-Eddine, 2006). In the current study, all the isolates grew well at 0.25% of bile salts. Aswathy et al. (2008) observed that most of the LAB isolates from vegetables, sour dough, milk products, sheep and human excreta exhibited good growth in presence of 0.3 - 0.8 % bile salts. Similarly, Reddy et al. (2007) observed that the growth performance of two native isolates *Lactobacillus plantarum* MTCC 5422 and *Bifidobacterium adolescentis* MTCC 5423 was quite appreciable in the presence of bile salts at concentration levels of 0.15%, 0.3%, and 0.45%, respectively. However, in the presence of 0.45% bile salt, the growth was slightly reduced. Ahmed et al. (2007) examined the growth of five strains of *Lactobacillus reuteri* in presence of different bile salts concentrations and reported that 0.3% bile salt significantly reduced the growth of *L. reuteri* strains. Further, the addition of Tween 80 enhanced the bile salt tolerance of *L. reuteri,* indicating that bile salt and Tween 80 have an influence on the biochemical properties of *L. reuteri* and should be considered for useful applications in enhancing the survival of *Lactobacillus*. Pereira and Gibson (2002) observed that most of the LAB and bifidobacteria isolated from human gut were sensitive to bile salts in the range of 0.2-0.4%., however a few of LAB isolates showed relatively good growth in presence of bile salts.

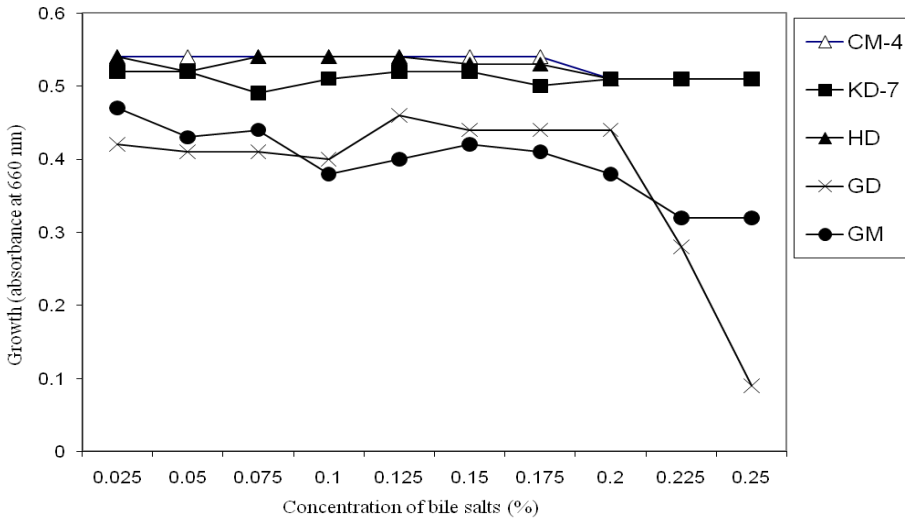

Figure 4 - Growth of LAB isolates at different concentration of bile salts

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Antibacterial activity of LAB isolates against potent pathogens

Another important probiotic attribute associated with LAB is their antibacterial activity against potential human pathogens. The selected isolates were screened for their antimicrobial activity against potential human pathogens such as *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas aeruginosa, P. alcaligenes, Staphylococcus aureus* and *Streptococcus* sp. Isolates CM-4 and KD-7 were remarkable as they exhibited antibacterial action against all the pathogens tested. Isolate CM-4 exhibited potent inhibitory activity against *Pseudomonas aeruginosa* (inhibition zone of 14 mm), *B. cereus* (13 mm), *B. subtilis, E. coli, S. aureus* (12 mm, each), *P. alcaligenes* (11 mm) and *Streptococcus* sp. (10 mm) as shown in Table 3. Isolate KD-7 significantly inhibited *B. subtilis* (21 mm) and *P. alcaligenes* (15 mm), while the other pathogens were inhibited to a lesser extent (11-13 mm), and *Streptococcus* sp. was the least inhibited one (9 mm).

Maximum antibacterial activity was observed after 72 h of cultivation, though lower level of activity was observed after 24-48 h (data not shown). All the LAB isolates showed inhibitory effect against most of the test pathogens excluding *S. aureus* and *Streptococcus* sp., however, LAB isolates CM-4 and KD-7 were remarkable as they showed antibacterial activity against these two pathogens also. Two LAB isolates (CM-4 and KD-7) also showed maximum inhibition zone against all the pathogens tested. The LAB isolate GM did not show antagonistic action against any of the pathogens tested. Sholeva et al. (1998) screened 46 strains, out of which only 28 possessed antimicrobial activities. The largest spectrum of inhibition was observed for *Lactobacillus casei* NBMCC300, which inhibited 5 test organisms. Roopashri and Varadaraja (2009) reported that all the 9 LAB isolates showed inhibition against *Bacillus cereus, Listeria monocytogenes* and *Yersinia enterocolitica*, however, only 4 LAB isolates could inhibit the growth of *Staphylococcus aureus*. In the current study, LAB isolates CM-4 and KD-7 were remarkable as they showed antibacterial activity against *S. aureus* and *Streptococcus* sp. These findings necessitate the further study of these two LAB isolates, and prompts for establishment of the active principle associated with antibacterial activity. Aswathy et al. (2008) reported that among a total 16 LAB isolates examined for their antibacterial activity against human pathogens, 14 inhibited *Escherichia coli*, 9 showed inhibitions against *Shigella sonnei*, 7 inhibited growth of *Shigella flexnerii* and 6 caused inhibition of *Staphylococcus aureus*. The inhibition zone size in most of the cases varied from 4-7 mm except in case of the isolate CB5 which showed an inhibition zone of 10 mm against *E. coil*. Similarly, Reddy et al. (2007) reported the antimicrobial property of six selected LAB isolates from *Kanjika,* a wellknown ayurvedic lactic acid-based fermented product, against known food-borne pathogens such as *B. cereus*, *L. monocytogenes*, *E. coli*, *S. aureus* and *Y. enterocolitica*.

Table 3 - Antagonistic action of the selected LAB isolates against pathogens of human significance *

	Diameter of the inhibition zone (mm) Pathogens tested						
LAB	***E. coli***	***B. subtilis***	***B. cereus***	***P. aeruginosa***	***P. alcaligenes***	***S. aureus***	***Streptococcus* sp.**
CM-4	12	12	13	14	11	12	10
KD-7	11	21	12	13	15	12	9
HD	8	12	8	9	8	ND	ND
GD	7	13	9	11	8	ND	ND
GM	ND	ND	ND	ND	ND	ND	ND

*ND= not detected

Enzyme producing ability of the LAB isolates

Enzyme (amylase, lipase, protease, phytase) producing ability of LAB isolates was determined by observing the formation of halos around the colonies on specific agar medium plates. All the bacterial isolates displayed varying level of phytase producing ability. Maximum phytase production was shown by CM-4 (zone size 14 mm, followed by KD-7 (13 mm), HD and GM (10 mm each) and isolate GD (5 mm) as shown in the Table 4. . However, lipase producing ability was expressed only by LAB isolates CM-4 and KD-7. Isolate CM-4 displayed considerable lipase activity (zone size 10mm) while KD-7 showed marginal level of lipase activity (Table 4). No extracellular protease and amylase activity was detected in the LAB isolates of this study. Application of enzymes in poultry started in early fifties and with the advent of technology several enzymes have become cheap and easily available. Application of enzymes of enzymes not only increases the nutritional value of the food but also keeps a regulation on the release of undigested waste material into the environment (Cmiljanic et al., 2005). Abriouel et al.(2005) reported that the enzyme extract from *E. faecium* RJ16 isolated from food was found to possess enzymes important in food industry like phytase and esterase lipase.

Table 4 - Enzymatic activity of the five selected LAB isolates*

Enzyme Activity	LAB Isolates, Zone size, mm				
	CM-4	**KD-7**	**HD**	**GD**	**GM**
Amylase activity	–	–	–	–	–
Phytase activity	14	13	10	5	10
Protease activity	–	–	–	-	–
Lipase activity	10	+	–	–	–

* indicates absence of enzyme activity, + indicates that the activity though present but not measurable

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Cell surface hydrophobicity

The selected isolates displayed variable level of hydrophobicity (Fig 5). Isolate CM-4 displayed maximum hydrophobicity i.e. 93.55% and was followed by KD-7 (76.28%). Isolate GD displayed the least hydrophobicity (63.24%) while rest of the isolates showed hydrophobicity ranging in between 93.55% and 63.24%. Cell-surface hydrophobicity is recognized as measurable physicochemical variables for evaluating bacterial adhesion to the surfaces. The high levels of hydrophobicity of microorganisms are usually associated with the presence of fibrillar structures on the cell surface (Mcnab et al., 1999) and specific cell wall proteins (Kos et al., 2003). Kaushik et al., (2009) reported that *L. johnsonii* LA1 and *L. acidophilus* LA7 showed hydrophobicity level of 47% and 57–58%, respectively.

Strain identification

The isolate CM-4 exhibited most of the desirable probiotic attributes, therefore its identification was done based on 16S rDNA sequencing. PCR amplification of 16S rDNA of isolate CM-4 was executed using universal primers and the amplicon obtained (Fig. 6) was sequenced. The sequence was analysed using NCBI Mega BLAST. The isolate CM-4 was identified to be an *Enterococcus* sp. based upon 16S rDNA sequence as it showed high resemblance (99%) with that of several other *Enterococcus* spp. available in the gene bank data base. Furthermore, the isolate was designated as *Enterococcus faecium* as it formed the part of same cluster as that of *Enterococcus faecium* GM3. The evolutionary history was inferred using the UPGMA method and boot strapping was done using MEGA 5 software (Fig. 7). Even though biochemical tests are quite reliable but for unambiguous and precise identification molecular techniques based upon 16S rDNA sequences are preferred.

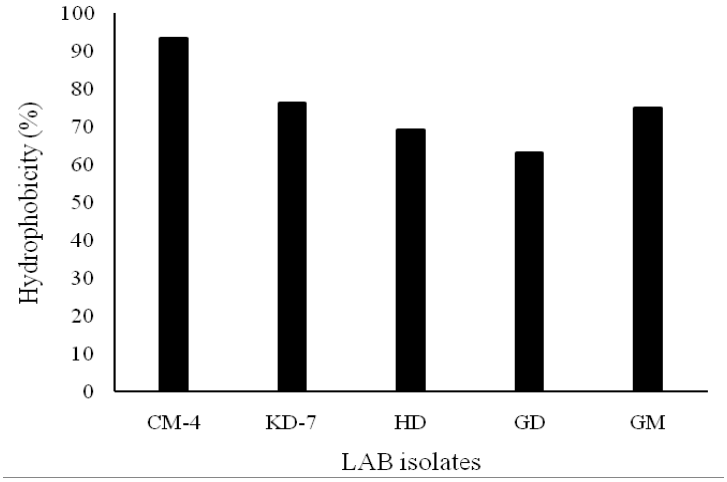

Figure 5 - Cell surface hydrophobicity of the selected LAB isolates

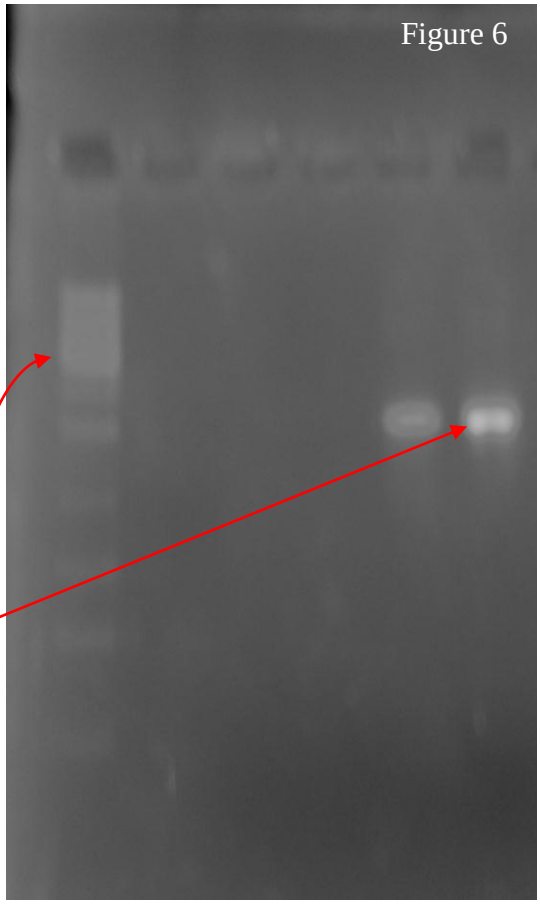

Figure 6 - Agarose gel electrophoresis showing the amplified 16SrDNA using universal primers

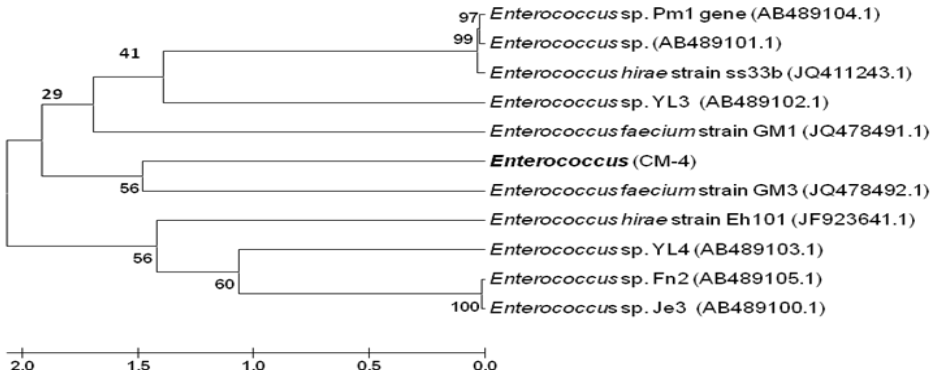

Figure 7 - Phylogenetic tree showing the comparison of 16S rDNA sequence of *Enterococcus* sp. CM-4 with those of other spp. of *Enterococcus* available in NCBI database. Numbers at the nodes are percentage bootstrap values based on 500 replications.

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CONCLUSIONS

The selected five isolates from different samples showed desirable probiotically important attributes like growth, and survival at varying pH, high bile salt and NaCl concentrations. All the isolates survived the low pH conditions of 2.0, and were not inhibited by the presence of bile (0.02-0.25 %) and NaCl (2-14 %) indicating their ability to survive in GI tract conditions and hence making them suitable candidates for probiotic application. Of the 7 potential pathogens (*Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas aeruginosa, P. alcaligenes, Staphylococcus aureus* and *Streptococcus* sp.) examined for their growth inhibition by 5 LAB isolates, growth of majority of pathogens was inhibited by LAB isolates. The isolates CM-4 and KD-7 were most remarkable as they inhibited all the pathogens tested including *S. aureus* and *Streptococcus sp.* Among all the LAB isolates examined CM-4 possessed most of the desirable probiotic properties, and therefore must be studied further to fully claim its potential for applications in poultry feed.

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REFERENCES

Abriouel H, Lucas R, Ben ON, Valdivia E, Maqueda M, Martínez-Cañamero M and Gálvez A (2005). Enterocin AS-48RJ: a variant of enterocin AS-48 chromosomally encoded *by Enterococcus faecium* RJ16 isolated from food. Sys Appl Microbiol, 28: 383-97.

Ahmed SA, Ibrahim SA, Kim C and Shahbazi A (2007). Significance of bile salt tolerant *Lactobacillus reuteri.* Proc Nat Conf Environ Sci Technol, Springer, New York.

Aswathy RG, Ismail B, John RP and Nampoothiri KM (2008). Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. Appl Biochem Biotechnol, 151: 244–255.

Bajaj BK and Wani MA (2011). Enhanced phytase production from *Nocardia* sp MB36 using agro- residues as substrates: potential application for animal feed production. Eng Life Sci, 11: 620-628.

Cmiljanic R, Pavlovski Z, Trenvoski S and Lukic M (2005). New trends in poultry nutrition. Biotech Anim Husbandary, 21: 241-245.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F and Collins JK (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr, 73 (suppl): 386S–392S.

Garrity GM, Bell JA and Lilburn TG (2004). Taxonomic Outline of the Prokaryotes. Bergey'sManual of Systematic Bacteriology, 2nd Ed. Springer-Verlag, New York: 1-399.

Holzapfel W, Haberer P, Geisen R, Bjorkroth J and Schillinger U (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr, 73: 365s-373s.

Mourad Kand Nour-Eddine K (2006). In vitro pre selection criteria for probiotic lactobacillus plantarum strains of fermented olives origin. Int J Probiotics Prebiotics, 1: 27-32.

Kaushik JK, Kumar A, Duary RK, Mohanty AK, Grover S and Batish VK (2009). Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. PLoS ONE, 4(12): e8099.

Khattak FM, Pasha TN, Hayat Z and Mahmud A (2006). Enzymes in poultry nutrition. J Anim Pl Sci, 16:1-2.

Kim EY, Kim YH, Rhee MH, Song JC, Lee KW, Kim KS, Lee SP, Lee IS and Park SC (2007). Selection of *Lactobacillus* sp. PSC101 that produces active dietary enzymes such as amylase, lipase, phytase and protease in pigs. J Gen Appl Microbiol, 53: 111-117.

Kos B, Suskovi´c J, Vukovi´c S, Simpraga M, Frece J and Matosi´c S (2003). Adhesion and aggregationability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol, 94: 981–7.

Ljungh A and Wadstrom T (2006). Lactic acid bacteria as probiotics. Curr Issues Intest Microbiol, 7: 73–90.

McNab R, Forbes H, Handley P S, Loach DM, Tannock GW and Jenkinson HF (1999). Cell wall-anchored CshA polypeptide (259 kilodaltons) in *Streptococcus gordonii* forms surface fibrils that confer hydrophobic and adhesive properties. J Bacteriol, 181: 3087-3095.

Pereira DIA and Gibson GR (2002). Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Appl Environ Microbiol, 68: 4689–4693.

Reddy KBPK, Raghavendra P, Basavanna GK, Misra MC and Prapulla SG (2007). Screening of probiotic properties of lactic acid bacteria isolated from *Kanjika*, an ayruvedic lactic acid fermented product: An *in-vitro* evaluation. J Gen Appl Microbiol, 53: 207–213.

Robert H, Marrec CL, Blanco C, Jebbar M (2000). Glycine, betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophila.* Appl Environ Microbiol, 66: 509-517.

Roopashri AN and Varadaraj MC (2009). Molecular characterization of native isolates of lactic acid bacteria, bifidobacteria and yeasts for beneficial attributes, Appl Microbiol Biotechnol, 83: 1115 –1126.

Rosenberg MD, Gutnick, and Rosenberg E (1980). Adherence of bacteria to hydrocarbons: A simple method for measuring cell surface hydrophobicity. FEMS Microbiol Lett, 44: 929–937.

Sholeva Z, Stefanova S and Chipeva V (1998). Screening of antimicrobial activities among Bulgarian lactobacilli strains. J Culture Collect, 2: 15-20.
Simon O (2005). Microorganisms as feed additives – Probiotics. Adv Pork Prod, 16: 161.
Tatsadjieu NL, Njintang YN, Kemgang Sonfack T, Daoudou B and Mbofung CMF (2009).
Characterization of lactic acid bacteria producing bacteriocins against chicken *Salmonella enterica* and *Escherichia coli.* Afr J Microbiol Res, 3 (5): 220-227.
Tinrat S, Saraya S and Chomnawang MT (2011). Isolation and characterization of Lactobacillus salivarius MTC 1026 as a potential probiotic. J Gen Appl Microbiol, 57: 365-378.

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