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Probiotic Potential and Antibiotic Resistance of Lactic Acid Bacteria Isolated from Curd in Sri Lanka

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ABSTRACT

Lactic acid bacteria (LAB) are organisms beneficial to the human beings as probiotics which the organism can remain viable under stress conditions in the gastrointestinal tract.The aim of this study was to characterize and evaluate probiotic properties, and antibiotic resistance of thirteen isolates of lactic acid bacteria obtained from curd. In order to characterize the lactic acid bacteria isolates from curd; Gram's staining, endospore staining, motility, catalase reaction, indole production, methyl red test, Voges-Proskauer test, citrate utilization, oxidase activity, urease activity, H2S production, arginine hydrolysis, growth at different temperatures, gas production from glucose and fermentation pattern of different carbohydrates were performed. In order to screen the probiotic potential of the isolates; tolerance to pH, bile, and phenol, survival in the presence of simulated gastric and pancreatic juices were examined. Antibiotic resistance of selected isolates was evaluated using 12 antibiotics. Results of biochemical tests and physiochemical tests revealed that all thirteen isolates (ITI_CD001, ITI_CD 002, ITI_CD 003, ITI_CD 004, ITI_CD 005,ITI_CD006, ITI_CD 009, ITI_CD 010, ITI_CD 011, ITI_CD 012 ITI_CD013, ITI_CD 014, andITI_CD 015) are belonged to the genus Lactobacillus. Species were identified as Lactobacillus plantarum, Lactobacillus brevis and Lactobacillus delbrukeii lactis. Most of the isolates were able to tolerate and grow well under low pH conditions (1.5 - 3.0), high pH (9.0), extremes of bile up to 0.5% and phenol up to 0.6%.The isolates were able to growin the presence of simulated gastric and pancreatic juices too. Results for the antibiotic resistance varied among isolates. Isolates ITI_CD004, ITI_CD 009, ITI_CD 010, ITI_CD 011, ITI_CD 012 and ITI_CD 015 were identified as higher probiotic isolates. The study revealed that the tested LAB isolates possess desirable probiotic properties. Hence, these isolates can be used to produce foods high in probiotics.

KEYWORDS: *Curd, probiotics, Lactobacillus, antibiotic resistance*

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1. INTRODUCTION

Curd is a traditional dairy product and a popular dessert in Sri Lanka. The curd-bacteria have been well accepted and generally recognized as safe food for human consumption (Shruuthy *et al*., 2011). Curd is used as a therapeutic agent in traditional medicine and documented in Ayurveda literature from 600 AD (Nanda *et al*., 2013). It has been found that active lactic acid bacteria in curd improve the quality of internal microflora of gut and also help to improve the digestion and cure intestinal disorders such as constipation, diarrhea and dysentery by their anti-pathogenic properties (Samuel and Shukla, 2014) and are involved in activation of the immune system, prevention of cancer cell growth and maintenance of mucosal integrity (Amraii *et al*., 2013).

Food and Agriculture Organization of the United Nations (UNFAO) and the World Health Organization (WHO) define probiotics as live microorganisms, when administered them in adequate amounts, bestow a health benefit on the host (Fijan, 2014). Many research on probiotic effect of Lactic Acid Bacteria (LAB) have been carried out over the past 10 years, especially on the genera *Lactobacillus* and *Bifidobacterium* (Amraii *et al*., 2013). LAB can be found in various habitats particularly rich in carbohydrates.

Generally, they can be found in plants, fermented foods, mucosal surfaces of humans, terrestrial and marine animals, etc. According to Florou-Paneri *et al*. (2013) they are part of the human and animal microbiota, inhabiting the gastrointestinal and genitourinary tracts. Lactic acid bacteria are very important in food industry as they can convert carbohydrates into organic acids (lactic acid and acetic acid) during their growth and create acidic condition that has preservative effect during manufacture and storage of fermented foods (Kumar and Kumar, 2014). Lactic acid bacteria are commonly found in fermented dairy products such as cheese, buttermilk and curd.

Several lactic acid bacteria such as *Streptococcus lactis* (now *Lactococcus lactis lactis*), *Streptococcus diacetylactis* (now *Lactococcus lactis lactis biovar diacetylactis*), *Streptococcus cremoris* (now *Lactococcus lactis cremoris*) and *Leuconostoc* spp. have been isolated from buffalo curd in Sri Lanka (Jayamanne and Adams, 2004). Although curd is a popular dessert in Sri Lanka, more studies have been concerned on isolation, but only few studies were on characterization. Furthermore, probiotic potential of lactic acid bacteria associated with curd in Sri Lanka has not been screened. This study aimed at characterization of lactic acid bacteria isolated from curd and assess their probiotic potential and resistance to antibiotics.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

Pure cultures of LAB isolates from curd were obtained from Industrial Technology Institute (ITI), Sri Lanka. Thirteen isolates referred to as ITI_CD001, ITI_CD002, ITI_CD003, ITI_CD004, ITI_CD005, ITI_CD006, ITI_CD009, ITI_CD010, ITI_CD011, ITI_CD012, ITI_CD013, ITI_CD014 and ITI_CD015 were used for this study.

2.2. Characterization of isolates

Gram's staining, endospore staining, motility and catalase test were performed as per the standard procedures for the preliminary identification of LAB. Gram positive, catalase negative, non- motile, non-sporing isolates were further characterized through biochemical tests; indole production in tryptone broth medium, methyl red (MR) reaction and Voges-Proskauer (VP) reaction in buffered peptone glucose broth medium, utilization of citrate in Simmons citrate medium, oxidase activity, urease activity, production of H_2S, arginine hydrolysis and gas production from glucose. Temperature sensitivity to growth was determined in De Man-

Rogosa-Sharpe (MRS) broth medium by incubating cultures at different temperatures for five days. Fermentation of carbohydrates (glucose, D-trehalose, D-mannitol, D-melizitose, D-mannose, L-arabinose, D-maltose, D-fructose, L-rhamnose, D-cellobiose, D-galactose, D-salicin, D-raffinose, D-sorbitol, D-melibiose and D-ribose) was tested in MRS broth (prepared without sugars) containing 1% solution of carbohydrate and 0.025% bromocresolpurple as pH indicator. Fermentation ability was examined after five days of incubation at 37ºC.

2.3. Determination of probiotic potential of isolates

pH tolerance

pH tolerance was determined according to the method described by Khalil *et al*. (2007) with some modifications. pH of MRS broth was adjusted to 1.5 and 3.0 using 1M HCl solution and to pH 9.0 using 1M NaOH solution. Exactly100 µL of 18 h old pure culture was pipetted under aseptic conditions into a 2 mL Eppendorf tube containing 1 mL MRS broth adjusted to required pH and mixed well. Cultures were transferred into 96 well microtiter plate and incubated aerobically at 37ºC over five hours and OD was measured at 600 nm at hourly intervals using a microplate reader (Spectra max plus 384).Growth of an isolate was assumed to be proportionate to absorbance.

Bile tolerance

Bile tolerance was determined using the broth assay described by Khalil *et al*. (2007) with some modifications. Bile (oxgall) was added to MRS broth to make the final concentration 0.1% (w/v) in the medium and pipetted 1 mL into each 2 mL Eppendorf tube. Exactly 100 µL of 18 h old pure culture was pipetted into each tube and mixed well under aseptic conditions. Cultures were transferred into 96 well microtiter plate and incubated aerobically at 37ºC and OD was measured at 600 nm at hourly intervalsover five hours using a microplate reader (Spectra max plus 384). Growth of an isolate was assumed to be proportionate to the absorbance value. Resistance to bile concentrations 0.3% (w/v) and 0.5% (w/v) was also determined using the above method.

Resistance to simulated gastric and pancreatic juices

MRS broth containing simulated gastric and pancreatic juices was prepared freshly. To make simulated gastric juice, NaCl was added to MRS broth to a final concentration 0.6% (w/v). Prepared medium was sterilized by autoclaving at 121ºC under 15 psi pressure for 15 min.After sterilizing and cooling, pepsin was added to the medium to make the final concentration 3 g/L. The solution was adjusted to pH 2 using 1 M HCl. Prepared broth (1 mL) was pipetted into each 2 mL Eppendorf tube. Exactly 100 µL of 18 h old pure culture was pipetted into each tube under aseptic conditions. Cultures were transferred into 96 well microtiter plate and incubated aerobically at 37ºC over five hours and OD was measured at 600 nm at hourly intervals using a microplate reader (Spectra max plus 384).The cell density of an isolate was assumed to be proportionate to absorbance. Cell density was determined by converting OD value to colony forming units per millilitre (CFU/ mL) [1.0 OD (600 nm) = $1x\ 10^8$ CFU/ mL].

Simulated intestinal fluid was prepared by dissolving bile (oxgall) in sterile MRS broth to a final concentration 0.3% (w/v), pancreatin to a final concentration 1 g/L and NaCl to a final concentration 0.85% (w/v). The medium was adjusted to pH 8.0 using 1M NaOH. Prepared broth (1 mL) was pipetted into each 2 mL Eppendorf tube. Under aseptic conditions 100 µL of 18 h old pure culture was pipetted into each tube and mixed well. Cultures were transferred into 96 well microtiter plate and incubated aerobically at 37ºC over five hours and OD was measured at 600 nm at hourly intervals using a microplate reader (Spectra max plus 384).The cell density of an isolate was assumed to be proportionate to absorbance. Cell density was determined by converting OD value to colony

forming units per millilitre (CFU/ mL) [1.0 OD (600 nm) = 1x 10^8 CFU/ mL].

Phenol tolerance

Phenol tolerance was determined as described by Divya *et al*. (2012) with some modifications. LAB isolates were tested for their ability to survive in the presence of growth inhibitory substance, phenol. Phenol was added to MRS broth to make 0.2%, 0.4% and 0.6% (w/v) final concentrations andpipetted 1 mL into each 2 mL Eppendorf tube. Exactly 100 µL of 18 h old pure culture was pipetted into the tube and mixed well under aseptic conditions. Cultures were transferred into 96 well microtiter plate and incubated aerobically at 37°C over five hours and OD was measured at 600 nm at hourly intervals. Isolates ITI_CD 004, ITI_CD 009, ITI_CD 010,ITI_CD 011,ITI_CD 012 and ITI_CD 015 which showed tolerance to pH, bile, simulated gastric and pancreatic juices were selected to evaluate the tolerant potential to higher concentrations of phenol (0.4% and 0.6% w/v). The cell density of an isolate was assumed to be proportionate to absorbance. Cell density was determined by converting OD value to colony forming units per millilitre (CFU/ mL) [1.0 OD (600 nm) = 1x 10^8 CFU/ mL].

2.4. Investigation of antibiotic resistance of isolates

Five isolates; ITI_CD 004, ITI_CD 009, ITI_CD 010, ITI_CD 012 and ITI_CD 015 that showed higher survival capacity during tolerance studies were selected to evaluate the antibiotic resistance. Antibiotic resistance of isolates was tested using disk diffusion method on Muller Hington agar using antibiotics (Himedia, India); ampicillin, erythromycin, sulphamethoxazole, norfloxacin, amikacin, chloramphenicol, gentamicin, amoxicillin, cefotaxime, cephalothin, vancomycin and tetracycline. Well isolated colonies of each isolate were picked from a sterile inoculation loop and dispensed into 0.85% (w/v) sterile NaCl solution. The bacterial suspension was prepared to reach a density similar to a McFarland standard value of 0.5. The bacterial suspension was spread in three directions on Muller Hington agar plates by using a sterile cotton swab. Care was taken to use agar plates with a layer thickness of 4±0.5 mm in order to standardize the diffusion of antibiotics. Standard antibiotic disks (6 mm diameter) were placed on the medium and plates were incubated at 37°C for 48 h. The diameter of inhibition zone including the disc was measured using a calibrated Vernier ruler (smallest unit= 0.1 mm). The results were expressed as sensitive (S ≥21 mm); intermediate (I, 16 to 20 mm) and resistant (R≤15 mm) respectively (Puphan *et al.*,2015).

3. RESULTS AND DISCUSSION

3.1. Characterization of isolates

All the isolates were Gram positive and catalase negative bacilli which appeared in chains or pairs or single cells. Therefore, they were identified as genus *Lactobacillus.*

All isolates were non-motile, non-spore forming, negative to citrate, indole, urease, and oxidase and H2S production tests (Table 1). These characteristics are similar to the characters of the genus *Lactobacillus* described by Vos *et al*. (2011). Six isolates (46.2%) which fermented both hexoses and pentoses and produced an acid were identified as facultative heterofermentative *Lactobacilli*. Six isolates (46.2%) which fermented hexoses into an acid and a gas and fermented pentoses into an acid were identified as obligatory heterofermentative *Lactobacilli*. One isolate (7.6%) which fermented only hexoses was identified as obligatory homofermentative *Lactobacillus* by sugar fermentation test (Tables 2 and 3).

All the isolates fermented glucose, sucrose, fructose, raffinose, maltose, melibiose, cellobiose, and galactose. Ribose was fermented by almost all the isolates except ITI_CD015 whereas Sorbitol was fermented by only ITI_CD 010, ITI_CD 011 and ITI_CD 012. Isolates were

grouped based on the sugar fermentation ability (Table 2).Species were identified based on the results of biochemical and physiological tests (Table 1) in combine with sugar fermentation results. Curd associated LAB were identified as *Lactobacillus brevis, Lactobacillus plantarum* and *Lactobacillus delbrueckii lactis* (Table 3). The characteristics are agreeable with the species isolated from fermented dairy products in Sudan, Sri Lanka and Bulgaria (Ali, 2011a; Dekumpitiya *et al*.,2016;Tserovska *et al*., 2002).

Table 1. Biochemical and physiological characteristics of isolates

Isolate	Gram's staining	Motility	Endospore staining	Catalase reaction	Indole reaction	Methyl red reaction	VP reaction	Utilization of citrate	Urease activity	Oxidase activity	H_2S production	Arginine hydrolysis	Gas from glucose	Growth at10°C	Growth at15°C	Growth at 45°C
ITI_CD001	+	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+
ITI_CD002	+	-	-	-	-	+	-	-	-	-	-	-	+	+	-	+
ITI_CD003	+	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+
ITI_CD004	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+
ITI_CD005	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+
ITI_CD006	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
ITI_CD009	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+
ITI_CD010	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+
ITI_CD011	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
ITI_CD012	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
ITI_CD013	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+
ITI_CD014	+	-	-	-	-	+	+	-	-	-	-	+	-	-	+	+
ITI_CD015	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+

+, Positive reaction; -, Negative reaction

Table 2. Observations on Sugar fermentation by LAB isolated from curd

Isolatte	Glucose	Sucrose	Fructose	Arabinose	Raffinose	Melazitose	Maltose	Rhamnose	Salicin	Melibiose	Cellobiose	Mannose	Trehalose	Galactose	Manitol	Sorbitol	Ribose
ITI_CD001	a,g	a,g	a,g	-	a,g	-	a,g	a	-	a,g	a	a,g	-	a	-	-	a
ITI_CD002	a,g	a,g	a,g	-	a,g	-	a,g	a	-	a	a,g	a	a	a,g	-	-	a
ITI_CD003	a,g	a	a,g	-	a,g	-	a,g	a	-	a,g	a	a,g	-	a,g	a,g	-	a
ITI_CD004	a	a,g	a	-	a,g	-	a,g	-	-	a,g	a	-	-	a,g	-	-	a
ITI_CD005	a,g	a.g	a	-	a,g	-	a,g	-	-	a,g	a	a	-	a,g	-	-	a
ITI_CD006	a	a	a	a	a	a	a	a	a	a,g	a	a	a	a	a	-	a
ITI_CD009	a,g	a,g	a,g	-	a,g	-	a,g	a,g	-	a,g	a	a,g	-	a,g	a	-	a
ITI_CD010	a	a	a	a	a,g	a	a	a	a	a,g	a	a	a	a	a	a	a
ITI_CD011	a	a	a	a,g	a,g	a,g	a	a	a	a	a	a	a	a	a	a	a
ITI_CD012	a	a	a	a,g	a,g	a,g	a	a	a	a	a	a	a	a	a	a	a
ITI_CD013	a,g	a,g	a,g	a	a,g	a	a,g	a.g	a	a	a	a	a	a,g	a	-	a
ITI_CD014	a	a	a	a,g	a,g	a,g	a	a	a	a,g	a	a	a	a,g	-	-	a
ITI_CD015	a	a	a	-	a,g	-	a	a	-	a	a	a	a	a	a	-	-

a, acid production; g, gas production; - , no reaction

Table 3. Sub groups and identities of isolates based on sugar fermentation, biochemical tests and physiological tests

Isolate	sub groups based on sugar fermentation	Identity
ITI_CD001	obligatory heterofermantative	*Lactobacillus brevis*
ITI_CD002	obligatory heterofermentative	*Lactobacillus brevis*
ITI_CD003	obligatory heterofermentative	*Lactobacillus brevis*
ITI_CD004	facultative heterofermentative	*Lactobacillus plantarum*
ITI_CD005	obligatory heterofermentative	*Lactobacillus brevis*
ITI_CD006	facultative heterofermentative	*Lactobacillus plantarum*
ITI_CD009	Obligatory heterofermentative	*Lactobacillus plantarum*
ITI_CD010	Obligatory heterofermentative	*Lactobacillus brevis*
ITI_CD011	facultative heterofermentative	*Lactobacillus plantarum*
ITI_CD012	facultative heterofermentative	*Lactobacillus plantarum*
ITI_CD013	obligatory heterofermentative	*Lactobacillus brevis*
ITI_CD014	facultative heterofermentative	*Lactobacillus plantarum*
ITI_CD015	obligatory homofermentative	*Lactobacillus delbrueckii lactis*

In many studies, the ability to ferment different sugars combine with biochemical and physiological tests have been used to characterize and identify Lactic Acid Bacteria to the species level (Ali, 2011a; Ali, 2011b; Hamed and Elattar, 2013; Khedid *et al*., 2009; Mithun *et al*., 2015)

3.2. Determination of probiotic potential of isolates

pH tolerance

Except the isolate ITI_CD 013 all the other isolates were able to survive at pH1.5 for 3h. However, growth of ITI_ CD 001, ITI_CD 002, and ITI_CD 013 decreased after 5h at pH1.5. The highest growth was observed in ITI_CD 006, but no significant difference in growth was observed among the isolates ($p>0.05$) (Figure 1A). According to Halder and Mandal (2015) and Shruthy *et al*. (2011) LAB isolates obtained from curd could not tolerate pH below 2 for 3h. However, in the present study all thirteen isolate survived at low pH level 1.5 for 3h.

Except ITI_CD 001, ITI_CD 002 andITI_CD 015 isolates were able to tolerate pH3.0 for the duration of both 3hand 5h (Figure 1B). The highest growth was observed in ITI_CD 004 but it was not significantly different from ITI_CD 005, ITI_CD 012, ITI_CD 009, ITI_CD 010 and ITI_CD 011($p>0.05$). In a study carried out to evaluate probiotic potential of lactic acid bacteria isolated from chicken intestine, found that isolates had moderate growth at pH 3.0 (Jin *et al*., 1998). In another study carried out to investigate the acid and bile tolerance properties of *lactobacilli* isolates from Koozeh cheese, the growth of isolates has significantly decreased at pH3.0 (Hassanzadazar *et al*., 2012).

Resistance to low pH is considered as one of the major selection criteria for probiotics as they have to pass through the low pH conditions of the stomach to reach the small intestine (Hamed and Elattar, 2013). Though the stomach pH can be as low as 1.0, pH 3.0 is often used in *invitro* assays to determine pH tolerance (Kavitha and Devasena, 2013). According to Hamed and Elattar (2013) the viability of many strains significantly decreased at pH 2.0.

Growth increased in all isolates at pH 9 kept over 5h (Figure 1C). The highest growth was observed in ITI_ CD 010 after 5 h and it was

significantly different from other isolates. However, a previous study on screening probiotic potential of lactic acid bacteria isolated from infant faeces reported the decreased of viability of isolates at pH 9.0 (Pelinescu *et al*., 2011).

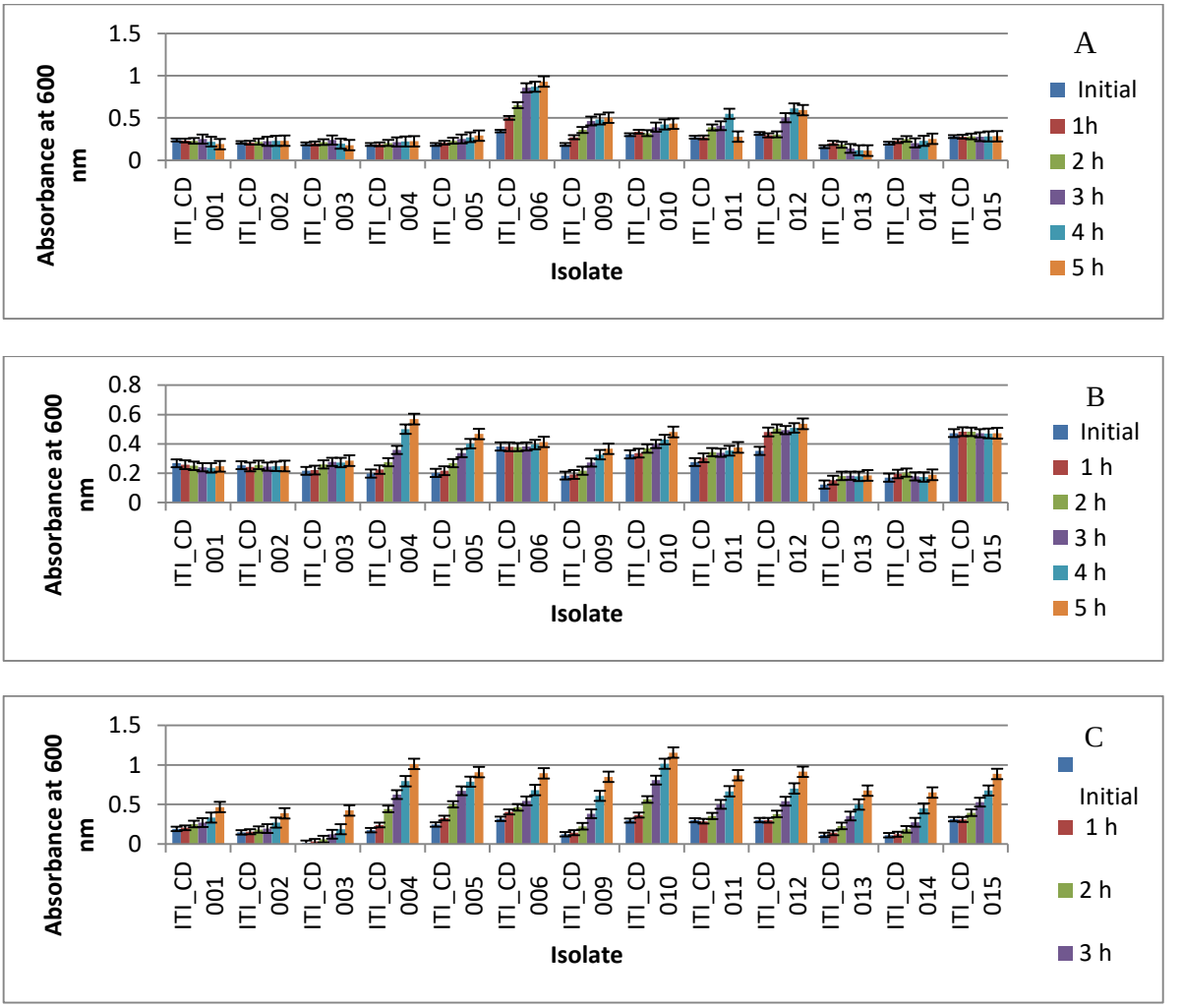


Figure 1: Tolerance of isolates to different pH. A: pH 1.5, B: pH 3.0, C: pH 9.0. (Values are means ± SE)

Bile tolerance

Bile concentration in the human gastrointestinal tract varies and hard to predict at a particular time (Chou and Weimer, 1998). When selecting a probiotic species for human consumption, 0.3% (w/v) bile concentration is taken as the mean intestinal bile concentration (Aswathy *et al*., 2008). In the present study growth of ITI_CD 001, ITI_CD 002 and ITI_CD 003 decreased with the time at 0.1% bile concentration while significant increase in growth ($p< 0.05$) was observed in ITI_CD 006, ITI_CD 010, ITI_CD 011, ITI_ CD 012 and ITI_CD 015 (Figure 2A).Only ITI_CD 010, ITI_CD 011, ITI_CD 012 and ITI_CD 015 were able to tolerate 0.3% bile concentration (Figure 2B). However, isolates ITI_CD 010, ITI_CD

011, ITI_CD 012 and ITI_CD 015 were able to tolerate bile up to 0.5% (w/v) concentration (Figure 2C). Significant increase in growth was observed in ITI_CD 010($p<0.05$). Bile tolerance is an important trait for a probiotic to be survived in small intestine (Hamed and Elattar, 2013).

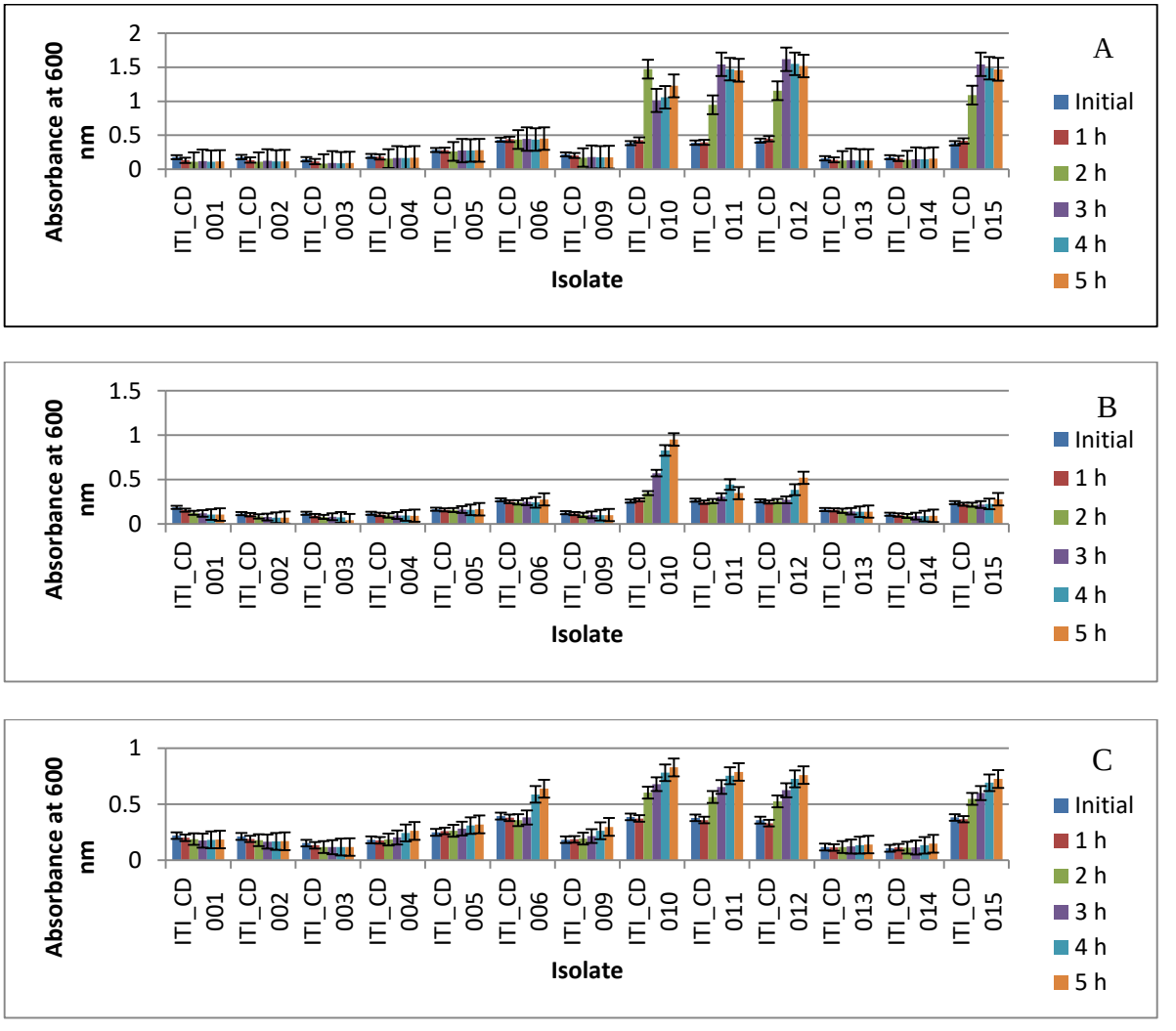


Figure 2: Tolerance of isolates to different bile concentrations. A: 0.1% (w/v), B: 0.3% (w/v), C: 0.5% (w/v). Values are means ± SE.

It has been reported that ten species of probiotic thermotolerant lactic acid bacteria from cooked meat products are tolerant to 0.1% (w/v), 0.3% (w/v) and 0.5% (w/v) bile concentrations (Ramirez-Chavarin *et al*., 2013). Shruthy *et al*. (2011) have identified three strains of curd lactic acid bacteria having higher survivability at the 0.3% (w/v) bile concentration.

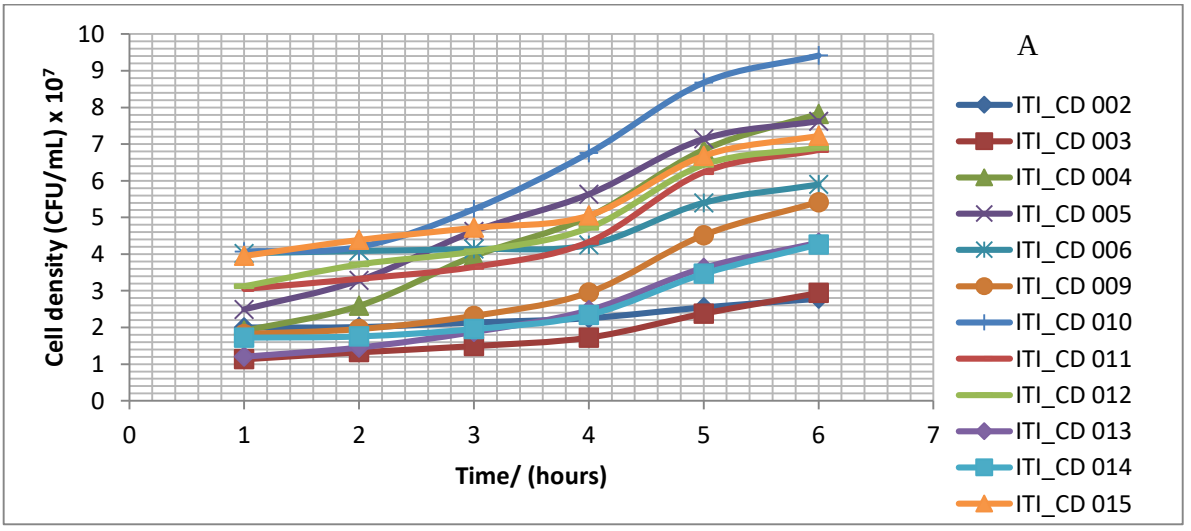
Resistance to simulated gastric and pancreatic juices

Only Eight isolates (ITI_CD 004, ITI_CD 005, ITI_CD 006, ITI_CD 009, ITI_CD 010, ITI_CD 011, ITI_CD 012 and ITI_CD 015) were able to tolerate simulated gastric juice for 5h while all the isolates tested were able to tolerate simulated

pancreatic juice for 5h (Table 4). Significant growth was observed in ITI_CD 004, ITI_CD 010, ITI_CD 011, and ITI_CD 012($p<0.05$). Similar results were observed by Monteagudo-Mera *et al*. (2012) in a study carried out with LAB of dairy and human origin.

Table 4. Resistance of LAB isolates from curd to simulated gastric juice and simulated pancreatic juice

Isolate	Simulated gastric juice		Simulated pancreatic juice	
	Initial cell density (CFU/ml)	Final cell density (CFU/ml)	Initial cell density (CFU/ml)	Final cell density (CFU/ml)
ITI_CD001	1.43×10^7	1.05×10^7	1.25×10^7	1.73×10^7
ITI_CD002	1.04×10^7	8.7×10^6	5.6×10^6	1.31×10^7
ITI_CD003	9.7×10^6	8.0×10^6	5.6×10^6	1.88×10^7
ITI_CD004	1.14×10^7	1.46×10^7	1.08×10^7	6.99×10^7
ITI_CD005	1.57×10^7	2.39×10^7	1.46×10^7	7.06×10^7
ITI_CD006	2.68×10^7	5.87×10^7	3.02×10^7	5.41×10^7
ITI_CD009	1.26×10^7	1.54×10^7	1.17×10^7	6.1×10^7
ITI_CD010	2.7×10^7	3.59×10^7	2.27×10^7	1.034×10^8
ITI_CD011	2.53×10^7	4.11×10^7	2.22×10^7	8.62×10^7
ITI_CD012	2.85×10^7	4.79×10^7	2.33×10^7	9.55×10^7
ITI_CD013	1.4×10^7	1.18×10^7	1.07×10^7	5.37×10^7
ITI_CD014	7.5×10^6	5.8×10^6	5.8×10^6	2.74×10^7
ITI_CD015	2.59×10^7	3.35×10^7	1.96×10^7	6.66×10^7



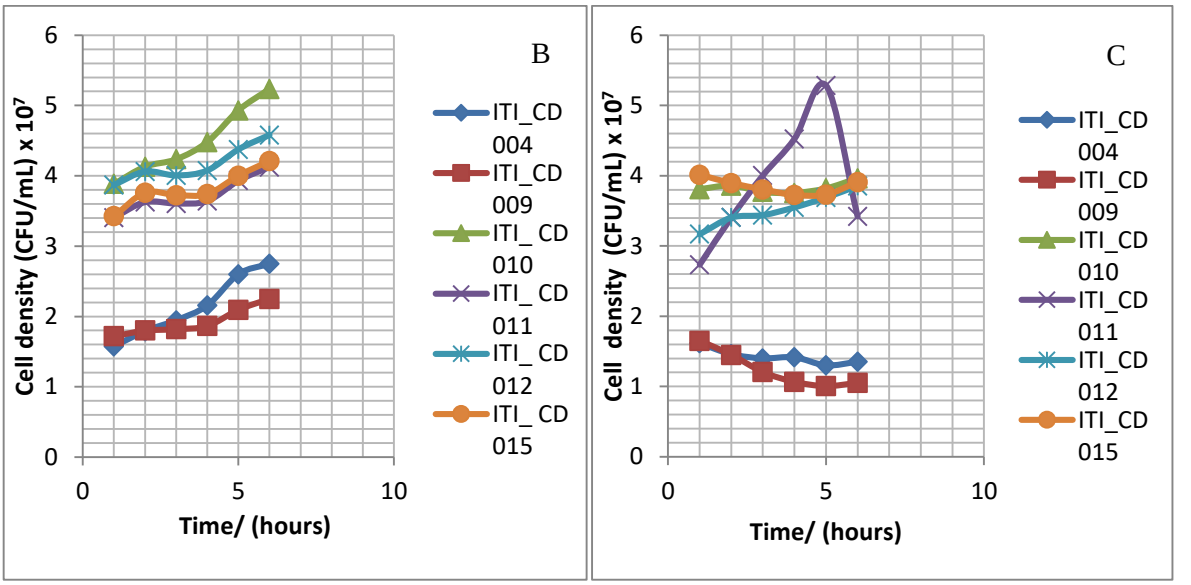


Figure 3: Growth of isolates at (A) 0.2% (w/v) and (B) 0.4% (w/v) and (C) 0.6% (w/v) phenol concentrations

Phenol tolerance

The results showed that all the isolates were able to tolerate 0.2% (w/v) concentration of phenol (Figure 3A). All selected isolates were able to tolerate 0.4% phenol (Figure 3B) while ITI_CD 010,ITI_011,ITI_CD 012 and ITI_CD 015exhibited higher tolerance to 0.4% and 0.6%. However, ITI_CD 004and CD 009 showed slightly low tolerance at 0.6% phenol (Figure 3C).These results are in agreement with those observed by Divya *et al*. (2012) where LAB isolated from milk samples tolerated phenol up to 0.6 %(w/v).

3.3 Antibiotic resistance

The ITI_CD 004 was succeptible to nine antibiotics that were used in this study except vancomycin. Intermediate results were given to sulphamethoxazole and norfloxacin. Resistance was shown to erythromycin,vancomyin and norfloxacin by ITI_CD 009. Isolate ITI_CD 010 was resistant to sulphamethoxazole, ampicillin, amikacin, cephalothin, vancomycin and norfloxacin. Resistance was shown to four antibiotics; sulphamethoxazole, ampicillin, norfloxacin and vancomycin by ITI_CD 012. ITI_CD 015 was resistant to only two antibiotics; vancomycin and norfloxacin. The highest antibiotic resistance was shown by the isolate ITI_CD 010 which was resistant to six out of twelve antibiotics (Table5).

Antibiotic resistance is an important property of a probiotic strain (Majhenič and Matijašič,2001). Normal intestinal microflora become unstable when using antibiotics. Therefore, it is advised to take probiotics containing food products to re-establish the normal intestinal microbiota.

Sometimes these resistance genes of probiotic strains can be transferred to pathogenic bacteria (Mathur and Singh, 2005). Therefore,probiotic strains should be carefully selected when preparing probiotic food products (Majhenič and Matijašič, 2001).

Some LAB strains of *Lactobacillus* have been reported to be intrinsically resistant to vancomycin (Karapetkov *et al*., 2011). In the present study also resistance was shown by all the isolates used in the test to vancomycin. The

resistance to vancomycin by *Lactobacillus* strains is due to the presence of D-Ala-D-lactate in their cell wall component peptidoglycan instead of the normal dipeptide D-Ala-D-Ala, which is the target of the antibiotic (Monteagudo-Mera *et al*., 2012). The tested isolates were highly resistant to vancomycin and norfloxacin and highly susceptible to amoxicillin, chloramphenicol, gentamycin, cepatoximeamikacin and erythromycin. Though Puphan *et al*. (2015) reported that LAB are, in general, sensitive to ampicillin, penicillin and erythromycin, in this study ITI_CD 010 and ITI_CD 012 were identified as amphicillin resistant bacteria. However, the basis of this resistance is not clear and different studies indicate that it can be an intrinsic property of the bacterium (Monteagudo-Mera *et al*., 2012). It is suggested that antibiotics along with probiotics intake would enhance the effectiveness of the treatment for bacterial infections and efficient recovery of affected intestinal microflora (Majhenic and Matijiasic, 2001). Future studies should be directed to investigate the effectiveness of LAB from curd to cure and prevent gastrointestinal infections and LAB product formulations.

Table 5. Antibiotic resistance of potential probiotic lactic acid bacteria

Antibiotic	Mean diameter of inhibition zone (mm)				
	CD 004	CD 009	CD 010	CD 012	CD 015
Sulphamethoxazole	19.0±0.1 (I)	35.1±0.2(S)	0 (R)	0 (R)	21.1±1.4 (S)
Ampicillin	47.5±0.4(S)	36.3±0.4(S)	0 (R)	0 (R)	29.8±0.6 (S)
Erythromycin	51.6±0.9(S)	0 (R)	35.3±0.4(S)	35.3±0.4(S)	39.5±0.7 (S)
Amoxycillin	47.3±0.4(S)	33.6±1.6(S)	28.0±0.7(S)	31.0±3.2(S)	32.6±1.24(S)
Amikacin	25.0±0.8(S)	30.8±1.3(S)	1.3±0.4 (R)	37.5±0.7(S)	31.3±1.5 (S)
Chloramphenicol	23.8±1.6(S)	29.6±0.4(S)	22.0±1.0(S)	37.1±0.6(S)	29.5±1.0 (S)
Gentamicin	27.8±0.2(S)	31.0±2.1(S)	27.0±0.4(S)	30.0±0.8(S)	32.6±0.9 (S)
Cefotaxime	39.6±0.6(S)	26.1±0.2(S)	24.0±0.8(S)	28.8±1.8(S)	34.0±1.4 (S)
Cephalothin	39.6±1.2(S)	31.8±0.6(S)	11.6±0.4(R)	16.8±0.2 (I)	17.8±0.2 (I)
Vancomycin	9.6±0.4 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Norfloxacin	20.5±0.4 (I)	0 (R)	0 (R)	0 (R)	0 (R)
Tetracycline	25.5±0.4(S)	25.5±0.7(S)	17.1±0.6 (I)	17.1±0.6 (I)	20.3±0.4 (I)

R, Resistant; S,Succeptible; I, Intermediate

4. CONCLUSIONS

LAB isolates in curd were identified as *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus delbrueckii lactis*. Potential probiotic isolates were screened evaluating their probiotic potential using promising probiotic characteristics.The results demonstrate the potential probiotic ability of the all isolates from curd. Varied response to 12 antibiotics observed among LAB isolates in the present study. However, all tested isolates showed resistant to vancomycin. LAB isolates from curd can be used to produce probiotic rich safe food products.

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