Evaluation of Probiotic Attributes of *Lactobacillus* Sp. Isolated from Different Cow and Buffalo Curd Samples Collected from Central Province

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Abstract

Curd is a potential source of probiotic *Lactobacilli* species. This study was carried out to isolate and characterize *Lactobacilli* species available in curd samples sold in the market.

Nine curd samples prepared from cow and buffalo milk were obtained from the local market in Kandy district. A total of seven isolates (LB 1-7) were identified based on their colony morphology and biochemical characteristics and evaluated for their probiotic attributes such as low pH tolerance, resistance to bile salt, antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa*, antibiotic activity against Erythromycin, Chloramphenicol and Norfloxacin, haemolytic activity and DNase activity.

It was observed that all the isolates were able to grow at low pH (pH=3.0) and able to survive at 0.3% bile salt, however, the viability decreased with time. LB7 showed very low viability to bile salt compared with the others. All isolates exhibited antimicrobial activity against the two pathogenic organisms tested and the maximum zone of inhibition (18±1.13mm) was observed against *E.coli* by two isolates (LB1 and LB2) and against *P.aeruginosa* by four isolates (LB1, LB2, LB6 and LB7). Only LB6 and LB7 exhibited resistance to all three antibiotics tested while the other isolates were found to be sensitive. In general, a higher sensitivity was shown against Erythromycin and Chloramphenicol compared with Norfloxacin. Furthermore, all the isolates exhibited δ-haemolysis (non-haemolysis) while none of the isolates showed any DNase activity.

Further investigations are in progress to identify the *Lactobacilli* strains available in these isolates.

Introduction

Probiotics are live microorganisms used as food supplements, which provide health benefits when consumed, through improving the intestinal microbial balance of the host. Most probiotic organisms belong to lactic acid bacteria (LAB) which comprise of a wide range of genera and include a considerable number of species especially *Lactobacillus*, *Bifidobacterium* and *Enterococcus* species. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also present in the gastrointestinal microflora. *Lactobacilli* are one of the most important genera of LAB which are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks and curd is one of the best-known foods that contain probiotics.

Lactobacilli comprise of a large and diverse group of gram positive, non-spore forming, catalase negative rod bacteria, able to produce lactic acid as the main end product of the fermentation of carbohydrates. They are considered as generally recognized as safe (GRAS) organisms and safely used as probiotics for food, medical and veterinary applications.

Probiotics should be resistant to specific conditions of the gastrointestinal tract (GIT), remain resistant for more than 4 hrs to proteolytic enzymes, low pH values (1.8-3.2) prevailing in the stomach and to bile concentration, pancreatic juices and mucus which are part of the small intestine. Furthermore, bacterial strains to be used in probiotics obtention are supposed to be resistant to antibiotics eventually administered in animal diets and, are also to be a producers of antimicrobial substances such as lactic acid, hydrogen peroxide, bacteriocins, etc.

Materials and Methods

Sample collection

Clay potted curd samples prepared from cow and buffalo milk were randomly collected from local market in Kandy area.

Isolation of Lactobacilli

A loopful of curd samples was streaked on the sterile Rogosa SL Agar by quadrant streaking

method under aseptic conditions. After streaking Petri plates were incubated at 37°C for 24 hrs under anaerobic conditions. Subsequently purified cultures were obtained by sub culturing on Rogosa SL agar by incubating at 37°C for 24 to 48 hrs.

Identification and characterization of pure culture

Morphological examination was carried out by using Gram's staining method described by H. C. Gram (1884).

Biochemical examination of culture

Endospore staining

Bacterial smear was prepared on microscopic slide and heat fixed. Malachite green (primary stain) was applied and heat fixed. Slide was removed from the flame and rinsed with water until water run clear. Then the slide was flooded with the counter stain diluted carbol fuschsin for 30 seconds and rinsed with water. After that slide was air dried and observed under the light microscope.

Motility Test

Motility of strains was examined by "hanging drop method". A drop of bacterial culture was placed in the centre of a coverslip. A drop of paraffin placed at each corner of the coverslip. A cavity slide was inverted over the coverslip so that it was stick to the slide and the drop of bacterial culture was suspended in the central depression of the cavity slide. Motile organisms were observed under microscope.

Catalase Test

A clean microscopic slide was taken. A drop of 3% H_2O_2 was taken on the microscopic slide aseptically. A loopful of bacterial culture was added on 3% H_2O_2 solution on the slide and allowed to react for 30 seconds. The presence of a bubble was recorded as catalase positive and absence as catalase negative.

Probiotic characterization of pure culture

Resistance to low pH

Lactobacilli isolate obtained from overnight culture was inoculated in MRS broth and incubated at 37°C for 24 hours. The culture broth was transferred into 10ml MRS broth adjusted to pH 3 with conc. HCl and incubated at 37°C under anaerobic condition. Resistance was assessed in terms of viable colony counts enumerated on Rogosa agar in triplicate at 0, 1, 2, 3 and 4 h. Plates were incubated at 37°C under anaerobic conditions for 48 h. The survival rate of *Lactobacilli* was observed for colonies grown on Rogosa agar compared with the initial bacterial concentration.

Bile state tolerance

1 ml of overnight culture was added to 10 ml of 0.3% bile containing broth and incubated at 37C under anaerobic condition. Viable colonies were counted for every hour of incubation time at 0, 1, 2, 3 and 4 h. Plates were incubated at 37C under anaerobic condition for 48 h.

Antimicrobial activity

Antimicrobial activity of the *Lactobacillus* sp. was determined using the disc diffusion method on nutrient agar against pathogenic strains *Pseudomonas aeruginosa* and *Escherichia coli*. The pathogenic strains were inoculated on nutrient agar plates using sterilized cotton swabs. Sterile paper blank discs of 9 mm dia. were dipped in fresh overnight culture and placed on the surface of agar plates. The plates were kept at 4°C for 30 min to permit diffusion on the assay material, and incubated at 37°C for 24 h. Zone of inhibition was measured in mm. The assay was repeated 30 times. Discs dipped in sterile water served as control.

Antibiotic Activity

Fresh overnight culture was spreaded evenly on the surface of Rogosa agar plates using sterile cotton swabs. Antibiotic discs of Streptomycin and Gentamycin were placed on agar plates and kept for 30 min at 4°C for diffusion of antibiotics. Discs dipped in sterile water served as control. The plates were then anaerobically incubated at 37°C for 24 hrs. Zone of inhibition diameters were measured inclusive of the diameter of discs.

Haemolytic activity

Blood Agar plates were prepared using Blood Agar Base and 10% human blood. Fresh overnight cultures were streaked on blood agar plates and incubated at 37°C for 24 hrs in anaerobic jars. After incubation, the agar plate was observed for zones around the colonies. The assay was repeated 30 times.

DNase test

Fresh overnight broth culture was inoculated on DNase agar and incubated at 37°C for 24 hrs. After incubation, DNase agar plate was flooded with HCl and excess was removed. After 5 min, agar plate was observed for a "halo" appearance surrounding the strains. The assay was repeated 30 times.

Data analysis

Data were analyzed by using analysis of variance (ANOVA) and Comparison of means was carried out. Some experiments were repeated in triplicates (n=3) and some were replicated for 30 times (n=30).

Results and Discussion

Isolation of Lactobacillus

A total of seven isolates were obtained from curd samples by streak plate method and classified as LB1, LB2, LB3, LB4, LB5, LB6 and LB7.Considering the storage condition, curd samples were divided into four groups as shown in Table 1.

Buffalo curd		Cow curd	
Refr.	**Non-Refr**	**Refr.**	**Non-Refr.**
LB1	LB4	LB7	No isolates
LB2	LB5		
LB3	LB6		

Table 1: Isolates from cow and buffalo curd samples
Refr. – Refrigerated, Non-Refr. – Non-Refrigerated

Identification of pure culture

Isolated colonies were characterized and identified based on their colony morphology and biochemical characteristics. Most of the colonies were small, smooth and white-cream in color while colony LB6 having a rough texture. All isolates were found to be gram positive, rod shaped, non-spore forming, non-motile and catalase negative while LB6 showed long rods compared to other isolates.

Based on these morphological and biochemical characteristics, the isolates tested were identified as *Lactobacillus* sp. The results were comparable with those reported .

Isolate	Colony Morphology	Gram staining
LB1	White-creamy, smooth	+ rods
LB2	White-creamy, smooth	+ rods
LB3	White-creamy, smooth	+ rods
LB4	Creamy, smooth	+ rods
LB5	White-creamy, smooth	+ rods
LB6	White-creamy, rough	+ Long rods
LB7	White-creamy, smooth	+ rods

Table 2: Morphological/Biochemical characteristics
All isolates showed negative results in endospore staining, motility and catalase tests

Resistance to low pH

In this study, the resistance to low pH was assessed in terms of viable colony counts which resist at pH=3, for 4 h of incubation. All isolates were able to grow at low pH while their viability decreased with time. Most of them showed significant growth at pH 3.0 even after 5 h of incubation. But the number of viable colonies decreased. However, all isolates were able to retain constantly at low pH as shown in Fig. 1.

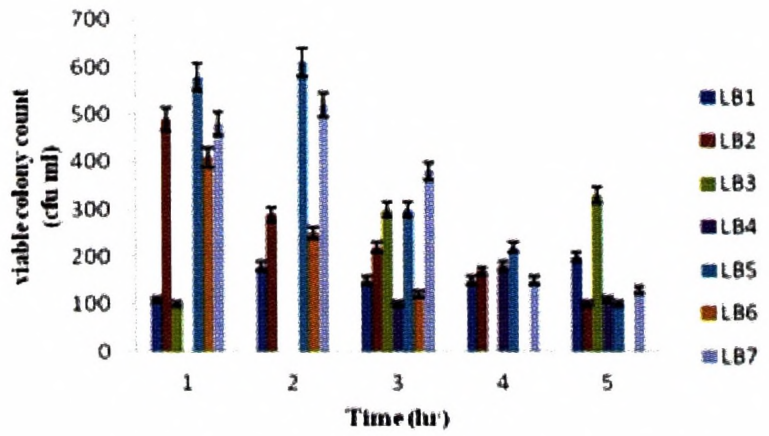

Fig. 1: Survival rate of isolates at pH 3.0

The probiotic isolate must travel through human stomach and survive in the acidic pH 1.5 to 2.0, before reaching the intestine to colonise and to establish metabolic activity. Survivability of isolates in in low pH and high levels of bile allows the probiotics to survive through gastric transient in the stomach and reach the intestine and thus maintain gut flora. As they establish in the GIT, microorganisms enhance their active metabolic pathways resulting in the release of some organic molecules which are beneficial to host. Therefore, pH 3.0 is set as the standard acid tolerance for screening the acid toleranceThis is also due to the fact that a significant decrease in viability of the strains is often observed at pH 2.0 and below. In a study, no viable cells were found at pH 2 after 30 min, but at pH 3, the number of viable *L. acidophilus* cells decreased with time, whereas in the case of *L. casei*, the number of viable cells were found to be constant at pH 3.

Resistance to bile salts

The study showed that all isolates were able to survive at 0.3% bile salt concentration, although viability decreased with time. After 2 h of incubation, a drastic reduction in viability was observed in all isolates. Fig. 2 illustrates the variation of the isolates in resistance to bile salt after 2h of incubation. LB6 showed highest growth compared to other isolates whereas, LB7 showed no growth at 2 h of incubation.

The tolerance against bile salt was carried out based on the intestinal bile concentration, 0.3% and staying time of food in small intestine is 4hr.[13]

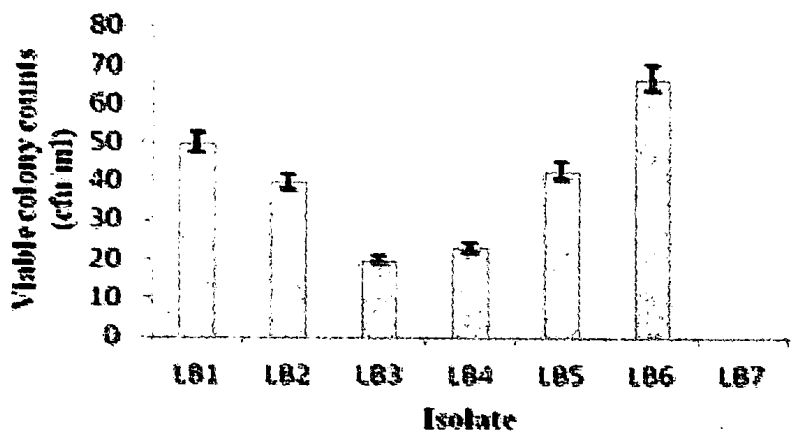

Fig. 2. Bile salt tolerance of isolates 2h of incubation

Antimicrobial activity

The study also showed antimicrobial activity of the isolates against pathogenic organisms *Escherichia coli* and *Pseudomonas aeruginosa*. The diameters of zone of inhibition were measured. Two isolates (LB1 and LB2) exhibited maximum zones of inhibition against *E.coli* while four isolates (LB1, LB2, LB6 and LB7) showed inhibition against *P. aeruginosa* (Table 3).

Lactic acid bacteria are known to produce antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl which are capable of inhibiting the growth of pathogenic and spoilage microorganisms.

Antibiotic Activity

In this study, resistance was tested by the disc diffusion method using antibiotic discs of Erythromycin, Chloramphenicol and Norfloxacin.

Table 3. Antimicrobial activity of isolates (Mean± SD). Means with same subscript are not significantly different

Isolate	Diameter of zone of inhibition (mm)	
	P. aeruginosa	***E.coli***
LB1	17.89±0.80^a	18.29±0.51^a
LB2	17.78±0.25^a	17.58±0.11^b
LB3	16.11±0.36^a	16.88±0.22^a
LB4	14.78±0.53^a	13.64±0.1^b
LB5	16.78±0.26^a	15.05±0.15^b
LB6	17.62±0.40^a	15.23±0.45^a
LB7	18.89±0.16^b	16.05±0.26^a

Only LB6 and LB7 exhibited resistance to all three antibiotics tested while the others were found to be sensitive. In general, a higher sensitivity was shown against Erythromycin and Ciprofloxacin compared with Norfloxacin.

As shown in Table 4, LB4 shows significant difference in antibiotic activity against both Erythromycin and Chloramphenicol compared with other isolates. A significant difference was also observed with LB2 against Norfloxacin. The resistance of probiotics tó antibiotics helps to replenish normal microflora in an individual after antibiotic therapy. Probiotics have been used to prevent antibiotic associated diarrhoea, which results from an imbalance in gut microflora caused by antibiotic therapy.

Table 4. Antibiotic resistance of isolates against antibiotics (Mean±SD) Means with same subscript are not significantly different; Chloram.=Chloramphenicol

Isolate	Diameter of zone of inhibition (mm)		
	Erythromycin	Chloram.	Norfloxacin
LB1	19±0.57^a	21±0.57^a	23±0.57^a
LB2	29±0.57^a	27±0.57^a	7±1.15^b
LB3	21±0.57^a	20±0.57^a	17±0.57^a
LB4	31±1.73^b	30±1.15^b	24±0.57^a
LB5	14±0.57^a	17±1.00^b	0
LB6	0	0	0
LB7	0	0	0

Lactobacilli are known to have a high natural resistance to some antibiotics including bacitracin, ciprofloxacin, gentamicin, norfloxacin and streptomycin.

Haemolytic activity

All the tested isolates exhibited δ-haemolysis (non-haemolysis) which is considered as a safety character for good probiotic bacteria. Non-haemolytic activity of probiotic isolates are considered as a safety prerequisite for the selection of a probiotic organism. *L. casei*, *L. delbruekii* and *L. lactis* have been reported to show δ-haemolysis.

DNase activity

DNase activity of *Lactobacilli* isolates grown on DNase agar was examined for the hydrolysis of DNA molecules. It was determined by observing a "halo" appearing round the isolates after applying HCl solution. In this study, none of the isolates showed DNase activity. *Staphylococcus aureus* was used as control strain. It has been suggested that DNase could be used to identify potentially pathogenic Staphylococci.

Conclusion

Isolated bacteria were found to be rod shaped gram positive, non-spore forming and non-motile and therefore confirmed as *Lactobacillus* species. And none of the isolates produced catalase enzyme. The isolates tested demonstrated probiotic attributes such as resistance to low pH, tolerance to bile salt, antimicrobial resistance, antibiotic activity, non-haemolysis and no DNase activity. No significant difference of probiotic attributes was observed between refrigerated and non-refrigerated conditions of curd. Further investigations are in progress to identify the *Lactobacilli* strains available in these isolates.

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