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Short Communication

Antibiosis of bacteriocins with domestic lactobacilli isolated from prepared curd

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Abstract: The antimicrobial activity of lactobacilli has been widely exploited for prevention of food-borne pathogens, food spoilage organisms, biotherapeutic agents and as probiotics. The present study tests antibiosis of lactobacilli isolated from domestically prepared curd because of bacteriocin activity against selected opportunistic pathogens, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Of the seven strains isolated from curd samples, three were identified to be lactobacilli; of which, two were found to have antibiosis against *E. coli* and *Ps. aeruginosa*. As *E. coli* and lactobacilli co-exist in the human intestines, with *E. coli* being the major cause of traveller's diarrhoea; and *E. coli* and *Ps. aeruginosa* also being the most common contaminants of water - proper utilization of the antimicrobial properties of lactobacilli isolated from curd, a very less expensive dairy food product. Thus, this could have a positive impact on the commercialisation of the use of curd as a low-cost probiotic or bacteriotherapeutic agent as a potential alternative to the established commercially available probiotics.

Keywords: Bacteriocins, Curd, *Escherichia coli,* lactobacilli*, Pseudomonas aeruginiosa*

مضاد بكتيريوسين بواسطة لالكلاتوباسلي المعزولة محليا عن طريق رائب مخثر معد مسبقا

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الملخص: يعتبر نشاط المضادات لالكلاتوباسلي مستغلة على نطاق واسع للوقاية من مسببات الأمراض التي تنقلها الأغذية والكائنات المسببة من تلف الأغذية ومعاملات البايوثرابوتك كما في البروبيوتك. في هذه الدراسة الحالية يتم اختبار لمضادات اللاكتوباسلى المعزولة محليا من مخثر مجهز ومعد وذلك بسبب نشاط البكتريوسين ضد مسببات الأمراض ***Staphylococcus*** **aureus,** ***Escherichia coli*** **and** ***Pseudomonas aeruginosa.*** ومن خلال سبع سلالات معزولة من العينات المخثرة حددت ثلاثة من اللاكتوباسلى منها وجد أنها مضادة لمعامل ال ***E. coli*** و ***Ps. Aeruginosa.*** وكما أنها تتعايش مع الإنسان ومع ***E. coli*** يعتبر ***E. coli*** سبب رئيسي للاسهالات للمسافرين وأيضا *E. coli* and *Ps. aeruginosa* تعتبر من الملوثات الأكثر شيوعا في المياه الاستخدام الأمثل لمضادات الميكروبات للالكلاتوباسلى المعزولة عن المخثر يعتبر اقل تكلفة من منتجات الألبان فهذا سوف يكون له اثر ايجابي على تسويق هذا المخثر كونه اقل تكلفة وأيضا معامل بروبيوتيك يعتبر بديل محتمل للبروبتك المتاح تجاريا.

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Introduction

Bacteriocins are a group of heterogenous compounds secreted extraneously by bacteria that has antagonists activity with widely varying molecular weight, biochemical properties, broad spectrum of host organisms and mode of action. Bacteriocins can be classified broadly as those synthesized by Gram-positive and those by Gram-negative organisms. Amongst those synthesized by Gram-positive organisms, lactobacilli bacteriocins are of commercial value. Since 1961, after the first detailed characterisation of bacteriocinogenic activity of lactobacilli was reported by (Coetzee and Klerk, 1961), much attention has been given to research on characterising bacteriocins from various sources like curd, sake, sausages, idli, cheese, wine, sour dough, etc. Lactobacilli bacteriocins are grouped as class-I (lantibiotics), class-II (heat-stable compounds), and class-III (heat-labile proteins). Although knowledge on bacteriocins and their activity has been limited, the use of food preparations using lactic acid starter cultures, and the use of bacteriocin producing lactic acid cultures or strains of lactic acid bacteria showing antimicrobial properties have been utilised for controlling food spoilage (Guerrieri et al. 2009), food-borne pathogens (Guerrieri et al., 2009; Randazzo et al., 2009), improve food fermentation processes (Varadaraj et al., 1990), and as biotherapeutic agents (Kim et al., 2008; Mishra and Lambert, 1996), and quite recently as antifungal agents too (Voulgari et al., 2010). As of to date, nisin from *Lactococcus lactis* (Delves-Broughton, 1990) and pediocin A from *Pediococcus pentosaceous* B61 and L-7230 (Daeschel and Klaenhammer, 1985) are the two bacteriocins that have been characterised for industrial use and as broad-spectrum antimicrobial agent against food-borne pathogens; nisin, being the only bacteriocin that has been licensed across forty-five countries as a food additive (Hurst, 1981). The present preliminary work aims to evaluate the use of lactobacilli strains isolated from domestically prepared curd for bacteriocin activity, and thereby discusses the prospect of using curd as a low-cost biotherapeutic agent.

Materials and Methods

Isolation of Lactic Acid Bacteria

Lactic acid bacteria (LAB), *Lactobacillus* sp. were isolated from commercial LAB capsules (spores dissolved in a small amount of distilled water) and domestically prepared curd on skim milk agar plates using streak plate technique, and incubated at 37°C for 24 hours. Skim milk agar was prepared following the procedure adopted in (Atlas, 2004).

Isolated, dissimilar colonies were selected from these plates, recultured into sterile milk from similar source and were re-isolated on the same medium. The pure culture thus obtained was subcultured on milk agar plates for further characterisation.

Identification of *Lactobacillus* sp.

Lactobacillus sp. were characterised following the step-by-step methodology described in Hendricks and Holt (1994); Sneath et al. (1986) and Cappucino and Sherman (1998).

Microscopical Observation

The seven strains thus isolated were microscopically screened for Gram's reaction, Acid-Fast Stain Reaction and motility by hanging drop method, using overnight broth culture.

Biochemical Tests

Strains thus identified as cultures of *Lactobacillus* sp. were further characterised for indole production, hydrogen sulphide production, catalase reaction, nitrate reductase reaction, and for

utilization of glucose and/or lactose as substrates for fermentation. The results of the biochemical tests were thus used to confirm *Lactobacillus* sp. and differentiate the biovariants.

Enhancement of the production of antimicrobial substances

In order to enhance the production of the antimicrobial substances, bacteriocin, and the selected strains were subcultured onto Paneer Whey Medium (Desai and Purandare, 1996) that was prepared, steamed for 60 minutes and filter-sterilised. The clear whey was supplemented with fortified substances and the pH was adjusted to pH 6.4. The sterile medium was then inoculated with the selected subcultures and incubated for 24 hours at 37°C.

Screening for bacteriocin producing strains

Primary screening using spot-assay method

Escherichia coli, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, the pathogenic test organisms and the different strains isolated were cultivated using broth culture technique at 37°C for 24 hours. The pathogenic test organisms were inoculated on the nutrient agar plate using swabs, and lawn culture were developed. Then a drop of 0.1 ml of the overnight broth culture of the isolated strains of *Lactobacillus* sp. were placed in the wells cut, and incubated at 37°C for 24 hours and observed for zones of inhibition.

Secondary screening for concentration dependant antibacterial activity

The overnight broth cultures of the screened strains of *Lactobacillus* sp. were tested for their concentration dependant antimicrobial activity by pre-inoculating the nutrient agar plates with those pathogenic species against which exhibited zones of inhibition. Wells were cut and differing volumes of overnight broth cultures of LB1 and LB2 were placed on filter paper discs, air-dried and placed on the medium under sterile conditions, and incubated for 24 hours at 37°C and observed for development of zones of inhibition of growth of pathogenic species.

Bacteriocin assay

A bacteriocin assay was performed to confirm that the antimicrobial activity was due to the extraneous secretion of bacteriocin into the medium and not due to the growth of the *Lactobacillus* sp. A two-fold dilution of the isolated and screened *Lactobacillus* sp., LB1 and LB2, emulsions were prepared in sodium phosphate buffer to stabilise the medium, pH 7.2; from the culture filtrate obtained by filtering freshly prepared overnight broth culture followed by centrifugation at 10, 000 rpm for 20 minutes. 0.1 ml of the diluted samples were placed in wells cut on nutrient agar plates pre-inoculated with the test pathogenic species using swab-culture technique. Plates were examined for zones of inhibition of growth after 24 hours of incubation period at 37°C. Antibacterial activity was defined as the inhibition of the highest dilution showing definite inhibition of bacteria and was expressed as arbitrary units (Au/ml) (Varadaraj et al., 1990).

Results and Discussion

Isolation of lactic acid bacteria

Lactic acid bacteria, *Lactobacillus* sp., were isolated from commercial LAB capsules and domestically prepared curd on milk agar plates. Seven distinct, smooth, white and mucoid colonies were identified, screened and, subcultures thus obtained from them were labelled and maintained as strain LB1, LB2, LB3, LB4, LB5, LB6 and LB7 and were further characterised for identification and confirmation as *Lactobacillus* sp.

Identification of *Lactobacillus* sp.

Lactobacillus sp. were characterised following the step-by-step methodology

described in (Hendricks and holt, 1994; Sneath et al., 1986 and Cappucino and Sherman, 1998).

Microscopical observation

The seven strains thus selected were microscopically observed for Gram's reaction, Acid-Fast Stain Reaction and Motility. Based on the response of the seven strains (Table 1), three strains, LB1, LB2 and LB3 that had non-motile, rod-shaped cells in chains and showed Gram positive, non-acid-fast reactions and were selected.

Table 1. Microscopical identification for *Lactobacillus* sp.

Morphological Features	LB 1	LB 2	LB 3	LB 4	LB 5	LB 6	LB 7
Shape and Arrangement	Rod, chains	Rod, chains	Rod, chains	Cocci, cluster	Cocci, chain	Cocci, cluster	Cocci, cluster
Gram's Staining	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Acid-fast Staining	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Motility	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Biochemical tests

Three strains thus screened as cultures of *Lactobacillus* sp. were further characterised for indole production, hydrogen sulphide production, catalase reaction, nitrate reductase reaction, and carbohydrate fermentation responses.

The results of the biochemical tests were used to confirm *Lactobacillus* sp. and differentiate the biovariants based on their response to sugar fermentation analyses. Table 2 summarises the results thus obtained confirms that the three strains of *Lactobacillus* sp. and that there is some variation amongst their response to carbohydrate fermentation, indicating that the three strains are different biovars.

Table 2. Biochemical Characterisation For *Lactobacillus* sp.

Biochemical tests		LB 1	LB 2	LB 3
Carbohydrate fermentation reaction	Glucose	Acid and Gas Production	Acid and Gas Production	Acid, but No Gas Production
	Lactose	Acid and Gas Production	Acid, but no Gas Production	Acid, but No Gas Production

LB 1 was positive for acid and gas production for both glucose and lactose. LB 2 was positive for acid and gas production for glucose and, not for gas while utilizing lactose. LB 3 utilized glucose and lactose by producing gas only and no acid. Thus, the results of carbohydrate fermentation reaction for *Lactobacillus* sp. indicates that the three strains thus isolated were different biovars. Similar variations in lactobacilli have been observed by Hassaine et al. (2008) and by Soda et al. (2003) for isolating variants for cheese making.

Antimicrobial activity of the isolates

Preliminary screening for antimicrobial activity

All the three strains were screened initially for antimicrobial activity using spot-assay method against the three test opportunistic pathogenic organisms, *E. coli, Pseudomonas aeroginosa,* and *Staphyloccocus aureus*. Preliminary screening of bacterial isolates showed that LB1 and LB2 had antibacterial activity against *E. coli* and *Ps. aeroginosa*, but not LB3. LB1 and LB3 had no antibacterial against *Staph. aureu*s, but LB2 did show some antibacterial activity, but not LB1 and LB3. Overall, LB1 and LB2 had antimicrobial effect, while LB3 seemed to be passive.

Secondary screening for concentration-dependant activity

Two of the four isolated strains, LB1 and LB2, were identified to have antagonistic activity against the selected opportunistic pathogen. Based on the area of zone of inhibiton as an indicator for selection of appropriate inoculum density for obtaining maximal antagonistic activity, *Lactobacillus* sp. Strains LB1 and LB2 showed no significant concentration dependancy against *E. coli*, while LB1 had no concentration dependency against *E. coli*, while the area of zone of inhibition was four to five times more under three-fold dilution of the inoculum, and decreased with increasing or decreasing density of the inoculum against *Ps. aeroginosa* (Table 3).

Table 3. Antimicrobial activity of Lactobacillus sp. strains at varying dilution.

Test organisms		**Zone of inhibition in mm**	
Strains	**Dilution**	***E. coli***	***Ps. aeroginosa***
LB 1	10^{-2}	1.0	1.3
	10^{-3}	1.3	1.2
	10^{-4}	1.0	1.4
LB 2	10^{-2}	1.3	0.6
	10^{-3}	1.4	1.5
	10^{-4}	0.7	1.0

Bacteriocin assay

This assay confirmed that the antagonistic activity against the tested opportunistic pathogens was due to the extracellular secretion of bacteriocin and not other organic acids. However, the extent of inhibition varied amongst the strains against the selected two pathogens, and each strain responded differently to the selected pathogens as evident in Table 4. LB1 strain seemed to decrease with increasing dilutions tested, indicating that either the syntheses of the bacteriocin is not in required quantities for efficient antimicrobial activity, and may require a medium with varying composition that has to be evaluated, or the activity of the compound is not very high. In order to answer these questions, further biochemical analyses for isolation, purification and characterisation of the compound, and evaluation thereof of their activity is required.

On the whole the current study carried out revealed that the lactobacilli strains isolated and screened from homemade curd samples had bacteriocins with antimicrobial activity, in specific bacteriocins and not the organic acids that are synthesized as a result of their growth activity, with properties to inhibit the test organisms, both being Gram-negative than Gram-positive that has been usually observed for strains isolated from curd (Varadaraj et al., 1990). Strains of lactobacilli producing bacteriocins, and the

industrially established bacteriocins like nisin, isolated from lactococcus, that are of wide use in the food and dairy industrial sectors have so far been known to possess antimicrobial activities against Gram-positive organisms (Fukasae et al., 1988 and Buchman et al., 1988), except for few cases like *Lactobacillus acidophilus*, starter culture in acidophilus milk has been known to have antagonistic activity against Gram-negative organisms (Mittal and Garg, 1995), and strains of *Lactobacillus casei* have been known to show antibiosis against *E. coli* and *Ps. aeroginosa* (Lozo et al., 2004). However, these reports do not clarify whether the antimicrobial activity is due to the organic acids produced by the activity of lactobacilli or is due to the bacteriocin activity. This study is the first to report the activity of bacteriocins produced by lactobacilli strains isolated from homemade curd against Gram-negative organisms.

Table 4. Antimicrobial activity of bacteriocin from lactobacillus sp. lb1 and *Lactobacillus* sp. lb2.

Test Organisms		**Zone of Inhibition in mm**	
Strains	**Dilution**	***E. coli***	***Ps. aeroginosa***
LB 1	4	2.0	3.0
	8	1.4	1.4
	16	1.3	1.2
	32	1.0	0.7
LB 2	4	1.8	1.7
	8	1.6	1.2
	16	1.5	1.9
	32	0.4	2.0

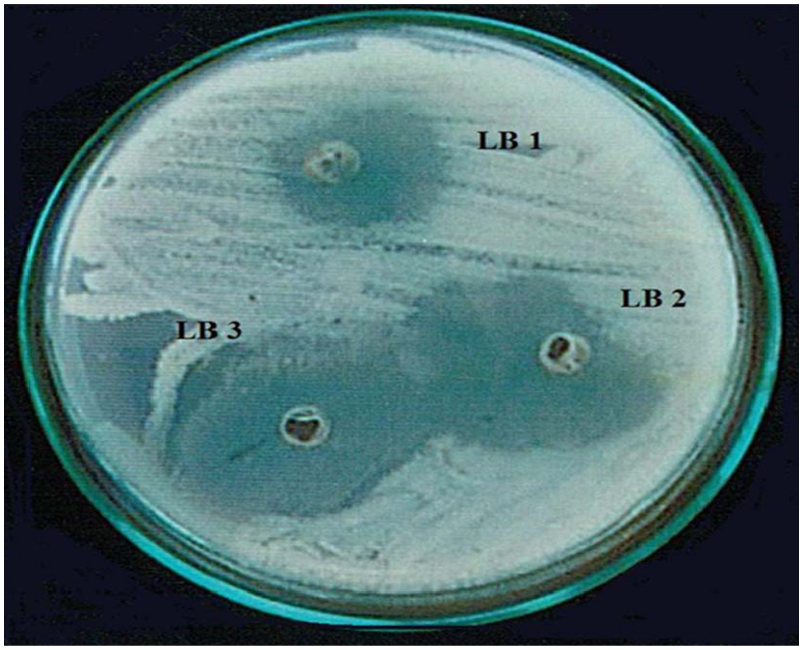

Figure 1. Preliminary screening of lactobacilli strain, LB 1, LB 2 and LB 3 were tested for antibiosis against one of the opportunistic test pathogen, *E. coli.* Zones of inhibition were measured and strains showing positive results were thus considered for further studies.

Lactobacilli and *E. coli* coexist in the human gut, but sometimes, *E. coli* being an opportunistic enteric pathogen can result in enteric diseases like Traveller's Diarrhoea. Moreover, *E. coli* and Pseudomonas *aeroginosa* are the most common contaminants of water and indicators of faecal contamination in drinking water. Reports of the presence of strains of lactobacilli producing bacteriocins with antimicrobial activity against Gram-negative organisms in homemade curd therefore will be of both industrial and medical significance, if further mass screening, evaluation, characterisation of bacteriocin producing lactobacilli strains, or isolation and characterisation of bacteriocins from lactobacilli strains, were used as starter cultures in commercial production of curd.

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