Original Article

Inhibitory potential of *Lactobacillus* species isolated from fermented dairy products against *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Background & Objectives: Probiotics exert a strong antagonistic activity against many microorganisms including food spoilage organisms and enteropathogens. Lactobacilli find increasing acceptance as probiotics by showing its beneficial effects. Keeping in view the benefits of probiotics in literature, the present study was planned to isolate *Lactobacillus* spp. from different fermented dairy products and to study the inhibitory potential against biofilm forming bacteria viz. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

Methods: *Lactobacillus* spp. were isolated from different fermented dairy products and characterized. The antimicrobial activity of each *Lactobacillus* spp. was checked by agar well diffusion and overlay methods. The strains showing inhibitions in these assays were further used to detect biofilm inhibition against *E. coli* and *S. aureus* by microtiter plate biofilm assay method.

Results: A total of 21 samples were studied of which pure growth was observed in 67% and mixed growth was observed in 33% of the samples. Of 18 isolates obtained, 45% belonged to *Lactobacillus plantarum (L. plantarum)*, 33% were *Lactobacillus acidophilus (L. acidophilus)* and 22% comprised of *Lactobacillus fermentum (L. fermentum)*. Ten isolates showed inhibition against *E.coli* and 9 isolates showed inhibitory activity against *S. aureus* in agar well diffusion assay and overlay method. In microtiter plate biofilm assay, the absorbance values were less in the wells where *E. coli* and *S. aureus* were mixed with the *Lactobacillus* broth.

Interpretation and Conclusions: The *Lactobacillus* strains showing inhibitory activities against the pathogens can be used as probiotics or as starter culture in food fermentations after confirming other attributes.

Keywords: Bacteriocins, Biofilm, *Lactobacillus*, Probiotics

INTRODUCTION

Probiotic bacteria are well known to have a positive effect on the maintenance of human health. Lactobacilli find increasing acceptance as probiotics which aid in stimulating immune responses, preventing infections by pathogenic bacteria, treating and preventing diarrhoea.[1] They can improve lactose digestion, play a role in preventing and treating diarrhea, helping the body to resist and fight infections.[2]

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Probiotics exert a strong antagonistic activity against many microorganisms including food spoilage organisms and enteropathogens. Probiotics are being tried as alternative to antibiotics in treating many gastrointestinal diseases.[3] Probiotics exert their beneficial effects by producing various compounds such as organic acids, hydrogen peroxide, diacetyl, bacteriocins and adhesion inhibitors such as biosurfactants.[4] Lactobacilli are important in the food and dairy industries because lactic acid and other organic acids produced by these bacteria act as natural preservatives as well as flavor enhancers.

Bacteriocins are extracellularly released proteinaceous antimicrobial compounds released which exhibit bactericidal effect against closely related bacteria.[5] Biosurfactant production by these bacteria can prevent biofilm formation by bacterial pathogens.[6] *Lactobacillus* spp. exhibiting antagonistic activities towards food spoilage bacteria or enteropathogens are especially

important as these microorganisms even at low levels pose a significant spoilage and public health threat.[7] Considering the beneficial effects of probiotics, the present study was planned to explore the inhibitory potential of *Lactobacillus* spp. isolated from fermented dairy products for their inhibitory potential against two biofilm forming microorganisms viz. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

MATERIAL AND METHODS

Lactobacillus species isolated from different fermented dairy products like curd, cheese, butter, fermented milk, lassi and milk were randomly collected from retail markets of Chandigarh, India and its periphery.

Isolation of Lactic Acid Bacteria: *Lactobacillus* spp. were isolated from various fermented dairy samples using 10 fold serial dilution. One ml of each dilution was plated on de-Mann Rogosa and Sharpe (MRS) agar (HiMedia, Mumbai) plate. Then plates were incubated at 37^{0}C for 48 hrs. After 48 hrs of incubation, the plates were checked for colony, color, size, morphology and texture. The isolated colonies were purified by streak inoculation on MRS agar plates and purified culture were stored on MRS agar slants and kept at 4^{0}C till further use. Gram staining was performed on the purified isolates for determining the cell morphology by standard procedure. Biochemical characterization of isolates was done carrying out catalase test, arginine hydrolysis, nitrate reduction, motility, sugar fermentation (glucose, sucrose and dextrose) and gas formation tests.[8]

Antimicrobial activity detection assay: Anti bacterial activity of the *Lactobacillus* spp. was determined by agar well diffusion and overlay method as described earlier.[9]

Agar well diffusion method: The antimicrobial activity due to bacteriocin produced by *Lactobacillus* spp. in broth against *E. coli* and *S. aureus* was checked by agar well diffusion assay. *Lactobacillus* culture was grown in MRS broth for 18-24 hrs at 37^{0}C. The cells were removed by centrifugation at 12000 rpm for 20 min at 5^{0}C. The culture supernatant thus obtained was sterilized by passing through a sterile 0.22 µm membrane filter. The supernatant was adjusted to pH of 7 by addition of 1N HCl and 1N NaOH and used in the assay.

Mueller Hinton agar (HiMedia, Mumbai) plates were inoculated with 0.1 ml of 24 hrs old culture containing approximately 10^8 CFU/ml of *S. aureus* or *E. coli*. Wells of 5 mm diameter were made in Mueller Hinton agar plates. Each well was filled with 100 µl of *Lactobacillus* spp. culture supernatant. The inoculated plates were incubated at 37^{0}C for 24 hrs. Zone of inhibition around the wells was recorded.

Overlay method: An overnight culture of *Lactobacillus* spp. was spotted onto the surface of MRS plate and incubated for 16 hrs at 37^{0}C to allow colonies to develop. Muller-Hinton agar inoculated with 0.1 ml of 24 hrs old culture containing approximately 10^8 CFU/ml of *S. aureus* or *E. coli* was overlaid onto MRS agar. The plates were incubated at 37^{0}C for 24 hrs. Zone of inhibition formed around the *Lactobacillus* culture spot was measured.

Inhibiton of biofilm formation by *E. coli* and *S. aureus*: The inhibition of biofilm formation by *Lactobacillus* spp. was studied by microtiter well plate biofilm assay.[10] Each *Lactobacillus* isolate and indicator strains *E. coli and S. aureus* were inoculated in a 3-5 ml of MRS and nutrient broth respectively and grown to stationary phase. *Lactobacillus* cultures were centrifuged at 12000 rpm for 20 min at 5^{0}C. The culture supernatant thus obtained was then sterilized by passing through a sterile 0.22 µm membrane filter (Millipore). Microtiter plate which has not been tissue culture treated was taken. The first well of micro titer plate was filled with 100 µl of distilled water and was taken as negative control. The second well of the plate was filled with 50 µl of *E. coli* culture and 50 µl of sterile distilled water and set as positive control. Then next ten wells of plate were filled with 50 µl of *E. coli* and 50 µl of the various *Lactobacillus* spp. broth. The same procedure was repeated for *S. aureus.*

The microtiter plate was then covered and incubated at 37^{0}C for 24 hrs. After incubation time, the planktonic bacteria were removed from each well and the wells were washed with sterile distilled water. Water was replaced when it became cloudy. Plate was washed three times. Then 125 µl of 0.1% crystal violet solution was added to each well and the plate was kept at room temperature for 10 min. After 10 min, each well of microtiter plate was washed three times with sterile distilled water to remove crystal violet solution. After drying, 200 µl of 95% ethanol was added to each stained well. The plate was incubated for 10 to 15 min at room temperature to solublize the dye. The contents of each well were mixed and then 125µl of the solution was transferred to another microtiter well plate. Optical density

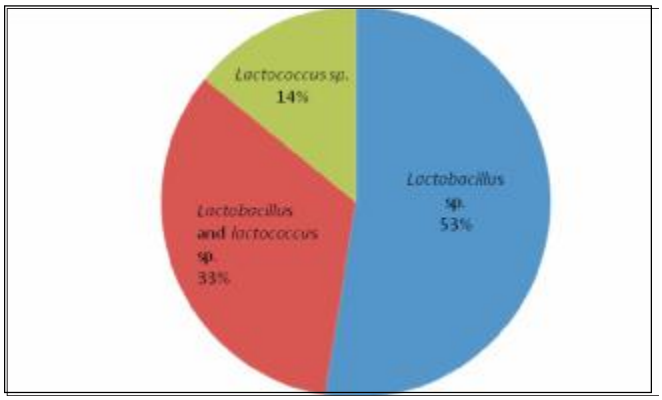
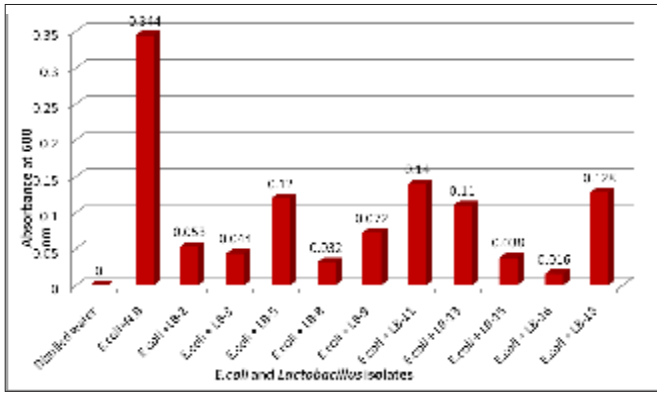


Figure I: Lactic acid bacteria isolated from fermented dairy products



FigureII: Effect of Lactobacillus spp. on biofilm formation by E. coli
N.B= Nutrient broth, LB Lactobacillus

of each well was taken at 600 nm using UV – Visible spectrophotometer (Systronics, India).

RESULTS AND DISCUSSION

A total of 21 different fermented dairy products were collected and processed for isolation of *Lactobacillus* spp. Pure growth was observed in 67% of the samples whereas mixed growth was observed in 33% of the samples. The 18 lactobacilli isolates were characterized on the basis of morphological and biochemical tests. Microscopic identification determined the presence of the rod shaped cells. On the basis of Gram's staining and catalase test it was found that the 18 rod shaped isolates were Gram positive and catalase negative which are the characteristics of lactobacilli. Out of 21 samples, pure growth of *Lactobacillus* spp. was isolated from 11 (53%) samples, mixed growth from 7 (33%) of the samples along with *Lactococcus* spp. whereas 3 (14%) isolates were observed as pure *Lactococcus* spp. as shown in Figure I.

All isolates were found positive for nitrate reductase production and sucrose fermentation. Apart from this, the isolates which were negative for arginine hydrolysis and gas production were identified as *Lactobacillus acidophilus* (*L. acidophilus*). The isolates which were positive for arginine hydrolysis and negative for gas production were identified as *Lactobacillus plantarum* (*L. plantarum*) whereas the isolates showing arginine hydrolysis and gas production in sugar fermentation were identified as *Lactobacillus fermentum (L. fermentum).* On the basis of biochemical results, it was observed that among these isolates 45% were *L. plantarum*, 33% were *L. acidophilus*, and 22% were *L. fermentum*. *Lactobacillus* strains have been isolated and characterized in many studies. Species belonging to *L. acidophilus*, *L. plantarum* and *L. fermentum* are frequently isolated from fermented food products.[11, 12]

All the *Lactobacillus* spp. isolated were studied for antimicrobial activity against *E. coli* and *S. aureus* by agar well diffusion method and overlay method. The bacteriocin activity in agar well diffusion assay by *Lactobacillus* isolates is shown in Table I. Of the 18 isolates only 10 (55%) isolates inhibited the growth of *E.coli* and 9 (50%) showed inhibitory activity against *S. aureus*. Among the ten isolates, 5 isolates of *L. plantarum* (LB- 8, LB-9, LB-13, LB-16 and LB-18), 3 isolates of *L. acidophilus* (LB-5, LB-11, and LB-15), and 2 isolates of *L. fermentum* (LB-2 , LB-3) showed inhibitory activity against *E. coli* (Figure II). Similarly 4 isolates of *L. plantarum* (LB-8, LB-9, LB-13, LB-16), 3 isolates of *L. acidophillus* (LB-5, LB-11, LB-15) and 2 isolates of *L. fermentum* (LB-2 and LB-3) showed inhibitory activity against *S. aureus.*

The antimicrobial activity of *Lactobacillus* spp. was also detected by overlay method. All the 18 isolates of *Lactobacillus* were used to study the inhibitory activity against *E. coli* and *S. aureus*. In overlay method, out of 18 isolates, 10 (55%) showed the zone of inhibition against the organisms studied as shown in Table I. The inhibitory activity in overlay method was shown only by the isolates which inhibited the growth of *E. coli* and *S. aureus* in well diffusion assay. Two isolates of *L. fermentum* (LB-2 and LB-3), two of *L. plantarum* (LB-8 and LB-16), and one of *L. acidophilus* (LB-15) showed strong inhibition zone (>2mm) as compared to other isolates which showed moderate zone of inhibition.

Table: I

Inhibitory pattern of *Lactobacillus spp.* against indicator strains by agar well diffusion assay and overlay method

Sample	*Lactobacillus* spp.	*Escherichia coli*		*Staphylococcus aureus*	
		Agar well diffusion assay	Overlay method	Agar well diffusion assay	Overlay method
Curd	*L. plantarum* LB-1	-	-	-	-
Curd	*L. fermentum* LB-2	+	++	+	++
Curd	*L. fermentum* LB-3	+	++	+	++
Curd	*L. plantarum* LB-4	-	-	-	-
Curd	*L.acidophillus* LB-5	+	+	+	+
Curd	*L. acidophilus* LB-6	-	-	-	-
Curd	*L. plantarum* LB-7	-	-	-	-
Cheese1	*L. plantarum* LB-8	+	++	+	++
Cheese	*L. plantarum* LB9	+	+	+	+
Cheese	*L. acidophilus* LB10	-	-	-	-
Fermented milk	*L. acidophilus* LB-11	+	+	+	+
Fermented milk	*L. fermentum* LB-12	-	-	-	-
Lassi	*L. plantarum* LB-13	+	+	+	+
Lassi	*L. acidophilus* LB-14	-	-	-	-
Butter	*L. acidophilus* LB-15	+	+	+	++
Butter	*L. plantarum* LB-16	+	++	+	++
Butter	*L. fermentum* LB -17	-	-	-	-
Milk	*L. plantarum* LB-18	+	+	-	+

Degree of inhibition: + = moderate inhibition zone (< 2mm); ++ = strong inhibition zone ;(> 2mm); - = no inhibition

The *L. fermentum* LB-2 and LB-3 showing more inhibitory activity, were isolated from curd sample, the isolates *L. plantarum* LB-8 was isolated from cheese sample whereas *L. acidophilus* LB-15 and LB-16 were isolated from butter sample.

Out of a total of 18 different fermented milk products, 10 *Lactobacillus* strains (55%) were found to produce bacteriocin like substance by agar well diffusion test thus inhibiting the growth of *E. coli* and *S. aureus*. The isolates showing zone of inhibition in agar well diffusion assay also inhibited the growth of *E. coli* and *S. aureus* in overlay method, however the zone of inhibition was more in overlay method. The inhibition was more shown by live bacteria as compared to sterile broth of *Lactobacillus*

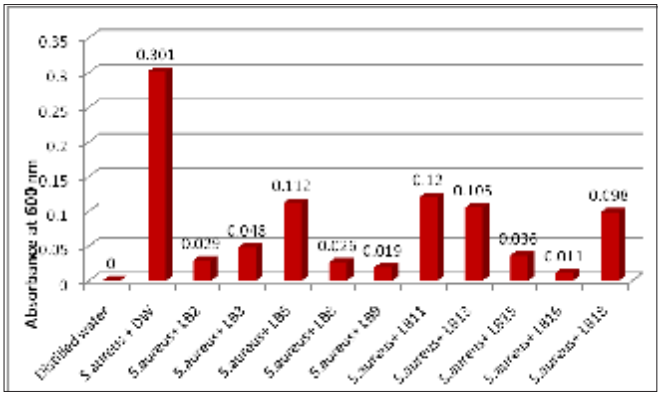


Figure III: Effect of Lactobacillus spp. on biofilm formation by S. aureus
DW= Distilled water, LB Lactobacillus

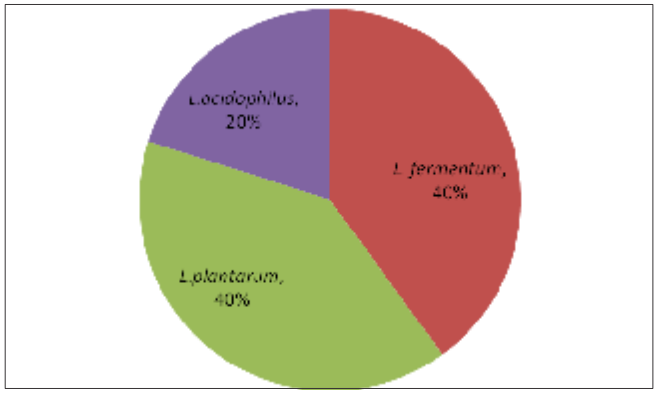


Figure IV: Lactobacillus spp. showing absorbance less than 0.05 in biofilm forming microtiter plate (E. coli)

culture in agar well diffusion assay suggesting other products produced by bacteria during growth like organic acid, hydrogen peroxide, diacetyl and low molecular weight antimicrobial substances could have contributed in inhibition of these pathogens. The antibacterial activity of *Lactobacillus* strains have been recorded in many studies. Antimicrobial peptides called bacteriocin produced by some microorganisms is one of the alternate methods for controlling pathogenic bacteria. Bacteriocins have attracted much attention due to their ability to act as a biopreservative agents which are used in foods particularly in dairy foods and also in human therapeutics.

The results obtained in our study regarding the production of bacteriocin against human pathogens which causes the gastrointestinal diseases, is in agreement with the work done by other workers.[13, 14]

Lactobacillus isolates that showed zone of inhibition in agar well diffusion assay and overlay method were used to determine inhibitory potential against biofilm formation by *E.coli* and *S. aureus* by microtiter plate method. Different isolates showed different absorbance at 600 nm. Ten bacteriocin producing *Lactobacillus* spp. isolates were taken for observation of biofilm inhibition produced by *E. coli*. Maximum absorbance was observed in sample containing *E. coli* only. The absorbance values were less in the samples where *E. coli* was mixed with broth containing *Lactobacillus* spp. All the isolates after incubation showed less absorbance, however 5 samples showed very less absorbance containing *L. fermentum* LB-3 (curd sample), *L. plantarum* LB-8 (cheese sample), *L. acidophilus* LB-15 and *L. plantarum* LB-16 (butter sample). Along with *E. coli* less absorbance indicated that *Lactobacillus* spp. broth inhibited the biofilm formation by *E. coli*.

Similarly ten bacteriocin producing *Lactobacillus* isolates were also studied for inhibition of biofilm formation by *S. aureus*. All the samples containing *Lactobacillus* spp. along with the *S. aureus* are shown in Figure III. The sample which contained only *S. aureus* isolates showed maximum absorbance of less than 0.30 nm whereas absorbance of less than 0.05 nm was observed in 6 samples which contained *Lactobacillus* spp. and *S. aureus*. The 6 isolates that showed minimum absorbance were LB-2 (curd sample), LB-3 (curd sample), LB-8 (cheese sample), LB-15 and LB-16 (butter sample). Less absorbance indicates that *Lactobacillus* spp. isolates inhibits the biofilm formation by *S. aureus*. *Escherichia* are human pathogens and are attached with epithelial cells of gastrointestinal tract. They divide rapidly and make biofilm there. These pathogens cause gastrointestinal diseases by secreting toxins. Lactobacillus produces bacteriocin compounds that are proteinaceous peptides.

The isolates showing absorbance even less than 0.05 against *E. coli* were *L. fermentum* 40%, *L. plantarum* 40%, and *L. acidophillus* 20% shown in Figure IV. The isolates showing absorbance even less than 0.05 against *S. aureus* were *L. fermentum* 34%, *L. plantarum* 50%, and *L. acidophillus* 16% shown in Figure V. Fracchia *et al* [6] observed that Lactobacilli isolated from fresh fruits and vegetables produced biosurfactants and antimicrobial compounds after 5 hours of growth in the mid-exponential phase which significantly ($p<0.05$) inhibited the adhesion of two *Candida albicans* pathogenic biofilm producer strains in pre-coating and co-incubation experiments indicating that the biosurfactant displayed anti-biofilm

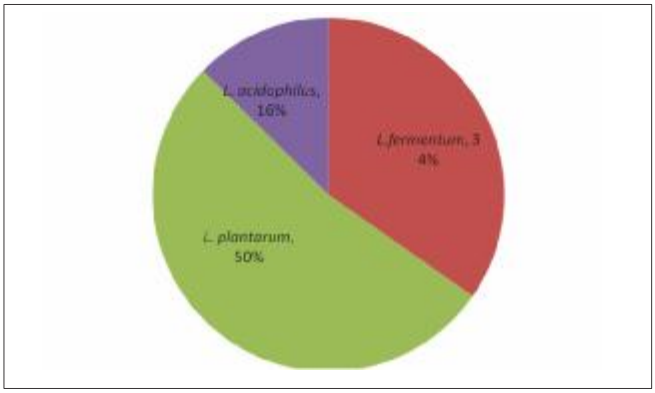


Figure V: Lactobacillus spp. showing absorbance less than 0.05 in biofilm microtiter plate (S. aureus)

formation. The mechanism of interference in biofilm formation is due to the release of biosurfactants.[15,16] Adsorption of biosurfactants to a substratum surface modifies its hydrophobicity, interfering in the microbial adhesion and desorption process.[15] In that sense the release of biosurfactant by probiotic bacteria *in vivo* can be considered as a defence weapon against other colonizing strains in the gastrointestinal tract.[17] In the present study, total 21 fermented dairy products were processed for *Lactobacillus* spp. isolation. A total of 18 isolates were speciated biochemically of which 45% of *L. plantarum*, 33% of *L. acidophilus* and 22% of *L. fermentum*. All 18 isolates were processed for antimicrobial activity by agar well diffusion and overlay method against *E. coli* and *S. aureus*. Ten isolates showed inhibitory activity against *E. coli* and *S. aureus* in agar well diffusion assay and overlay method. Of the 10 isolates, 5 isolates also prevented the biofilm formation by these pathogens. The beneficial attributes of lactic acid bacteria have been reported in many studies. The isolates showing inhibitory activities against the pathogens tested can be used as probiotics or as starter culture in food fermentations.

ACKNOWLEDGEMENTS

The study was carried out by the internal grants of the Institute. We are indebted to the Management of Shaheed Udham Singh Group of Institutes for providing the infrastructure and grants required for conducting the study.

Conflict of interest: None

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