

Antibacterial Spectrum, and Mode of Action of Bacteriocin produced by *Lactobacillus* sp., isolated from Traditional Dairy products

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Abstract: Lactic acid bacteria commonly used as a natural food preservative to improve the food safety and stability. These organisms produce certain antimicrobial substance such as bacteriocins. The present study was focused on isolation and characterization of bacteriocin producing *Lactobacillus* sp., from a traditional milk product the isolates were identified, based on characteristics of the strains of *Lactobacillus* spp., as present in Bergey's manual of determinative bacteriology. The bacteriocin was extracted from the isolated *Lactobacillus* LBC and antagonistic activity against *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, *Shigilla dysenteriae* .*E.coli*. The arbitrary unit of bacteriocin LBC against *Staphylococcus aureus* was 3000AU/mL⁻¹. Its production with simultaneous measurement of activity was monitored and was found to produce maximum amount of bacLBC after 24 hours of incubation. Mode of action of the bac LBC on the sensitive cells was bactericidal rather than bacteriolytic.

Key words: Dairy Products, *Lactobacillus* sp., Bacteriocin, Food Pathogen, Antagonistic activity.

INTRODUCTION

Lactic acid bacteria, particularly those belonging to beneficial and non-pathogenic genera (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*) are widely used in food industry. Among lactic acid bacteria; *Lactobacilli* are the most important group and are gaining increasing attention in food fermentation industry because of their potential biotechnological interest. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substance such as organic acids, hydrogen peroxide and bacteriocins.¹ Bacteriocins are small proteins with bactericidal or bacteriostatic activity.²

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria.³ The term bacteriocin-like inhibitory

substances (BLIS) is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocins. They have been reported to inhibit a wide range of both Gram-positive and Gram-negative bacteria. In recent years, interest has been shown on the microbiology, Biochemistry & molecular biology of bacteriocin like inhibitory substances (BLIS) because they are medically, industrially & agriculturally very important.^{4,5} Bacteriocins have been effective in controlling bacterial infections and their extensive use in combinations as natural food bio-preservatives and health care products has attracted many researchers.^{6,7} The bacteriocin-producing *Lactobacillus* may be used as protective culture to improve the microbial safety of foods.^{8,9} The antagonistic effects of bacteriocins against food spoilage which is usually achieved by

inhibition of *Pseudomonas*, *Staphylococcus aureus*, *Salmonella Spp.*, and *monocytogenes*.¹⁰ and they have great potential as biopreservatives for food. Understanding the significance particularly with reference to the therapeutic potential of *Lactobacillus* species,¹¹ we have decided to determine the spectrum, production and mode of action LBC in order to explore the applied aspects and a possibility of this bacteriocin for its extra laboratory application.

MATERIAL AND METHODS

Dairy Product:

Five samples of the Milk products, Curd, Cheese, Milk peda, Ghee and Butter, were collected from local market.

Viable Microbial Count:

Lactobacilli Counts were determined on MRS agar with glucose a source of energy. Appropriate dilutions were plated on MRS agar (Himedia Mumbai) and incubated aerobically at 37°C for 48hrs. Yeast and Mould were enumerated by surface plating on Potato Dextrose agar (Himedia Mumbai) and incubated aerobically at 25°C for 3days. Coliform counts were enumerated by MacConkey agar (Himedia Mumbai). Characteristic colonies were counted by dilution factor and expressed as colony forming units per milliliter (cfu mL⁻¹).

Pathogenic bacteria strains:

The bacteria used were *Bacillus cereus*, *Staphylococcus aureus*, *E.coli*, *Salmonella spp.*, *Shigella sp.*, all the cultures were maintained as per the recommended practices.

Isolation and identification of bacteriocin producing *Lactobacillus* sp:

The bacteriocin producers from naturally traditional milk products were isolated by pour plate method technique as per the conventional method using MRS agar. After incubation for 24 hrs at 37°C, typical colonies were isolated and purified. The isolated were differentiated on the basis of their morphological, cultural and physiological characteristics such as oxidase test, utilization of sugars and catalase test and accordingly were tentatively identified up to the genus level.

Extraction of bacteriocin:

The *Lactobacillus* isolates were propagated each in 250ml MRS broth (pH 6.8) for extraction of bacteriocin, a culture supernatant was obtained by centrifuging (6,000 rpm for 30min, at 4°C). The cell free solution was precipitated with ammonium

sulphate (40% saturation). The mixture was rotated for 2h at 4°C and later centrifuged at (10,000 rpm for 20 min). The precipitates were obtained and resuspended in 10ml of 0.05M potassium phosphate buffer (pH7.0).

Determination of bacteriocin activity:

Antimicrobial activity of the *Lactobacillus* isolates against all the food pathogenic bacteria was determined by agar well diffusion method (Jack et al., 1995). Under aerobic conditions. Agar plates were inoculated with 100µl of each target bacteria after growing them in a broth and diluting appropriately. Wells (5mm) were cut into the plated and 10µl of cell free culture supernatant was placed into each well. Plates were kept at cool temperature for 1 hr and then incubated at 37°C for 24hrs. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

Mode of action of *Lactobacillus* LBC on indicator/sensitive cells

a) Effect of *Lactobacillus* LBC on stationary phase cells of *S.aureus*:

S.aureus was grown overnight in BHI broth at 37°C harvested by centrifugation (10,000xg for 10 min) and re-suspended in 50mM sodium phosphate buffer (pH.7). Two-fold serial dilution of bac LBC was made in the same buffer and 0.2mL of *S.aureus* cells was added to each dilution (1.8mL) of bac LBC. Control was 0.2mL culture without adding bacLBC, instead making up the volume with 1.8mL buffer. The mixture was incubated, and the samples were drawn at 0, 0.5, 1, 1.5, 2.0 and 4.0 hours. Absorbance of each sample was read at 600nm¹²

b) Effect of LBC on growing cells of *S.aureus*:

Actively growing cells *S.aureus* was diluted 10 times in tempered BHI broth and incubated at 37°C. After 2h of incubation, 1mL (of each two-fold dilution) of LBC was added to 10mL of the *S.aureus* culture and incubated at 37°C. The mixtures were incubated, and the samples were drawn at 0, 0.5, 1.0, 2.0 and 4.0 hours. Absorbance of each sample was read at 600nm.

RESULTS

In this study of *Lactobacillus* spp, isolated from different milk products such as curd, cheese, milk peda, butter and ghee were characterized and identified. Their antimicrobial activity was evaluated against different food borne pathogens. The total microbial populations of different milk products were enumerated and the results in Table 1. The highest microbial population was found in the curd sample, which recorded a *Lactobacillus* population of 4.5

± 0.05 and yeast population of 1.8 ± 0.02 . This was closely followed by the cheese sample, which recorded Lactobacillus population of 3.9 ± 0.10 and yeast population of 1.1 ± 0.14 . The least Lactobacillus population was recorded in the Butter sample 2.5 ± 0.05 . However, the Yeast populations were found to be below not detectable level in butter samples. Based on the morphological, physiological and biochemical studies in Table 2, the isolates (LBC, LBL, LBB, LBH, LBF). All the five isolates were found to be gram positive. Catalase-negative, non spore-forming and rod shape. The optimum temperature of the isolates varied from $37-40^{\circ}\text{C}$; pH ranged from 6.0-6.8. Three of the isolates i.e., LBL, LBB and LBF were found to belong to heterofermentative group while the other two isolates i.e., LBC and LBH were found to belong to homofermentative group. The five isolates were characterized based on the carbohydrate utilization and the results are presented in Table 3. All the five

isolates i.e., LBC and LBH were found to be positive for utilization of glucose, lactose, maltose, galactose and fructose. While none of the two isolates were able to utilize D-xylose. The extracts of five isolated of lactobacillus gave zones of inhibition onto the indicator food pathogenic tested. The table 4 gives the results of inhibition; one activity unit (AU) of bacteriocin was defined as the reciprocal of the last serial dilution demonstrating inhibitory activity, Lactobacillus LBC titer against *S.aureus* was estimated to be 3000AU/mL. The growth curve of LBC was run to find out the critical phase of growth cycle offering maximum Bacteriocin production.(not shown data). It production starts during early logarithmic phase, as the culture supernatant was found to contain LBC after 4th hours of incubation and activity Agar-well diffusion method demonstrating bacteriocin activity in terms of activity units per mL (AU/mL) against *S. aureus*.

Table 1. Microbial viable counts log 10cfu g⁻¹ and pH of milk products

| S.No | Microbial Count | Total Microbial Population | | | | |
|------|-----------------|----------------------------|----------------|----------------|----------------|----------------|
| | | Curd | Cheese | Butter | Milk peda | Ghee |
| 1 | Lactobacilli | 4.5 ± 0.05 | 3.9 ± 0.10 | 2.5 ± 0.05 | 3.0 ± 0.15 | 3.5 ± 0.05 |
| 2 | Yeast | 1.8 ± 0.02 | 1.1 ± 0.14 | ND* | 1.2 ± 0.16 | 1.1 ± 0.38 |
| 3 | Coliform | 3.8 ± 0.05 | 3.1 ± 0.15 | 2.6 ± 0.09 | 2.8 ± 0.02 | 3.2 ± 0.28 |
| 4 | pH | 6.8 ± 0.15 | 6.8 ± 0.15 | 6.8 ± 0.15 | 6.8 ± 0.15 | 6.8 ± 0.15 |

*=Below detectable level of 1×10^2 cfu/ml

Values are mean \pm SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

Table 2. Morphological and Physiological characterization of the Lactobacillus isolates

| S. No. | Characterization | LBC | LBL | LBB | LBH | LBF |
|--------|----------------------------|----------------------------|------------------------------|------------------------------|----------------------------|------------------------------|
| 1. | Gram strain | + | + | + | + | + |
| 2. | Cell morphology | rod | rod | rod | rod | rod |
| 3. | Size | $1.2 \times 2 \mu\text{m}$ | $2.9 \times 0.2 \mu\text{m}$ | $2.7 \times 0.1 \mu\text{m}$ | $1.2 \times 2 \mu\text{m}$ | $0.5 \times 0.9 \mu\text{m}$ |
| 4. | Catalase | - | - | - | - | - |
| 5. | Gas from glucose | - | - | - | - | - |
| 6. | Mode of fermentation | Homo | Hetero | Hetero | Hetero | Homo |
| 7. | Optimal pH for growth | 6.8 ± 0.2 | 7 ± 0.1 | 6.8 ± 0.1 | 7 ± 0.2 | 6.9 ± 0.3 |
| 8. | Optimal growth temperature | 37°C | 37°C | 37°C | 38°C | 40°C |

+ = Positive , - □ = Negative

Values are mean \pm SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

Table 3. Isolates based on characterization of carbohydrate utilization from different *Lactobacillus spp*

| S. No. | Sugar | LBC | LBL | LBB | LBH | LBF |
|--------|-----------|-----|-----|-----|------|-----|
| 1. | Glucose | + | + | + | + | + |
| 2. | Maltose | + | + | - | **** | + |
| 3. | Lactose | + | + | + | + | + |
| 4. | Galactose | + | * | - | + | + |
| 5. | Fructose | + | + | + | * | + |
| 6. | Mannitol | - | - | - | - | - |
| 7. | Sucrose | + | + | - | - | + |
| 8. | D-xylose | - | - | - | - | - |

Studied at an optimum temperature of $28 \pm 2^\circ\text{C}$.

+ = Good growth, -□ = No growth, *** = Poor growth

Table 4. Evaluation of *Lactobacillus* isolates or its antimicrobial activity against different food borne pathogens

| S. No. | Strain | Diameter of inhibition zone(AU/mL) | | | | |
|--------|--------|------------------------------------|------------------|-----------------|-----------------|------------------------|
| | | <i>S. aureus</i> | <i>B. cereus</i> | <i>E. coli</i> | <i>S. typhi</i> | <i>S. dysenterinea</i> |
| 1. | LBC | 3000 \pm 0.50 | - | - | 2100 \pm 0.2 | 1800 \pm 0.50 |
| 2. | LBL | - | 1800 \pm 0.25 | 2800 \pm 0.5 | 2600 \pm 1.0 | - |
| 3. | LBB | 1800 \pm 0.20 | - | 2200 \pm 1.2 | 2000 \pm 0.8 | 2100 \pm 0.50 |
| 4. | LBH | 2800 \pm 0.20 | - | 2150 \pm 0.25 | - | 2200 \pm 0.50 |
| 5. | LBF | - | 2200 \pm 0.25 | 2610 \pm 0.10 | - | 1850 \pm 0.25 |

□ = No inhibition growth

Values are mean \pm SD of three replicates from one representative experiment. Within a column different letters after values indicate that there is a significant difference at P value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

DISCUSSION

Lactobacilli have been used for many centuries in food fermentation process. These lactobacilli are a diverse group of genera, which can be characterized as gram-positive, catalase negative, non-sporulating, non-pigmented bacteria.¹³ Lactic acid bacteria (LAB), particularly those belonging to beneficial and non-pathogenic bacteria (*Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Streptococcus*) have traditionally been used in the food industry. They also play an essential role in the dairy industry due to the tremendous level of human consumption of several important fermented products, mainly cheese and acidified or fermented milks.¹⁴ A detailed study on the total microbial population of different milk products studied revealed that the highest bacterial population was found in the curd (dahi) sample. This was closely followed by the cheese sample. While least microbial population was recorded in the butter sample. The incidence of different *Lactobacillus* sp., i.e., *L. bulgaricus* in yogurt.¹⁵ *L. casei* in cheese¹⁶, *L. helveticus* in butter¹⁷ *L. lactis* and *L. fermentum* in sour cream and other milk products¹⁸ has been reported earlier.

The results of our present study are in line with the earlier findings of¹⁹ they reported a high incidence of microbial load in curd (dahi) sample, when compared with other dairy products like Milk peda and cheese. Lactic acid bacteria are known for their ability to produce antibacterial substances such as organic acids, hydrogen peroxide and bacteriocins^{20, 21}. Bacteriocins, antimicrobial peptides, and bacteriophage have attracted attention as potential Substitutes for, or as additions to, currently used antimicrobial compounds. They are proteinaceous compounds of bacterial origin that are lethal to bacteria other than the producing strain. It is assumed that some of the bacteria in the intestinal tract produce bacteriocins as a means to achieve a competitive advantage, and bacteriocin-producing bacteria might be a desirable part of competitive exclusion preparations. Purified or partially purified bacteriocins could be used as preservatives or for the reduction or elimination of certain pathogens. Currently only nisin, produced by certain strains of *Lactococcus lactis* subsp. *lactis*, has regulatory approval for use in certain foods and its use for poultry products has been studied extensively^{22,23} The present research work associated with activity spectrum, production and mode of action of LBC

produced by *Lactobacillus sp.*. In our studies the inhibitory spectrum of LBC was exhibited against Gram-positive bacteria and the producer strain was resistant to its own bacteriocin. *Lactobacillus sp.*, besides inhibiting *S. aureus*, *Bacillus cereus*, *Escheria coli*, *Saemonella typhi* and *Shigella dysenteriea*. These results indicate that *Lactobacillus LBC* has a broad spectrum of activity. Earlier,²⁴ Activity unit of *Lactobacillus LBC* was found to be 3000/mL in two-fold serial dilution using *S.aurues* as sensitive culture. Maximal bacteriocin yields in a culture may occur at a different phases of the growth cycle. In our studies production of *Lactobacillus LBC* in MRS broth starts from 4th hour of incubation, its activity reaches to maximum level at 7th hour and then remained stable

throughout the incubation period. This prolonged stability of LBC in the growth medium is similar to that of other Gram-positive bacteriocins.^{25, 26, 27} *Lactobacillus* produces inhibitory substance that showed a bactericidal²⁸ or a bacteriolytic²⁹ mode of action. In our case, the effect of *Lactobacillus LBC* on growing and stationary phase cells of *S.aureus* was bactericidal, as the optical density of the cell suspension remains constant throughout the course of experimentation. The present study demonstrates that *Lactobacillus LBC* produced a bacteriocin-like inhibitory substance. With a broad spectrum of antimicrobial activity directed against Gram-positive indicator organisms. It can be used in future as chemotherapeutic agent.

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