# ISOLATION, PARTIAL PURIFICATION OF PROTEINS PRODUCED BY LACTOBACILLUS BIFERMENTANS AND ITS ANTIBACTERIAL PROPERTIES

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## Abstract

*The Antibacterial properties of many Lactic Acid Bacteria were exhaustively studied by many researchers, but little information is known about Lactobacillus bifermentans. This study aims to comprehend the effect of Lactobacillus bifermentans on various Gram Positive and Gram Negative bacteria like Escherichia coli, Bacillus subtilis and Staphylococcus aureus. Testing of antibacterial activity of crude as well as partially purified bacterial sample by Size Exclusion Column Chromatography was performed using Disc Diffusion method, the results of which were comprehended by the measurement of inhibition zones observed. Isolated protein showed antibacterial activity against Bacillus subtilis and Staphylococcus aureus and no effect was observed on Escherichia coli. The inhibition zone diameters obtained were between 8 mm and 12 mm.*

***Keywords:*** *Lactobacillus bifermentans, Bacteriocin, Size Exclusion Chromatography, Disc Diffusion method, Antibacterial activity, Escherichia coli, Bacillus subtilis, Staphylococcus aureus.*

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## 1. INTRODUCTION

The lactic acid bacteria (LAB) comprise a clad of Gram-positive, low GC content, acid-tolerant(able to grow at pH 4.4) generally non-sporulating and non-respiring rod or cocci. Lactobacillus, also called Doderlein's bacillus, is a genus of Genus of Gram-positive facultative anaerobic or microaerophilic rod shaped bacteria. (21). They are catalase lacking organisms occur in naturally fermented food and drink (23, 16).

They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. In humans they are present in the vagina (9) and the gastrointestinal tract, where they make up a small portion of the gut flora (19). They are usually benign, except in the mouth where they have been associated with cavities and tooth decay (dental caries). Many species are prominent in decaying plant material. The production of lactic acid makes its environment acidic,which inhibits the growth of some harmful bacteria. Several members of the genus have had their genome sequence (20).

Lactobacilli, produce special antimicrobial compounds such as bacteriocins which are highly specific antibacterial proteins (2)prevents food spoilage and provides additional protection against *Bacillus, ,S.aureus, Clostridial* spores in canned foods. Many mechanisms have been postulated by which *Lactobacilli* could produce antimicrobial activity. In addition to their competitive inhibition of the epithelial and mucosal adherence of pathogens and inhibition of epithelial invasion by pathogens, *Lactobacilli* and *Bifidobacteria* show antimicrobial activity by producing antimicrobial substances and/or stimulating mucosal immunity.(26)

### 1.1 LACTOBACILLUS BIFERMENTANS:

Bifermentans means "doubly fermenting". A subgroup called L.coryneformis comprised of L.bifermentans and L.rennini. L.bifermentans is found to causing small cracks by gas formation in Edam and Gouda cheeses,has been isolated and described by Pette and Van Beynum(1943).

The biological origin of Lactobacillus bifermentans was from spoiled Blown Dutch cheeses (ie) Edam and Gouda cheese. It is an obligate aerobic bacterium. It has the exotic ability to carry out homolactic fermentation at high glucose concentration and also the ability to ferment lactate at pH>4.0 to acetic acid, ethanol, traces of propionic acid, Co2 &H2.

Cells are non-motile, irregular rods with rounded or often tapered ends (0.5-1.0 or 1.5-2.0μm) occurring singly, in pairs, or irregular short chains, often forming clumps. It belongs to biohazard group I. The G ₊C content (mol %) is 45 and the lactic acid isomer is DL. The carbohydrates fermented by more than 90% of the strains of L.bifermentans are Mannitol, Sorbitol & Ribose.

L.bifermentans was used to produce the intracellular enzymes L-arabinose isomerises and D-xylose isomerise. The medium used to grow L.bifermentans is MRS medium at 30℃ at pH 6.2-6.6.

The strain used for our study is MA: RS. It is thus far the only species known to ferment lactic acid (anoxic degradation of lactic acid to acetic acid) without requiring an external electron acceptor and produce H2 gas to get rid of its excess of reducing equivalents.

## 1.2 BACTERIOCINS:

Bacteriocins are proteins which show bactericidal activity towards closely related species(27). There are 2 main reasons for studying bacteriocins in lactobacilli. Firstly, bacteriocin producing starter cultures may result in a more reliable fermentation process preventing growth of spoilage bacteria. Secondly, the genetic determinants for bacteriocin production and resistance to bacteriocins have great potential as genetic markers in rDNA technology for application in the future production of food additives or supplements from micro-organisms. (3)

Plasmid encoded bacteriocins are commonly observed among both Gram - negative (12, 4) &Gram-positive (28, 24, 5, 15). Bacteriocin produced by strains of Lactobacillus has been reported by(7,30,1,22,6,14,10,31,25)

## 2. EXPERIMENTAL PROCEDURES:

### 2.1 BACTERIAL STRAINS AND MEDIA:

Lactobacillus bifermentans MA:R5 strain of MTCC No.3818 was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh. The medium used to culture Lactobacillus bifermentans is MRS medium as it is growth specific and the optimum temperature for growth is 300c. The indicator strains used to check antibacterial property of L.bifermentans were Escherichia coli, Bacillus subtilis and Staphylococcus aureus.

### 2.2 ISOLATION OF ANTIMICROBIAL ACTIVE PROTEIN FROM MRS BROTH CULTURE:

Lactobacillus bifermentans MA: R5 MTCC 3818 was grown on MRS medium for 48hrs at 300c. Colonies were transferred into 20ml of MRS broth and incubated (aerobic conditions) at 300c for 48hrs. This culture was used to inoculate 2litres of MRS broth. The bacterial cells were harvested by centrifugation at 10,000xg for 15minutes at 40c. The supernatant containing the extracellular protein was collected and used further for checking the antibacterial effect on the indicator strains.

### 2.3 ESTIMATION OF PROTEIN CONCENTRATION BY LOWRY'S METHOD:

Five test tubes marked as A, B, C, D, E of 0.2, 0.4, 0.6, 0.8 and 1.0 ml of BSA working standard was taken and made up to 1.0 ml using distilled water. A test tube with 1.0 ml distilled water serve as a blank and marked as F. 1.0ml of centrifuged bacterial supernatant was taken in another test tube G. 4.5ml of Reagent I (48ml of 2% sodium carbonate in 0.1N NaOH, 1ml of 1% NaK Tartarate in H2O, 1ml of copper sulphate penta hydrate in H2O) and incubate for 10min. After incubation 0.5ml of Reagent II (1 part Folin Phenol [2N]: 1 part water) was added to all the test tubes and incubated for 30 min. After 30min incubation absorbance was taken at 660nm and the standard graph was plotted. From the standard graph, the concentration of protein in the bacterial sample was estimated.

## 3. TESTING THE ANTIBACTERIAL EFFECT OF THE CRUDE BACTERIAL SAMPLE BY DISC DIFFUSION METHOD:

8 test tubes were taken and marked as A, B, C, D, E, F, G, H. Test tube A was filled with 10ml of E.coli liquid culture and all the other 7 test tubes were filled with 9ml of distilled water. 1ml of E.coli culture was taken from test tube A and transferred to test tube B. The test tube B was diluted by a factor of 10^-1. Again 1ml of solution was taken from test tube B and transferred to test tube C. Test tube C was diluted by a factor of 10^-2. Similarly 1ml of solution was transferred to next test tube and diluted till I obtained a dilution factor of 10^-7. Similar procedure was repeated for other two indicator strains i.e. Bacillus subtilis and Staphylococcus aureus. Nutrient Agar medium was prepared and autoclaved. Three autoclaved Petri plates with Nutrient Agar medium were taken. The 1st Petri plate with nutrient agar medium was swabbed evenly with 10^-7 fraction diluted Escherichia coli culture, the 2nd one with 10^-7 fraction diluted Bacillus subtilis and the 3rd one with 10^-7 fraction diluted Staphylococcus aureus culture using sterilized L-Rod. Autoclaved what man filter paper discs of 5mm were taken and aliquots of 50, 75 and 100 µl (3 of each) of crude bacterial sample were applied onto the discs and placed on each Petri plate that were previously inoculated with the indicator strains. The what man filter paper disc absorbed with 50µl of autoclaved distilled water was placed on 3 Petri plates and was used as a negative control. Zone of Inhibition were observed and measured after 24hrs incubation at 370c.

## 4. PURIFICATION OF CRUDE BACTERIAL SAMPLE BY SIZE EXCLUSION COLUMN (SEC) CHROMATOGRAPHY:

The matrix (stationary phase) was prepared by soaking 15grams of Sephadex G50 powder in 0.05M TRIS-HCl buffer of pH-7 with 0.1N NaCl for overnight. The bottom of the column was plugged with Glass wool and the stop cock was closed. The gel was poured into the column by avoiding air bubbles and was packed 50cm high. To the other end of the opening of column, separating funnel was attached with a rubber tube. 500ml of wash buffer composed of 50mM Tris buffer and 125mM NaCl was prepared and autoclaved. The separating funnel was filled with the elution buffer. The column was washed thrice with the 3 column volumes of elution buffer. Using syringe, 1ml of Blue Dextran was added at the top of the column and the stop cock was opened so that the dye Blue Dextran moves through the column. The stop cock of the separating funnel was also opened and the flow rate was set at 1.5ml/3min. At a flow rate of 1.5ml/3min, the volume eluted from the column was collected in a graduated cylinder and the volume at which the dye starts to elute from the column was noted. After all the Blue Dextran is past, again the volume was recorded. After the standardization of column with Blue Dextran, the column was again washed thrice with the column volume of buffer. After washing, the bacterial sample was filtered using 0.2µm filter and 1ml of sample was applied at the top of the column. The flow rate was set at 1.5ml/3.30min and 67 fractions were collected in a 1.5ml eppendorf tubes. The stop cock was closed after collecting 67 fractions and the absorbance at 280nm was recorded for all the 67 fractions.

## 5. TESTING THE ANTIBACTERIAL EFFECT OF THE COLUMN PURIFIED SAMPLE BY DISC DIFFUSION METHOD:

3 autoclaved petri plates with nutrient agar medium were taken and inoculated with 10^-7 fraction diluted E.coli, B.subtilis and S.aureus culture. After recording the absorbance at 280nm for the collected fractions, the fraction which has obtained the highest absorbance was selected to check the antibacterial effect on indicator strains because it has the highest concentration of protein in it. The discs were saturated with 50, 75 and 100µl of the selected fraction and were placed on the plates. The autoclaved distilled was used as a negative control. The petri plates were incubated at 370c for 24hrs and the zone of inhibition was measured and recorded after 24hrs incubation.

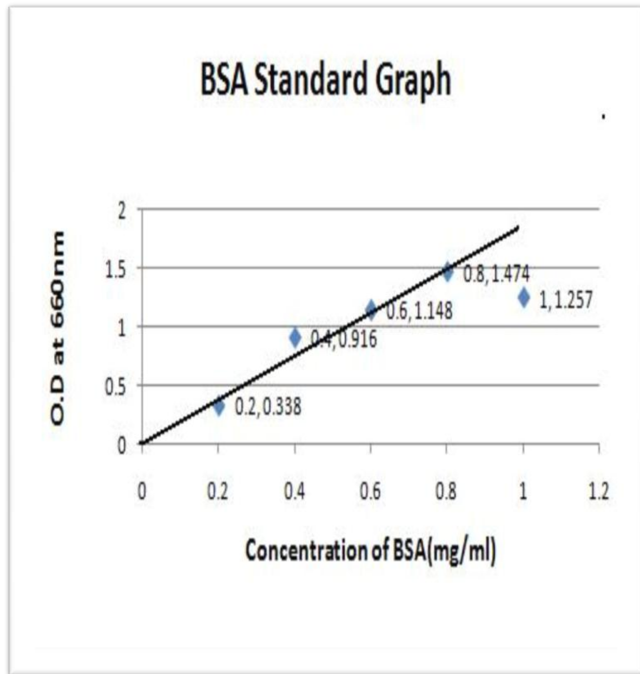
## 6. RESULTS:

### 6.1 ESTIMATION OF PROTEIN CONCENTRATION BY LOWRY'S METHOD:

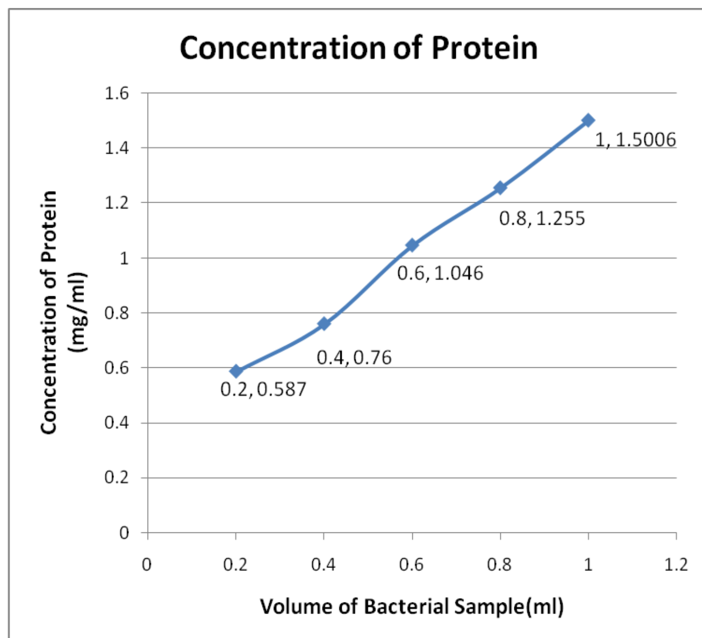
On performing Lowry's method, the following O.D values at 660nm were observed.

| S.NO | VOLUME OF BSA (ml) | O.D at 660nm |
|---|---|---|
| 1 | 0.2 | 0.368 |
| 2 | 0.4 | 0.916 |
| 3 | 0.6 | 1.148 |
| 4 | 0.8 | 1.474 |
| 5 | 1.0 | 1.257 |
| Blank | - | 0 |
| Bacterial Sample/ Test (1 ml) | - | 2.446 |

With the above O.D values, the standard graph was drawn between concentration of BSA (mg/ml) and the O.D at 660nm on X-axis and Y-axis respectively. The slope obtained from the standard graph was 1.63.




The Graph was drawn between the volume of sample (ml) and the concentration of protein (mg/ml) present in the respective volume of sample on X-axis and Y-axis respectively.



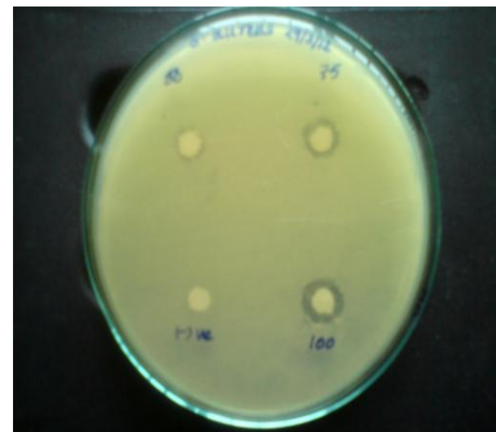

On plotting the graph, it was observed that the concentration of protein in the sample increased as the volume of sample increased.

The Concentration of protein present in the bacterial sample was 1.5006 mg/ml

## TESTING OF ANTIBACTERIAL EFFECTS OF CRUDE PROTEIN

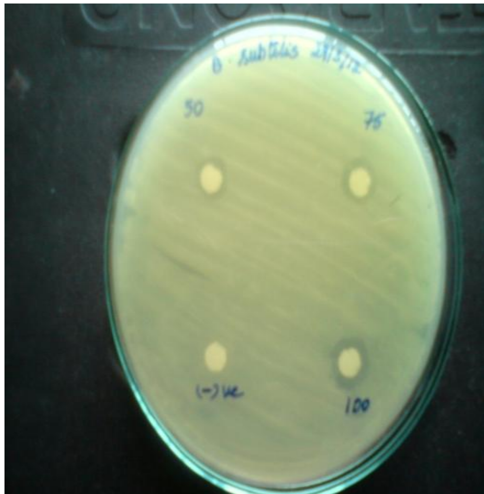
On testing the antibacterial effect of centrifuged L.bifernmentans MA:R5 MTCC 3818 supernatant, the Zone of Inhibition was observed after incubation for 24hrs at 370c for Bacillus subtilis and Staphylococcus aureus. The Zone of Inhibition was not seen for Escherichia coli.

50, 75 and 100µl are the volumes of bacteriocin containing sample saturated on whatman filter paper discs. The negative control used here is 50 µl of autoclaved distilled water.



| **VOLUME OF BACTERIOCIN CONATAINING SAMPLE (µl)** | **DIAMETER OF ZONE OF INHIBITION OF *Staphylococcus aureus* (mm)** |
|---|---|
| 50 | 8.0 |
| 75 | 9.0 |
| 100 | 11.0 |

ZONE OF INHIBITION FORMED ON Staphylococcus aureus by L.bifermentans

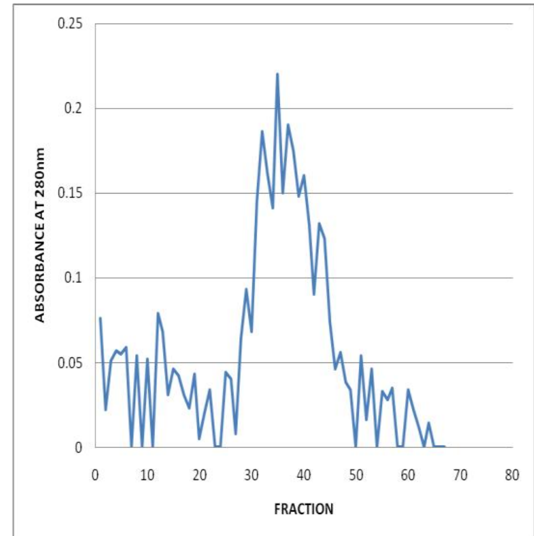



ZONE OF INHIBITION FORMED ON Bacillus subtilis by L.bifermentans

| VOLUME OF BACTERIOCIN CONATAINING SAMPLE (µl) | DIAMETER OF ZONE OF INHIBITION OF *Bacillus subtilis* (mm) |
|---|---|
| 50 | 9.0 |
| 75 | 10.0 |
| 100 | 11.0 |

**PARTIAL PURIFICATION BY SIZE EXCLUSION COLUMN (SEC) CHROMATOGRAPHY:**

The Void Volume obtained for Blue Dextran was 23ml. On running the column at a flow rate of 1.5ml/3.30min loaded with 1ml of crude bacterial sample, 67 fractions were collected among which highest amount of protein was present in 35th fraction collected after 54ml was eluted.



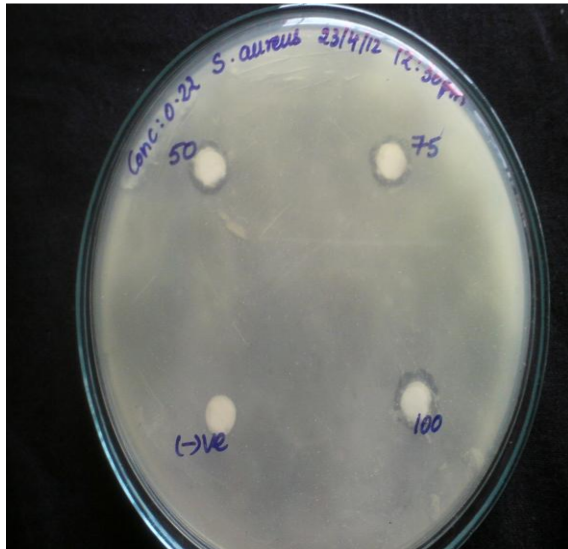

SIZE EXCLUSION COLUMN CHROMATOGRAM

**TESTING OF ANTIBACTERIAL EFFECT OF SIZE EXCLUSION COLUMN (SEC) CHROMATOGRAPHY PURIFIED BACTERIAL SAMPLE:**

The fraction No.34 and 35 which has an absorbance of 0.19 and 0.22 obtained on performing Size exclusion column chromatography were tested for antibacterial activity against Bacillus subtilis and Staphylococcus aureus. The Zone of Inhibition was observed after incubation for 24hrs at 370c.

**ZONE OF INHIBITION FORMED ON *Staphylococcus aureus* WHEN TESTED**

**WITH FRACTION NO.34 WITH ABSORBANCE OF 0.199**




**ZONE OF INHIBITION FORMED ON *Staphylococcus auereus* WHEN TESTED**

**WITH FRACTION NO.35 WITH ABSORBANCE OF 0.22**

50, 75 and 100µl are the volumes of bacteriocin containing purified sample saturated on whatman filter paper discs. The negative control used here is 50 µl of autoclaved distilled water. No Zone of Inhibition was observed for Negative control.

| VOLUME OF BACTERIOCIN CONTAINING PURIFIED SAMPLE (µl) | DIAMETER OF ZONE OF INHIBITION (mm) |
|---|---|
| 50 | 9.0 |
| 75 | 10.0 |
| 100 | 11.0 |

INDICATOR STRAIN: Staphylococcus aureus
FRACTION NO. : 34
ABSORBANCE : 0.19

INDICATOR STRAIN : Staphylococcus aureus
FRACTION NO. : 35
ABSORBANCE : 0.22

| VOLUME OF BACTERIOCIN CONTAINING PURIFIED SAMPLE (µl) | DIAMETER OF ZONE OF INHIBITION (mm) |
|---|---|
| 50 | 8.0 |
| 75 | 8.0 |
| 100 | 9.0 |

**ZONE OF INHIBITION FORMED ON Bacillus subtilis WHEN TESTED**

**WITH FRACTION NO.34 WITH ABSORBANCE OF 0.199**

INDICATOR STRAIN : bacillus subtilis
FRACTION NO. : 34
ABSORBANCE : 0.199

| VOLUME OF BACTERIOCIN CONTAINING PURIFIED SAMPLE(µl) | DIAMETER OF ZONE OF INHIBITION (mm) |
|---|---|
| 50 | 10.0 |
| 75 | 10.0 |
| 100 | 12.0 |

INDICATOR STRAIN : bacillus subtilis
FRACTION NO. : 35
ABSORBANCE : 0.22

| VOLUME OF BACTERIOCIN CONTAINING PURIFIED SAMPLE(µl) | DIAMETER OF ZONE OF INHIBITION (mm) |
|---|---|
| 50 | 9.0 |
| 75 | 11.0 |
| 100 | 13.0 |

## DISCUSSION:

In the present study, bacteriocin, a protein that inhibits the growth of bacteria produced by Lactobacillus bifernentans was recovered, partially purified and tested for its antibacterial effect against selected bacterial strains. The lowry's method was performed to determine the concentration of protein present in the sample and the concentration was found to be 1.5mg/ml. The Size Exclusion Column chromatography was standardized by running Blue Dextran and the void was found out. With the void volume of Blue Dextran, the flow rate required for running the sample was found out. In general, bacteriocins found in lactobacilli have been characterized as proteinaceous antagonists, displaying a narrow range of inhibitory activity towards closely related species within Lactobacillaceae (18). Results obtained by testing the crude and partially purified L.bifermentans sample against selected indicator strains suggest high antibacterial properties.The bacteriocin described in this study inhibited Staphylococcus aureus and Bacillus subtilis. Inhibitory activity was not shown against Escherichia coli. Increased activity has been observed upon purification of bacteriocin by Size Exclusion Column chromatography.

Bacteriocins have been reported to be inhibitory against several other bacteria (17,11). Nisin,the best known LAB bacteriocin has been repeatedly shown to be safe and effective for use in foods over the past 30 years.(8,13). Bacteriocin production was strongly dependent on pH,nutrient sources and temperature(29).

Ever since the era of Louis Pasteur and Robert Koch, there has been scientific recognition of an essential need to control detrimental microorganisms in our environment as well as in food industry. As therapeutic antibiotics are prohibited for use in foods, the utilization of antagonistic additives with preservative or antimicrobial properties has since become a trademark approach in food safety and preservation. Bacteriocins are produced by bacteria and possess antibiotic properties, but bacteriocins are normally not termed antibiotics. Bacteriocins differ from most therapeutic antibiotics in being proteinaceous and generally possessing a narrow specificity of action against strains of the same or closely related species (Tagg and others 1976). Because LAB and their metabolites have been consumed in high quantities by countless generations of people in cultured foods with no adverse effects, the LAB continue as the preferred source for food-use bacteriocins, either in the form of purified compounds or growth extracts.

Our study revealed that Lactobacillus bifermentans showed antibacterial activity against some common pathogenic and food spoilage microorganisms by the production of bacteriocins. Lactobacillus bifermentans is especially important in fermentation industry. bifermentans means "Doubly Fermenting", therefore L.bifermentans finds an exhaustive application in the field of fermentation and food processing.

Since much information is not available on Lactobacillus bifermentans till today, further research must be done by researchers to explore its potential use as an antimicrobial agent to prevent the proliferation of other pathogens and food-spoilage microorganisms. Further research is required for better understanding of interactions between various bacteria.

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