



Bacteriocin Producing *Lactobacillus* from Different Cattle Feed Sample

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ABSTRACT

Lactic acid bacteria and their antimicrobial metabolites have potential as natural preservatives to control the growth of spoilage and pathogenic bacteria in foods. So the aim of the study was to present some data on *Lactobacillus* as probiotic bacteria. Lactic acid bacteria were isolated from animal feed sample. Antimicrobial activity was done by agar well diffusion method. The isolated bacteriocin exhibited antimicrobial activity against, *Staphylococcus aureus*, *Streptococcus pneumonia* MTCC-655, *E.coli* MTCC-1583, *Klebsiella pneumonia* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC-98. The inhibition diameters obtained with bacteria are between 5 to 8 mm. Gram positive bacteria were most inhibited. The maximum production of bacteriocin was obtained at 25 – 35°C at 6.5 pH. The bacteriocin had a molecular weight of (S1 5.5, S2 3.5, S3 7.0 & S4 6.5 S5 10, kDa). The study revealed the possibility of using bacteriocin as a food preservative.

Keywords: Ammonium sulphate precipitation, Anti-Bacterial activity, Isolation of *lactobacillus*, Tricine SDS.

INTRODUCTION

Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations.¹ The bacteriocins from the generally recognized as safe (GRAS) lactic acid bacteria (LAB) have arisen a great deal of attention as a novel approach to control pathogens in food-stuffs.

Microbes compete for the limited space and nutrients present in natural ecological niches, therefore they have developed several strategies in order to survive: production of antimicrobial agents such as bacteriocin is one of them. Gram-positive bacteria, and mainly lactic acid bacteria (LAB), are now being increasingly studied for their production of bacteriocin-like substances.² In the last two decades several reports focused on the production of bacteriocin from lactic acid bacteria isolated from different fermented products, vegetables, fruits, meat, fish, human and animal gastrointestinal tract (GIT).³ Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.⁴ Probiotic *Lactobacillus species* have been implicated in a variety of beneficial roles for the human body, including maintenance of the normal intestinal microbiota, pathogen interference, exclusion and antagonism, immune stimulation and immune modulation, anti carcinogenic and anti mutagenic activities, deconjugation of bile acids, and lactase release *in vivo*.⁵⁻⁸ Lactic acid bacteria produce a variety of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocin or bactericidal proteins during lactic fermentations.^{9,10} Most of bacteriocins produced by gram positive bacteria are from lactic acid bacteria.^{11,12}

Bacteriocins are ribosomally synthesized antibacterial peptides and are usually active against genetically related species. They have been grouped into 4 classes based on their structure and mode of action.² *Lactobacillus* bacteriocins are found within each of the four major classes of antimicrobial proteins produced by lactic acid bacteria. Class I bacteriocins (antibiotics) were discovered in the *lactobacillaceae*.¹³ These bacteriocins are small membrane-active peptides (<5 kDa) containing an unusual amino acid, lanthionine. The class II bacteriocins are small heat stable, non-lanthionine containing and membrane-active peptides (<10kDa). The class III bacteriocins, have been found in *Lactobacillus*, include heat labile proteins of large molecular mass. The class IV bacteriocins are a group of complex proteins, associated with other lipid or carbohydrate moieties, which appear to be required for activity. They are relatively hydrophobic and heat stable.¹⁴

In this study, a strain having bactericidal activity against food pathogenic microorganisms. Purification and identification of a bacteriocin produced by *lactobacillus sps* isolated from animal feed sample.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Five strain of lactic acid bacteria were isolated from different brands of animal feed samples briefly, 1g of feed was mixed and vortexed into MRS broth medium, incubated at 37°C for 24 hrs. Growth from MRS broth cultures were used to streak on MRS agar plate. *Lactobacillus* species of these isolates were identified by staining, biochemical and sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic Bacteriology.¹⁵

Test bacterial strains

The microorganism used were *Staphylococcus aureus*, *Streptococcus pneumonia* MTCC-655, *E.coli* MTCC-1583, *Klebsiella pneumoniae* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC 98.

Concentration of the bacteriocin by ammonium sulphate precipitation

To begin the characterization of a new antimicrobial peptide, it is important to decide and to implement a purification strategy. Many LAB bacteriocins are not produced in high amounts by the producer strain; therefore it is very important to concentrate the supernatant that contains the antimicrobial substance at the very initial steps.

Extraction of bacteriocin

The lactic acid bacterial strains were propagated each in 1000 ml MRS broth (pH7). For extraction of bacteriocin, a cell-free solution of bacteriocin was obtained by centrifuging (10,000 rpm for 20 min, at 4°C) the culture and the pH was adjusted to 7.0 by means of 1M NaOH to exclude antimicrobial effect of organic acid. The cell-free solution obtained was precipitated with ammonium sulphate (80% saturation). The mixture was stirred for overnight at 4°C and later centrifuged at 20,000 rpm for 30 minutes at 4°C. The precipitates were resuspended in 25 ml of 0.05 M potassium phosphate buffer (pH7.0) and used in agar well diffusion assay.

Determination of bacteriocin activity

A agar well diffusion assay procedure was used.¹⁶ Aliquot of 50µl from each (extract) were placed on Mueller Hinton the agar medium. The plates were incubated overnight at 37°C (aerobically) against the indicator pathogenic bacteria. The plates were incubated aerobically at 30°C (Gram-negative) or 37°C (Gram-

positive) for 24 h. The diameters of the inhibition zones were measured.¹⁷

Influence of growth condition on the production of bacteriocin

Bacteriocin production level in different incubation time, temperature, pH was performed with 100ml of MRS broth in 500 ml of Erlenmeyer flasks. The media were inoculated (1%, v/v) with an overnight culture of LAB and incubated at different temperatures (25, 30, 35, 40 and 45), different pH (4.5, 5.0, 5.5, 6.0 and 6.5) and different incubation time (6, 12, 18, 24, 30, 36, 42 and 48 h). Samples were collected after 48h (expect for incubation time effect) and examined for bacteriocin production.

Determination of protein concentration

Protein concentration of the bacteriocin in supernatant was determined by the method of ¹⁸, using bovine serum albumin as the standard.

Molecular weight determination in tricine SDS-PAGE

A 10µl aliquot of the partially purified supernatant of lactic acid bacteria and 5 µl aliquot of the molecular weight standard were loaded to the gel. Electrophoresis was¹⁹ performed in a vertical slab gel apparatus at a constant voltage based on the gel thickness for approximately 5hrs. The gel was stained with 0.025% coomassie dye in 10% acetic acid for 1.5 to 5.5hrs. The gel was destained twice in 10% acetic acid, for 15-60min. After washing the gel was observed in white light.

RESULTS

Isolation and characterization of strains

The bacteriocin producing strain was isolation from the different animal feeds and the selected strain was identified as LAB based on its physiological and biochemical characteristics (table 1 & 2).

Table 1: Morphological, cultural and physiological characteristics of isolated strains

Strains	Cell form	Cellular arrangement	Growth in 6.5% NaCl	Gram staining	Catalase	Gas from glucose	sporulation
S1	Cocobacilli	single and chains	-	+	-	-	-
S2	Spherical	Pairs and tetrad	-	+	-	-	-
S3	Rods	single and chains	-	+	-	-	-
S4	Cocobacilli	single and chains	-	+	-	-	-
S5	Rods	single and chains	-	+	-	-	-

Table 2: Carbohydrate fermentation reactions of the isolated *Lactobacillus sps* bacteria

Sugar fermentation	S1	S2	S3	S4	S5
Arabinose	+	+	+	-	-
Melebiose	+	+	+	+	+
Raffinose	+	+	-	-	-
Salicin	+	+	+	-	-
Lactose	+	+	+	+	+
Xylose	+	+	-	+	+
Dextrose	+	+	+	+	+



Determination of inhibitory spectrum

The susceptibility of various Gram-positive and Gram – negative bacteria to growth inhibition by the supernatant of *Lactobacillus* spp are presented in Table 3. It showed inhibitory activity against *Staphylococcus aureus*, *Streptococcus pneumoniae* MTCC-655, *E. coli* MTCC -1583, *Klebsiella pneumonia* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC-98, Among these, maximum activity was observed against *Klebsiella pneumonia* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC-98, and minimum activity was observed against *Staphylococcus aureus*, *Streptococcus pneumoniae* MTCC-655, and *E. coli* MTCC-1583.

Table 3: Inhibition of various indicator organisms by bacteriocin produced by lactic acid bacteria

Lactic acid bacteria strains	Test organisms inhibited	Inhibitory action of CCE
S1	<i>Staphylococcus aureus</i>	+
	<i>Streptococcus pneumonia</i>	+
	<i>E. coli</i>	+
	<i>Klebsiella pneumonia</i>	++
	<i>Shigella</i>	++
	<i>Salmonella typhimurium</i>	++
S2	<i>Staphylococcus aureus</i>	+
	<i>Streptococcus pneumoniae</i>	+
S3	<i>E. coli</i>	+
	<i>Klebsiella pneumonia</i>	++
	<i>Shigella</i>	++
S4	<i>Staphylococcus aureus</i>	+
	<i>Streptococcus pneumoniae</i>	+
	<i>E. coli</i>	+
	<i>Klebsiella pneumonia</i>	++
	<i>Shigella</i>	++
	<i>Salmonella typhimurium</i>	++
S5	<i>Staphylococcus aureus</i>	+
	<i>Streptococcus pneumoniae</i>	+
	<i>E. coli</i>	+
	<i>Klebsiella pneumonia</i>	++
	<i>Shigella</i>	++
	<i>Salmonella typhimurium</i>	++

Symbols: +: minimum inhibition activity (within 1 to 4 mm)

Symbols: ++: maximum inhibition activity (within 4 to 6 mm)

CCE: Concentrated Crude extract obtained from a *Lactobacillus* spp culture.

Influence of growth condition on the production of bacteriocin

The bacteriocin was secreted into the growth medium, and production was started from late log phase itself and maximum was obtained in the early stationary growth phase at 18th h of the culture. Growth beyond the

stationary phase results in a decrease in bacteriocin production. Incubation temperature and pH played important role in cell growth as well as bacteriocin production. Furthermore were production recorded at 35°C and lower level were recorded at 45 °C. Regarding pH, maximum bacteriocin level was observed at pH 6.5 and minimum bacteriocin production level was observed at pH of 4.5.

Molecular weight determination in tricine SDS

Molecular weight of the bacteriocin was determined by SDS-PAGE gel electrophoresis. Single protein band was observed when stained with Comassie blue and it clearly indicated the purity of the protein. The molecular weight of the purified bacteriocin was calculated to be a (S1 5.5, S2 3.5, S3 7.0 & S4 6.5 S5 10kDa).

DISCUSSION

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by *Lactobacillus* spp from animal feed samples. It is rich in nutrient and organic matter. The isolate *Lactobacillus* spp was tested for antibacterial activity against gram-positive and gram-negative bacteria such as The microorganism used were *Staphylococcus aureus*, *Streptococcus pneumoniae* MTCC-655, *E. coli* MTCC-1583, *Klebsiella pneumoniae* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC 98.

The highest activity was demonstrated against, *Klebsiella pneumoniae* MTCC-39, *Shigella* MTCC-2957 and *Salmonella typhimurium* MTCC 98. While the least activity was demonstrated against *Staphylococcus aureus*, *Streptococcus pneumoniae* MTCC-655, *E. coli* MTCC-1583. The inhibitory effect demonstrated by *Lactobacillus* spp against these bacteria is an indication of antibacterial activity.

Results also revealed the presence of the compound bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria.²⁰⁻²⁴ Possession of bacteriocin by *L. lactis* is an indication that the bacteria can be used as probiotic and as bio preservative.

Optimal recovery was achieved by including ammonium sulphate precipitation. Its molecular weight was estimated to be S1 5.5, S2 3.5, S3 7.0 & S4 6.5 S5 10, kDa). by Tricine SDS-PAGE. Similar results were recorded.^{25,24} In conclusion therefore, the peculiar antimicrobial characteristics of *Lactobacillus* spp can positively have an impact on their use as starter cultures for traditional probiotic.

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