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Antagonistic activity and antibiotic sensitivity of Lactic acid bacteria from fermented dairy products

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ABSTRACT

Lactic acid bacteria (LAB) were isolated from different fermented dairy food and identified as Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus casei and Lactobacillus brevis. The cell free supernatant of the selected Lactic acid bacteria were able to inhibit the growth of all organisms, used in this study (E. coli, Klebsiella, Pseudomonas, Streptococcus, Proteus). The antibiotic sensitivity was investigated. The tested Lactic acid bacteria produced various antimicrobial compounds such as organic acid, Hydrogen peroxide and di-acetyl.

Key words: Lactic acid bacteria, *E. coli, Klebsiella, Pseudomonas, Streptococcus, Proteus* Antibiotics, Antagonistic effect.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of bacteria that can preserve dairy foods by producing a number of organic compounds that are antagonistic to other microorganisms [12],[6]. Among these compounds proteinaceous-bacteriocins have gained much attention especially regarding their role in the dairy foods where they are known to strongly inhibit the growth of pathogens [18], [5]. Much research has focussed on utilizing bacteriocins as novel food preservatives, but there is also interest in using them for the control of bacterial diseases in human and animals. The probiotic potential of these bacteria is also vastly investigated. [7] ,[14], [8].

Lactic acid bacteria (LAB) are the most prominent non-pathogenic bacteria that play a vital role in our everyday life, from fermentation, preservation, and production of wholesome foods, and vitamins to prevention of certain diseases and cancer due to their antimicrobial action. Lactic acid fermentation is generally inexpensive often requiring little or no heat in the process, making them fuel-efficient as well [11]. These microorganisms do one of the prominent bacteria inhabit the gastrointestinal tract, and the importance of these non-pathogenic bacteria has recently been

more noticed [9]. A lot of Lactobacilli have been noted to have nutritional benefits, improved lactose utilization, have anti-cholesterol, anti-carcinogenic activities, and protection against other diseases [9] , [17], [1].

Apart from the above-mentioned medicinal importance of Lactobacilli, it also helps in the control of intestinal pathogens. For instances *L. acidophilus* has been shown to be effective in the treatment of different type of diarrhoea in human. It has been reported to have effect on diarrhoea caused by *Salmonella* or *Shigella* [23]. While *L.casei* was reported to have curative effect on infections caused by *Salmonella typhimurium* and *E. coli* [16].

Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bacterial proteins during lactic fermentations [22], [15] .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.

Innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using live biotherapeutic agent such as bacterial isolates[15]. Lactic acid bacteria exert strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens. In addition, some strains may contribute to the preservation of fermented foods by producing bacteriocins. Research on bacteriocins from lactic acid bacteria has expanded during the last decades to include the use of bacteriocins or the producer organisms as natural food preservatives [19].

Bacteriocins are produced by some strains of LAB; they are antimicrobial peptides with activity against strains closely related to the producer microorganism. Some bacteriocins are also active against Gram-positive food-borne pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis* and spores of *Clostridium perfringens*. For this reason, they have received much attention for use as natural or so-called 'biopreservatives' in food in recent years[20], [19].

However, studies relating to the antibacterial properties of these organisms have been limited and not fully exploited for use. The most important aspects in the study is antimicrobials characterization. Therefore, the aim of this work was to isolate the different strains of Lactobacillus sp, from various food source like cheese, butter, curd, paneer, milk and to determine their antimicrobial activity and antibiotic resistance of these isolates and to estimate lactic acid and H_2O_2 produced by the test isolate.

MATERIALS AND METHODS

Collection of lactic acid bacteria from fermented foods:

A 25 g sample of each fermented dairy food products like cheese, butter, curd, paneer, milk were taken aseptically and transferred to sterile plastic bags and then homogenized in 225 ml of sterile buffered peptone water. Five 10 fold dilutions of the homogenates were then prepared and these were inoculated on plates of MRS agar, acidified with glacial acetic acid to P^H5.7 and incubated anaerobically for 48 hours at 32^0C colonies were tested for Gram staining and were identified using biochemical and sugar fermentation pattern.

***In vitro* Inhibition Test**

The antimicrobial activity of the isolated LAB (cell free filtrate) against *(Escherichia coli, Pseudomonas aerouginosa, Klebsiella pneumoniae, Proteus* sp, *Streptococcus pneumoniae* and *Staphylococcus aureus*), that obtained from IMTECH Chandigragh was performed by the well diffusion assay. The pathogenic test bacteria were incubated in Nutrient broth at appropriate temperature for 24 hrs. Petri dishes containing 20 ml of Muller Hinton agar were prepared previously and inoculated with 0.1 ml of 24 hrs. broth culture of pathogenic bacteria. Once solidified the dishes were stored for 2 hrs. in a refrigerator. Four wells were made and filled using different concentration like 25 µl , 50 µl ,75 µl ,100 µl of cell-free filtrate . Incubate Petri dishes at 37°C for 24 hrs. Then the diameter of the inhibition zone was measured with calipers in mm. The antimicrobial activity was determined by measuring the clear zone around the wells

Determination of antibiotic resistance of the isolates

In the study, the 7 antibiotic discs were used to determine antibiotic resistance of lactobacilli strains. These antibiotic discs were Rifampicin, Ketoconazole, Novobiocin, Fluconazole, Gentamycin, Amphotericin, and Chloramphenicol. The susceptibility tests for each isolates were performed using disc diffusion method [4]. The discs were placed on the solidified agar surface. The plates were incubated aerobically for 24 hours at 37^0C. The resistances were determined according to the zone formation.

Determination of lactic acid and hydrogen peroxide

For these measurements, the test organisms were grown on MRS broth for 72 hours and samples taken at 12 hours interval.

Lactic Acid

To 25 ml of broth culture of organisms, 3 drops of phenolphthalein were added as indicator. From the burette 0.1 N NaOH was slowly added to the sample until pink Colour appears. Each ml of 0.1 NaoH is equivalent to 90.08mg of lactic acid [3].

Hydrogen peroxide

Twenty five milliliter of dilute sulphuric acid were added to 25ml of the broth culture of test organisms. Titration was carried out with 0.1N potassium permanganate. Each ml of 0.1N potassium permanganate is equivalent to 1.070 mg of H202. A decolourization of the sample was regarded as end point [3].

Diacetyl

Twenty five milliliter of broth cultures were transferred into conical flasks and 7.5ml of hydroxylamine solution were used for the residual titration. The flasks were titrated with 0.1N HCl to a greenish-yellow end point using bromophenol blue as indicator. The equivalence factor of HCl to diacetyl is 21.5mg [3].

RESULTS

Table:1 LAB species isolated from different food samples

S. No	Fermented food sample	Code	LAB isolated
1.	Cheese	C1 C2 C3 C4	*L.fermentum* *L.casei* *L.plantarum* *L.brevis*
2.	Butter	B1 B2 B3 B4	*L.casei* *L.fermentum* *L.plantarum* *L.brevis*
3.	Paneer	P1 P2 P3 P4	*L.fermentum* *L.casei* *L.fermentum* *L.plantarum*
4.	Milk	M1 M2 M3 M4	*L.casei* *L.plantarum* *L.fermentum* *L.brevis*
5.	Curd	C1 C2 C3 C4	*L.fermentum* *L.plantarum* *L.casei* *L.brevis*

TABLE:2 Biochemical and sugar fermentation test

No	***Lactobacillus spp***	**Indole**	**MR**	**VP**	**Nitrate reduction**	**Catalase**	**Oxidase**
1.	*L. plantarum*	-ve	+ve	-ve	+ve	-ve	-ve
2.	*L.fermentum*	-ve	+ve	-ve	+ve	-ve	-ve
3.	*L.casei*	-ve	+ve	-ve	+ve	-ve	-ve
4.	*L.brevis*	-ve	+ve	-ve	+ve	-ve	-ve

Sugar Fermentation

Lactobacillus	Xylose	Cellubiose	Glucose	Galactose	Mannitol	Maltose	Rhamonase	Raffinose	Ribose	Sorbitol	Trehalose	Fructose
L.plantarum	-	+	-	+	+	+	+	+	+	-	-	-
L.fermentum	+	+	+	+	+	+	-	+	+	+	+	+
L.casei	+	+	-	+	+	+	+	+	+	-	+	+
L.brevis	+	-	-	-	-	-	-	-	-	-	-	-

Table:3 Antibiotic sensitivity of different Lactobacillus species

Lactobacillus bacteria	Novobiocin	Flucozonazole	Gentamycin	Chloramphenicol	Amphotericin	Rifampicin	Ketoconozole
L. plantarum	S	R	S	S	R	S	S
L.fermentum	S	R	R	R	R	R	R
L.casei	R	R	R	R	R	R	R
L.brevis	S	R	S	S	R	S	S

20 strains of LAB were isolated from different fermented dairy foods and identified based on biochemical test and sugar fermentation pattern as *Lactobacillus brevis, L.plantarum, L.fermentum, L.casei* (Table 1,2). The sensitivity of different pathogen to antibiotics was

investigated.(Table3).The antagonistic activity and other antimicrobial agent production was observed (Table 4 and 5)

Table: 4 Antagonistic activity of selected LAB against UTI pathogens (Zone of inhibition in 100 µl)

Pathogen	*L.fermentum*	*L.plantarum*	*L.brevis*	*L.casei*
E.coli	10mm	6mm	14mm	8mm
Klebsiella	10mm	7mm	7mm	14mm
S.aureus	13mm	6mm	10mm	9mm
Proteus	12mm	5mm	15mm	7mm
Pseudomonas	15mm	25mm	14mm	8mm
Streptococcus	5mm	20mm	12mm	7mm

Table 5:Antimicrobial production by the isolates

Isolates	Lactic acid	Hydrogen peroxide	Diacetyl
L. plantarum	11.6	12.0	10.4
L.fermentum	8.7	15.0	18.5
L.casei	11.0	20.0	3.0

DISCUSSION

As the results indicate, the diameters of the inhibition zones were varied it ranged between 0.6 to 4 mm. This revealed that the LAB inhibited all the pathogenic bacteria tested according to [21] whose mentioned that inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 0.5 mm or larger. Similar study was carried out in Morocco by Kalalou whose studied the activity of LAB on some gram positive and negative pathogenic bacteria such as *E.coli, Pseudomonas aeroginosa, Klebsiella pneumonia, Staphylococcus aureus* and *Bacillus cereus* and the inhibition zones were in the range of 1.4 to 2.8cm [10].

Lactic acid bacteria were isolated from different dairy food and characterized. The pathogens like *E.coli, Klebsiella, S.aureus, Proteus, Streptococcus, Pseudomonas* varies in their sensitivity to different antibiotics. Isolates from this part of the world have been reported to be more resistant to antibiotics than those from other parts of the world. This widespread resistance could be attributed to excessive or indiscriminate use of antibiotics in this part of the world or due to chromosomal resistance.

The metabolites of selected LAB have antagonistic activities against all organisms used in this work. The largest zone of inhibition was produced by *L.plantarum* (13mm) against *S.aureus.*The isolated LAB produced antimicrobial compounds to varying degree, the increase in the production of lactic acid with time have been attributed to lowered pH which permit the growth of LAB. The antimicrobial effect of lactic acid is due to undissociated form of acid which penetrate the membrane and liberate hydrogen ion in the neutral cytoplasm thus leading to inhibition of vital cell functions. The inhibitory effect of hydrogen peroxide produced by LAB has also been reported. The inhibition of *S.aureus* and *Pseudomonas* by hydrogen peroxide produced by certain LAB strains which contribute to their inhibitory activity against other microorganism.
Earlier, Lactobacillus bacteriocins are found within each of the four major classes of antimicrobial proteins produced by LAB and the lactobacilli produce many different bacteriocin activity [2]. Among the lactobacilli, there has been great interest in *L.plantarum*, due to the

potential applications of the microorganisms as a starter bacterium for a variety of fermented foods.The bacteriocin produced from *L.plantarum* have been found to be inhibitory towards closely related LAB, particularly the mesophilic and thermophilic lactobacilli [13].

It has been demonstrated from this study that LAB has a high potential for the treatment of UTI with the use of LAB from indigenous fermented dairy foods as probiotic organisms. The bacteriocin produced by *L.plantarum* and *L.fermentum* exhibited a wide spectrum of inhibition compared to the bacteriocin produced by L.brevis. The potential of these bacteriocins to inhibit the food pathogens such as *E.coli, Klebsiella, S.aureus, Proteus, Streptococcus, Pseudomonas spp* makes it of crucial interest especially in processed foods where there is risk of food pathogens. Due to the phenomenon of immunity the bacteriocin from the produced organism were resistant to the organism producing it.

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