Antagonistic Activity of Bacteriocins produced by *Lactobacillus* isolates against Multidrug Resistant Pathogens

Background: Multi drug-resistance pose a great threat to public health and are responsible for various life-threatening ailments. There is a crucial need to control the outbreaks by finding alternatives to the conventional drugs available. Over the last few years, the usage of probiotics, including *Lactobacillus spp.* and their bacteriocins has gained much attention to ward off various diseases. **Method:** This study was focused on characterizing bacteriocins extracted from *Lactobacillus spp.* and assessing their antagonistic effect against multi-drug resistant bacteria. **Fifteen *Lactobacillus spp.*** were isolated and identified from Pakistani dairy and fermented products (raw milk, cheese, butter milk, pickle and yoghurt). All the isolates were preliminarily screened by the antagonism method of agar well diffusion method, and the bacteriocins were isolated by ammonium sulphate method. Afterwards, to evaluate the release of bacteriocin in liquid medium, the Cell-Free Supernatant Fluid (CFSF) of the best producer strains were tested by agar well diffusion assay. To assess the thermostability of the bacteriocins, they were subjected to temperatures of 40^{O}C, 60^{O}C, 80^{O}C and 100^{O}C. **Results:** The study allowed the selection of **five bacteriocin** producing strains *Lactobacillus acidophilus* KAL1, *Lactobacillus casei* KAL3, *Lactobacillus plantarum* KAL5, *Lactobacillus reuteri* KAL6 and *Lactobacillus spp. delbrukei* KAL7, endowed with the strongest and broadest inhibitory ability against both Gram-positive (*Methicillin Resistant Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. Bacteriocins isolated were significantly thermostable with activity at 80^{O}C (30, 20 min) respectively. Moreover, all the bacteriocins were considerably stable at a range of pH (4–8) but all the activity was eliminated against proteolytic enzyme Proteinase K. **Conclusion:** From this study, it was concluded that bacteriocin extracts from five isolated *Lactobacillus spp.* can be considered a preferable candidate against multi-drug resistant pathogens. These partially purified bacteriocins should be further processed to attain purified product that could be useful for further studies for the control of pathogens, food spoilage and preservation purposes

Keywords: Lactobacillus spp., Bacteriocin, Antimicrobial Resistance, Antagonistic activity, Staphylococcus, Pseudomonas.

1. INTRODUCTION

The world faces a pressing problem of growing multi drug resistance in the pathogenic world owing to the exploitation of the antimicrobial drugs available in the market. The researches have hence, shifted to the natural metabolites to cater to this growing concern. Staphylococcus aureus is a gram-positive, pathogenic strain belonging to Micrococcaceae family. It is responsible for a wide range of infections in humans ranging from small skin problem; pimples, boils, impetigo or scalded skin syndrome to life-threatening diseases; pneumonia, meningitis, endocarditis and toxic shock syndrome. It has since become a pathogen that is a major health threat globally [1].

The most challenging strain of Staphylococcus aureus is the one that is resistant to the antibiotics that are commonly used namely, Methicillin-resistant *Staphylococcus aureus* which is resistant to a number of antibiotics like Penicillin, Vancomycillin, Oxacillin, and Methicillin among others; all having reduced efficacy against this pathogenic strain [2]. Staphylococcus aureus has hence become a serious hazard to human health owing to their ability to transfer this property to other pathogenic strains by the means of food chain, DNA fragments, bacteria's genetic pool or bacteriophage. Thus, a rapid alternative is needed against Methicillin resistance *Staphylococcus aureus* to treat infection without increasing the risk of antibiotic resistance being developed in them against the new drugs [3]. Pseudomonas is another pathogenic strain, gram negative in nature, belonging to Pseudomonadaceae family. Their reservoirs in environment are plants, soil and water from where they can enter human body can be a cause of infections. Pseudomonas strains have become resistant to many commonly used antibiotics making it a tough strain to be treated [4].

Bacteriocins are peptides that are released by certain bacterial strains and have inhibitory effect against similar other strains. Bacteriocins are diverse in all structural, functional and ecological means. Bacteriocin producer organisms are prokaryotes and target other prokaryotes by inhibiting their functions to give them a better chance of survival against the other microbes in the near vicinity. Out of all bacterial strains having the ability to produce bacteriocins, Lactobacillus spp. has received major focus of studies and researches [5]. Lactobacillus spp. is a major part of human body; present in digestive tract, urinary tract and genital tract as well as some others and also helps in treating many health problems related to digestive track like diarrhea. Lactobacillus spp. is a probiotic, so the bacteriocins released by Lactobacillus spp. can be used as alternative for pathogenic strains; MRSA and Pseudomonas, treating the infections caused by these globally challenging strains. Bacteriocins produced by gram-positive bacterial strains can be classified into five classes:

Class I: includes small protein inhibitors. Nisin and Lantibiotics belong to this group. They are modified post-translational peptides containing lanthionine amino acid in their structure [6].

Class II: (<10kDa) they are small peptides that are linear but are not post-translationally modified peptides [7].

Class III: (>10 kDa) they have high prospect as bioperservative in food industries [8].

Class IV: (<10 kDa) are small peptides, the peptides are circular having a peptide bond between C and N terminal.

Class V: (<5kDa) are also small peptides, linear or circular containing post-translationally modified peptides to extensive extend in their structure with addition to a thioether bridge between alpha-carbon of another amino acid [9].

In recent years of research, many useful bacteriocin have being isolated from various Lactobacillus spp. strains such as Pentocin, Lactococcin A, Lacticin Q, and Plantaricin. Other bacteriocins are still being isolated and under study to obtain and maximize the advantage from them; either in food industries or in health sectors like as potential antibiotics.

Bacteriocins by *Lactobacillus spp.* are being studied rapidly now-a-days by researches as bio preservatives in food industries. There are, as of yet, not many studies focusing on bacteriocin as alternate for antibiotics; to stop

resistance of antibiotics in bacterial strains. The main aim of our study is to use bacteriocin as an alternative for antibiotics and check its ability of inhibition against two major globally concerning pathogenic strains; Methicillin-resistant Staphylococcus aureus and Pseudomonas. The bacteriocin in our study will be isolated from *Lactobacillus spp.* major sources being dairy and fermented products, and checking its ability to inhibit the pathogenic strains major focus on MRSA and Pseudomonas. Along with measuring the zone of inhibition given by bacteriocin and comparing it with zones given by antibiotics [10].

Bacteriocins are antimicrobial proteins which can be taken into account as substantial alternate to antibiotics because of their ability to show pronounced activity against bacteria which are resistant to multiple drugs. They are seen to have both narrow and broad spectrum activity against some organisms [11].

World health organization (WHO) states that microbial resistance to antibiotics have posed a serious threat to the world. Antibiotics which had been widely used for the treatment of various illnesses have now lost their efficacy as effective modes of treatment because of resistance of certain disease causing microorganism against them. Bacteriocins are proteins having 19-37 amino acids while larger ones have molecular weight up to 90,000. They are seen to show narrow as well as broad spectrum activity. Unlike antibiotics, bacteriocins are produced naturally because they are obtained from a variety of food sources we consume. 'Nisin' which is a bacteriocin produced by the Lactic acid bacteria which performs probiotic activity in the body is considered GRAS (generally recognized as safe).

It is seen that E.coli which is present in the gut produces bacteriocin called microcin which provides protection against Salmonella typhimurium [12]. It is believed that administration of bacteriocin producing organism is cost effective instead of consuming the bacteriocin itself. However, in order to administer the bacteriocin individually, it is needed to properly develop the producer strain [13].

Many countries in the world have implemented ban on the use of antibiotic as growth promoters in livestock. Hence, to promote growth and reproduction in animals and to avoid infections, probiotics are widely being used [14]. Bacteriocins produced by Lactic acid bacteria is arising as a novel approach to antibiotics due to their extracellular as well as intracellular activity [15]. Bacteriocins produced by Lactic acid bacteria are seen to target selected species in the ecosystem. They are able to show bacteriostatic as well as bactericidal activity towards the targeted organism [16]. On an industrial scale, the cost of producing bacteriocins decreases from month to month and may become less than the cost of producing antibiotics.

Nisin produced by Lactic acid bacteria is used in 50 different countries as food preservatives because of its bactericidal activity against microbes present in food that can cause spoilage. Bacteriocins produced by *Enterococcus facieum* has been effective against Vancomycin resistant strains of *Enterococcus* [17]. Considering the fact that small molecular sized bacteriocins are able to survive heat and ultra violet light, larger sized bacteriocins, however, can be destroyed by enzymes like proteases, heat and other environmental stresses [18].

One of the advantages of bacteriocins are that they have a decreased risk of losing their efficacy against an organism as compared to antibiotics because resistance of organism to bacteriocin has not been reported yet. This is because of its narrow spectrum activity as it targets selected disease causing organism [19].

In addition to show bacteriostatic and bactericidal activity, bacteriocins have also seen to be performing fungicidal activity. They are narrow spectrum as compared to antibiotics and no side effects [20].

The study mainly focuses on the isolation, identification and characterization of bacteriocins isolated from probiotics and to determine potential antimicrobial activity of bacteriocins against multi-drug resistant bacteria.

2. MATERIAL AND METHODS

2.1 Isolation and Identification of bacterial strains

Strains of *Lactobacillus spp.* were isolated from dairy and fermented products including yogurt, cheese, milk and sauerkraut. The particular selected samples were converted in a semi-solid suspension and streaked selective

MRS media for *Lactobacillus spp.* ; De Man, Rogosa and Sharpe agar. After the streaking was done than the plates were incubated for 48 hours in incubator set at 37°C.
The identification and further characterization of Lactobacilli isolates grown on MRS agar was done mainly with the help of the following tests: microscopic examination (Gram staining),catalase test, growth at different temperatures 10 + 1 ºC and 42 + 1 ºC), growth under aerobic and anaerobic conditions, growth at different NaCl concentration, fermentation of different carbohydrates, etc.

a) Microscopic Examination: The purity morphological identification of the isolates as Lactobacilli was confirmed microscopically by performing Gram staining, for which single colony of each isolate was picked up and stained as per the standard protocol and viewed under oil immersion for similar type of cells.

b) Physiological Characterization of Isolates: After confirming the purity of culture, each isolate was further assessed for growth at two different temperatures.
i) Growth of isolates at (10 °C and 42 °C): The isolates were tested for their ability to grow in MRS broth at 10 + 1 °C for 7 days and 42 °C by incubating for 24–48 h. For this, 10 mL of MRS broth tubes were inoculated @ 1% of Lactobacilli cultures. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at 10 °C and 42 °C and results were noted as positive or negative.
ii) Oxygen requirement of the isolates: All the isolates were inoculated in MRS broth and were kept differently under oxygenated condition; in dessicator with burned candle (for micro-aerophilic condition) and in anaerobic jar with gas pack at 37 °C for 24–48 h to determine the impact of oxygen on the growth of the Lactobacilli isolates and results were noted as positive or negative.

c) Effect of NaCl Concentrations on Growth of Isolates: The isolates were inoculated in MRS broth having different NaCl concentration (2.0%, 4.0% and 6.5%) and incubated at 37 °C for 24–48 h. The culture tubes were observed for the presence or absence of growth.

d) Biochemical Characterization of Isolates:
i) Catalase Test: The test was performed in order to determine the ability of the isolated cultures to degrade the hydrogen peroxide by producing the enzyme catalase. The test was carried out as the slide method, using an inoculating needle. For this, culture from a typical colony was placed onto a clean grease-free glass slide and drop of 3% hydrogen peroxide solution was added onto the culture and closely observed for the evolution of bubbles. The production of bubbles indicated positive catalase reaction and was recorded accordingly for the presence or absence of enzyme.
ii) Gas from Glucose: Sterile test tubes of 10 mL glucose broth containing Durham's tube (inverted and dipped), were inoculated with Lactobacilli cultures at the @1% and incubated at 37 °C for 24–48 h. Gas production that appeared in the form of a hollow space in Durham's tube was recorded as a positive result.
iii) Nitrate Reduction Test: Nitrate reduction is an important criterion for differentiating and characterizing different types of bacteria. Therefore, the isolates were incubated at 37°C for 24 h in trypticase nitrate broth. After incubation, 0.5 mL each of sulphanilic acid (0.8%, in 5N Acetic acid) and α-naphthylamine (0.5%, in 5N Acetic acid) were added into the tubes. The appearance of red or pink color indicated the positive test for nitrate reduction and was recorded accordingly for the isolates tested.
iv) Citrate Utilization Test: The isolates were inoculated in Simmons citrate agar incubated at 37 °C for 24 h. After incubation, the appearance of blue coloration indicated the positive test for citrate utilization and was recorded accordingly for the isolates tested.

The strains of *Staphylococcus aureus* and *Pseudomonas* were isolated from soils; different soil samples were used in the study. The soil samples were dissolved in distilled water to makes a suspension. MSA was used for the growth of *Staphylococcus aureus* for both clinical and non-clinical samples for in-vitro studies and for *Pseudomonas,* the selective media used was Cetrimide agar. The plates were then placed in incubator for 48 hours, temperature set at 37°C.

2.2 Mc Farland standard
McFarland standards were used as referral solutions with the desired optical density to compare with the bacterial inoculums used during agar well diffusion. The OD of the indicator strains was compared using a McFarland standard of 0.5 OD which corresponds to the cell count of 1.5 x 10^{8}. The McFarland Standard of 0.5 OD was made by the addition of 9.95 ml of a solution of 1% H 2 SO 4 to 0.05 ml of 1.175% of BaCl 2 .2H 2 O

2.3 Antagonistic activity of producer strains against indicator strains

Before isolation and purification of bacteriocin, whole *Lactobacillus spp.* was tested for antimicrobial activity and the potential bacteriocin producing strains were carried further in the project. Culture of *Lactobacillus spp., P. aeruginosa* and *MRSA* was inoculated in Tryptone Soya Broth (TSB) and incubated in shaking incubator for 24 hours. In order to evaluate the inhibitory activity, agar-well diffusion was performed. 200ul of inoculum of indicator organism was poured in petri plates. Later, cooled liquid Mueller Hinton agar (MHA) was poured in the plates such that no lumps are formed and the temperature is not high enough to kill the organisms. Plates were rotated clock wise and anti-clockwise keeping on the slab to ensure uniform spread of organism in the media avoiding spill. After the agar solidifies, wells are punched into it. Inoculums of *Lactobacillus spp.* are then poured into these wells. The plates were then incubated for 48 hours to assess the activity of *Lactobacillus spp.* against *Methicillin resistant Staphylococcus aureus* and *Pseudomonas* [21].

2.4 Bacteriocin Production

For significant production of Bacteriocins, the producer strains were cultured at a pH of 6.5 for 48h in MRS broth at 37^{O} C on shaking Incubator. 1M NaOH was added and stirred for half an hour to facilitate the adsorption of bacteriocins on the producer cells. It was then subjected to a temperature of 70^{O} C for another half an hour [22].

2.5 Extraction of bacteriocin from Lactobacillus spp.

Bacteriocins were purified using modified protocol of Muriana and Kleinhammer 1991 [23]. 24 – 48 hr cultures of *Lactobacillus spp.* in MRS broth were centrifuged at 7500rpm for 15 minutes at 4^{o}C. The pellet discarded and Ammonium sulphate (40%, 50%, 60%, 70%) added to the supernatant. The pH was adjusted to 6.5 using 1M HCl and 10M NaOH. After being kept overnight in the shaking incubator, it was centrifuged at 7500rpm for 1 hr at 4^{0}C. The supernatant discarded and the pellet dialyzed in 2-3 ml PBS Buffer [24].

2.6 Antagonistic activity of crude extract against indicator strain

The isolated and purified proteins from the above mentioned procedure was then used to evaluate antimicrobial activity against *Methicillin resistant Staphylococcus aureus* and *Pseudomonas*. To assess the effect of bacteriocin, agar well diffusion was done with control run alongside. To confirm that the inhibition is due to the presence of bacteriocin and is not because of any other chemical, TSB was used as control.

Inoculums of the producing strain and indicator strains was made in TSB. 200ul of the inoculum of the indicator strain was poured in the plate. Later, cooled liquid Mueller Hinton agar (MHA) was poured in the plates such that no lumps are formed and the temperature is not high enough to kill the organisms. Plates were rotated clock wise and anti-clockwise keeping on the slab to ensure uniform spread of organism in the media avoiding spill. After the agar solidifies, wells are punched into it. Crude bacteriocin purified from the above mentioned protocol was poured in each petri dish and results were analyzed after 48 hours of incubation at 37^{o}C.

2.7 Characterization of partially purified bacteriocins

2.7.1 pH

Different pH was maintained to confirm that bacteriocin will show activity at neutral pH i.e. 6.5 – 7. To countercheck whether protein has been degraded by low pH, pH was maintained to 2,3,4,5,6,7,8,9,10 and 11. pH meter was used to constantly monitor pH. Acidic pH i.e. 2, 3, 4, 5, 6 was maintained by adding 1 molar HCl and basic pH i.e. 8, 9, 10 and 11 was maintained by adding 1 molar NaOH. Plates were poured via Agar well diffusion method was used to check bacteriocin activity set at different pH.

2.7.2 Temperature

In order to check bacteriocin activity via temperature, different degrees of temperature was set to determine best bacteriocin activity. Temperatures varied from high to low i.e. 40^{o}C, 60^{o}C, 80^{o}C, 100^{o}C and 120^{o}C to check the most optimum temperature at which bacteriocin showed best activity.

2.7.3 Proteinase K

1% solution of proteinase K was prepared and culture was inoculated in it which was incubated for 2 hours. 600 micro liters of this solution was poured in wells via agar well diffusion method to check whether protein was inactivated by the addition of proteinase K.

2.8 Protein estimation of cell free extract

2.8.1 Lowry's method

The protein estimation for Lowry method is used for cell fraction, enzyme preparation, and chromatographic fraction [25]. This method is used entirely at room temperature and it improves the sensitivity with some proteins and is less likely to be compatible with the salt solutions to provide linear response and less likely to become saturated. Bovine-Serum albumin was used to make standards of 1^{-1} to 10^{-5}. In a glass test tube, 0.2ml of the standards and sample were added and to that, 0.8 ml of dH_2O, 5ml of Lowry's Reagent and 0.5 ml of Folin-Ciocalteau Reagent were added sequentially. The blank can be prepared by adding 5ml of Lowry's Reagent and 0.5 ml of Folin-Ciocalteu Reagent to 1ml of dH_2O. Incubate for at least 30 minutes but no more than 60 minutes, since color appears after 30 minutes. All standards, samples, and blank's absorbance can be read on a spectrophotometer at A_{650nm}. Lowry's method requires precise timing due to color instability. Proteins having high or low percentages of tyrosine, tryptophan, or cysteine residues will give high or low errors, respectively.

3. RESULTS & DISCUSSION:

3.1 (i). Isolation & Identification of Producer and Indicator strains

Twenty samples of potential sources of Lactobacillus spp were collected and cultured on MRS agar for 48 hrs. Out of several colonies developed on agar plates, 15 isolates based on colonial morphology (i.e., color, size, margin and shape of the colony) such as white, greyish white or cream color, with entire or undulate margins were collected and cultured on MRS agar for 48 hrs. For identification, gram staining, biochemical tests were performed to confirm the presence of Lactobacillus spp.. Lactobacilli successfully isolated are enlisted in table 1 below. Apart from biochemical testing, carbohydrate fermentation tests were performed. Phenol red indicator was used to interpret the results; color change from red to yellow indicated a positive result.

Table I: Colonial Morphology of the Isolated Lactobacilli Cultures.

Isolate(s)	Colonial Morphology		
	Color	Shape	Margin
KAL 01	Cream	Pin point; circular; smooth; compact and convex	Entire
KAL 03	White	Circular	Entire
KAL 05	White	Circular; large; rough and irregular	Undulate
KAL 06	Creamish white	Circular	Entire
KAL 07	Grayish white	Pin point; circular; compact and convex	Entire

In Gram reaction and cell morphology, the isolates were found to be purple colored Gram-positive rods (i.e., straight rods, irregular rods, rods with rounded ends) and arrangements such as rods in single, or in chains (2–5 cells), under oil-immersion microscope (Table II).

Table II: Cell Morphology of the Isolated Lactobacilli.

Isolate No. (s)	Cell Morphology	
	Gram Reaction	Shape and Arrangement
KAL 01	G +ve	Irregular rods with rounded ends
KAL 03	G +ve	Rods
KAL 05	G +ve	Rods
KAL 06	G +ve	Rods in chains
KAL 07	G +ve	Rods

3.1 (ii). Physiological Characterization

Growth at 10 °C and 42 °C: The isolated Lactobacilli cultures were assessed for their growth at two different temperatures (i.e., 10 °C and 42 °C). For this, cultures were incubated in MRS broth at 10 °C for 7 days and the turbidity in broth was observed as an indication of microbial growth. On the other hand, isolates were also incubated at 42 °C for 24 to 48 h and turbidity was observed in tubes containing isolates. The other Lactobacilli could not grow at the elevated temperature of 42 °C. Hence, it can be concluded form Table III that 37 °C is the optimum temperature for all the isolates and few of these could either survive or grow at 10 °C/42 °C or at both the temperatures away from the optimum. After assessing the growth of Lactobacilli at different temperatures, they were exposed to the growth in oxygenic, reduced oxygen and anoxygenic environments. For this, all the isolates were incubated in MRS broth and incubated at 37 °C for 24 h under aerobic, micro-aerophilic and in anaerobic gas jars. In aerobic and micro-aerophilic conditions all the isolates tested showed turbidity in the medium indicating the occurrence of growth. Under anaerobic condition, the turbidity was not observed with isolates KAL 06 & KAL 07; however, all other isolates showed turbidity and/or growth. It can be concluded from Table III that all the isolates were facultative anaerobes and can grow in the presence and absence of oxygen that failed to grow in anoxygenic condition. Hence, it can be stated that the isolates have both the mechanisms of oxidative and fermentative processes for energy generation.

Table III: Physiological Characterization of the Lactobacilli Isolates.

Isolate No. (s)	Physiological Characteristics							
	Growth at Temperature		Oxygen Requirement			Effect of NaCl (%)		
	10 °C	42 °C	Aerobic	Micro-aerophilic	Anaerobic	2	4	6.5
KAL 01	+	+	+	+	+	+	-	-
KAL 03	+	+	+	+	+	+	+	-
KAL 05	-	+	+	+	+	+	+	-
KAL 06	-	+	+	+	-	+	-	-
KAL 07	-	+	+	+	-	+	-	-

Symbols: + = able to grow; - = not able to grow

3.1 (iii). Biochemical Characterization:

a. Catalase Production: Catalase, an extracellular enzyme secreted by several microorganisms, helps in degradation of hydrogen peroxide produced during carbohydrates utilization for energy production, thereby its presence or absence in a microbial cell can be used as a significant diagnostic tool. The catalase is involved in catalyzing the breakdown of toxic hydrogen peroxide to produce molecular oxygen that generates vigorously while producing effervescence, when a microbial culture is mixed with an equal volume of 3% solution of hydrogen peroxide. Absence of effervescence is taken as indicative as negative for catalase enzyme production. In the present study, all the isolates were found to be catalase negative (Table IV). These results obtained for catalase further support the identification of isolates as Lactobacilli and pave the way in further characterization of the isolates and are in agreement with [14, 15].

b. Gas from Glucose: The microorganisms use carbohydrates in a different pattern depending on their enzyme complement. In fermentation, substrates such as carbohydrates and alcohols undergo anaerobic dissimilation and produce organic acids that may be accompanied by the production of gases such as hydrogen or carbon-dioxide. Therefore, all the Lactobacilli were subjected to glucose fermentation test in order to see the production of gas. It was observed from Table IV that isolates produced gas in broth containing glucose as the sole source of carbon while other isolates could not produce any gas as shown by a hollow space in the inverted Durham's tubes and, therefore, resulted as negative for gas production from glucose. Thus, the above isolates showed fermentation of glucose in the medium for their growth. These variations in results were also reported [13].

c. Nitrate Reduction Test: In microbial taxonomy, nitrate reduction is an important criterion for characterization and identification of different types of bacteria. This is due to the fact that certain bacteria have the capability to reduce nitrate to nitrite while others are capable of further reducing nitrite to ammonia. The formation of ammonia changes the pH of media to alkaline thus, changing the color of media from yellow to cherry red. In the nitrate reduction test, all the isolates tested showed negative reactions, as there was no formation of red/pink color after incubation of isolates in the nitrate broth (Table IV), a characteristic of Lactobacillus group of organism [12].

d. Citrate Utilization Test: For further characterization and identification, all the isolates were subjected for their potential to utilize citrate as the sole carbon source. Further, it also attributes to the better technological property of the product as citrate utilization leads to a better flavor production in fermented milk production. Certain bacteria utilize citrate with the help of enzyme citrate permease and citrase-producing diacetyl, a flavoring compound as end product. Following incubation on Simmon's citrate agar, citrate-positive cultures were identified by the presence of growth on the surface of slant, accompanied by blue coloration whereas negative cultures did not show any growth and medium remained green. From Table IV, citrate utilization was confirmed in isolates (KAL 01, KAL 03 & KAL 05) whereas isolates (KAL 06 & kal 07) were found to be citrate negative and are in agreement with [13].

Table IV: The Biochemical Characterization of the Isolated Lactobacilli.

Isolate No. (s)	Catalase Test	Gas from Glucose	Nitrate Reduction	Citrate Utilization
KAL 01	-	-	-	+
KAL 03	-	-	-	+
KAL 05	-	-	-	+
KAL 06	-	+	-	-
KAL 07	-	+	-	-

Symbols: + = able to ferment; - = not able to ferment; v = variable fermentation; w = weak fermentation.

3.2. Antagonistic Activity of Producer strains against indicator strains

Inhibitory activity of *Lactobacillus spp.* was first tested before isolating the proteins following agar well diffusion assay to observe and calculate the zones of inhibition produced by *Lactobacillus spp.* For control TSB without any culture was used. The petri plates were observed after 48 hours of incubation at 37˚. Zones of inhibition produced by the strains of *Lactobacillus spp.* are listed in table 2.

3.3. Antagonistic Activity of crude extract against indicator strains

The aforementioned purification procedures were performed on all 05 strains of *Lactobacillus spp.*. The best results were obtained at 60% Ammonium Sulphate solution within 48 hours of incubation at 37˚C. Agar-well diffusion technique was performed to evaluate the inhibitory activity of the crude protein extract potentially containing the bacteriocins. After 48 hours incubation clear zones of inhibition were observed on the plates of various diameters which were then measured in millimeters using a measuring scale as shown in table 3.

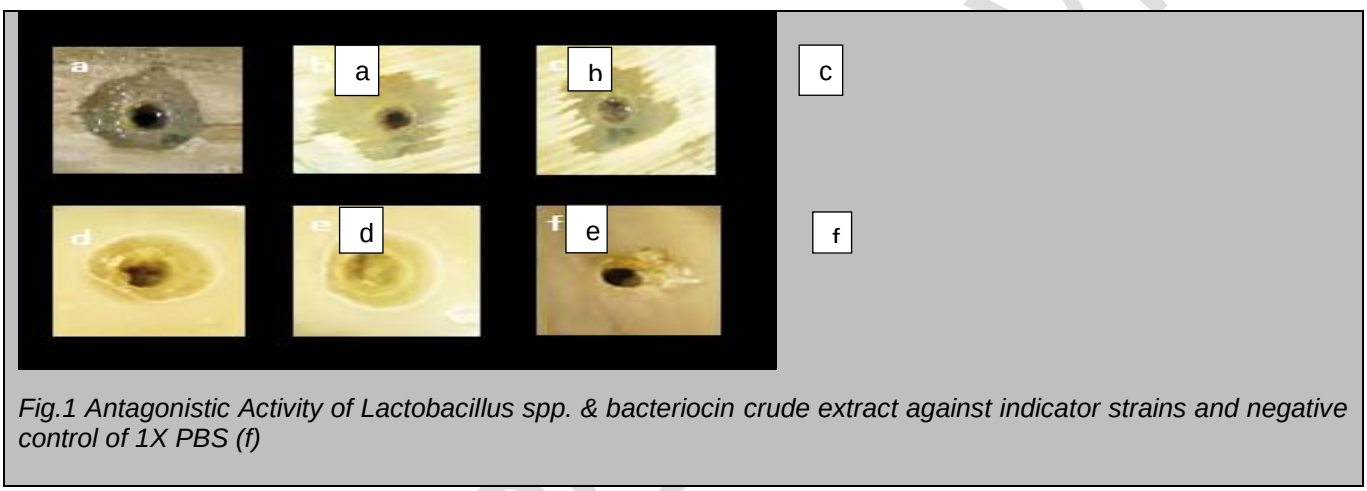


Fig.1 Antagonistic Activity of Lactobacillus spp. & bacteriocin crude extract against indicator strains and negative control of 1X PBS (f)

Table 2: Inhibition zones produced by partially purified proteins against indicator strains.

P. Strain	KSA1	KSA2	KSA3	KSA4	PA1	PA2	PA3
KAL 01	+ + +	+ + + +	+ +	+ + +	+	+ +	+
KAL 03	+ + +	+ + +	+	+ +	-	+	-
KAL 05	+ +	+ + + +	+ +	+	+ +	-	+
KAL 06	-	+ +	+ +	+ +	+	+ +	+ +
KAL 07	+ +	+ + +	+ + +	-	+ +	+	-

**Zone of inhibition: 3-10mm: +; 10.5-15: ++; 15.5-20: +++; >20: ++++*

***KSA: Staphylococcus aureus strains; PA: Pseudomonas aeruginosa strains*

Table 3: Inhibition zones produced by cell free extract against indicator strains.

P. Strain	KSA1	KSA2	KSA3	KSA4	PA1	PA2	PA3
KAL 01	+ +	+ +	+ + + +	+ + +	+ + +	+ + + +	+ + + +
KAL 03	+ + +	+ + +	+ +	+ + +	+ + +	+ + + +	+ + + +
KAL 05	+ + +	+ + +	+ + + +	+ +	-	+	+ + +
KAL 06	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + + +
KAL 07	+ +	+ +	+ + + +	+ + +	+ +	-	+ +

**Zone of inhibition: 3-10mm: +; 10.5-15: ++; 15.5-20: +++; >20: ++++*
***KSA: Staphylococcus aureus strains; PA: Pseudomonas aeruginosa strains; KAL: Lactobacilli strains*

Table 4: Characteristics of isolates

Test	KAL 01	KAL 03	KAL 05	KAL 06	KAL 07
Optimum pH [Antagonistic Activity]	5-7	7	6.5	6.5	6.5
Optimum Temperature (˚C)[Antagonistic Activity]	20- 60	40-80	40-100	20-80	20-80
Proteinase K	-	-	-	-	-
Catalase	-	-	-	-	-
Glucose	+	+	+	+	+
Ribose	+	+	+	+	+
Galactose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+

Positive test: +; Negative test: -

3.4 Characterization of partially purified bacteriocin

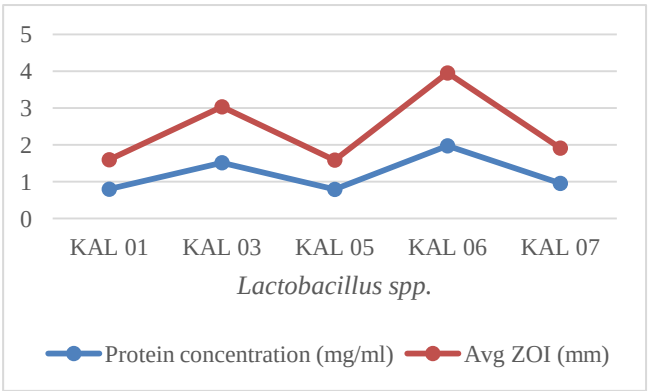
Stability of bacteriocin at various pH, temperatures and enzyme was checked to confirm that it is protein in nature and is active within specific parameters. All protocols were evaluated by agar-well diffusion technique. The values were altered to determine the effect of these parameters on antimicrobial activity. No difference in antibacterial activity was found with the optimum conditions; from 5.5 to 7 pH and 35-37˚C. But minimum or no activity was seen when pH was dropped to acidic (2, 3, 4) or basic (8, 9, 10, 11). For temperature, notable activity was observed at 20˚C, 40˚C, and some activity retained at 60˚C, 80 ˚C and 100˚C in some isolates. It might be concluded that alterations in pH and temperature denature the protein structure or inactivates it and results in its loss of activity. Inactivity and denaturation of protein eventually produces no result and no zone of inhibition thereof. The last treatment was done with the protease enzyme; 1% of the solution of proteinase K was incubated with the purified protein sample for 2 hours then poured in the wells; no inhibitory activity was observed. This concluded the fact that the purified sample was protein in nature and was sensitive to protease due to which it lost its antimicrobial properties.

3.5. Protein Estimation

The total concentration of proteins in the different samples following the different purification protocols after its estimation using Lowry's Method are listed in the following chart.

Concentration of standard = 1mg/ml
Concentration of Sample = (Absorbance of Sample/ Absorbance of Standard) * Concentration of Standard.
Absorbance of Standard = 0.209

Fig 2. Concentration of proteins (mg/ml) present in the samples



In the present study, the bacteriocins partially purified from different strains of *Lactobacillus spp.*, isolated from dairy and fermented products exhibited inhibitory activity against both pathogenic strains of *MRSA* and *Pseudomonas*. The isolated bacteriocins were sensitive to protease enzyme; proteinase K showing its proteinaceous nature. Along with these attributes, the bacteriocins isolated where sensitive to more acidic and alkaline pH; major zones appeared on neutral Ph. The stable pH range of partially purified bacteriocins was between 5 to 7 pH. The isolated bacteriocins isolated were proven to be thermostable with activity at 100^{O}C (30, 20 min) respectively**.**

Moreover, the protein estimates in the samples of crude extracts were proportional to the zones that they produced against the indicator strains. Significant zones, greater than even 20mm were observed in most cases against both the most notorious gram negative and gram positive bacteria namely *Pseudomonas* and *MRSA.* Thus with few alterations the bacteriocin can be used as potential natural antibiotic. These bacteriocins are of great importance for their future prospects and for them to be utilized as a source of antibiotics. They can also be of peculiar importance in catering to finding the solution of the growing resistance that the microbial world is now achieving at a rapid rate against the various antibiotics available today. Hence, a better insight into the study of these moieties is of supreme importance in order to find a better and more prosperous relevance or significance of theirs.

4. CONCLUSION

Lactobacilli are probiotic organisms that have been broadly utilized in different areas inferable from the advantages that they grant to the hosts. Production of antimicrobial peptides called Bacteriocins, helps in giving them an edge to endurance against the contending organisms in the close vicinity. Bacteriocins have acquired a ton of consideration in the scientific world since their revelation attributed to their antimicrobial properties. They have since been concentrated to propose their utilization as more fruitful antimicrobial specialists in contrast with the more traditional ones like antibiotics against which, the greater part of the pathogenic microscopic organisms have developed resistance. In-depth studies of a select few bacteriocins opened exiting new research fields and broadened the application of these antimicrobial peptides. The possibility of developing bacteriocins into next generation antibiotics, accompanied with the rapid development in genetics and nanotechnology, paves the way to even more fascinating applications such as novel carrier molecules (delivery systems) and the treatment of cancer.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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