

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°C, 60°C, 80°C and 100°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°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, Vancomycin, 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 biopreservative 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 faecium* 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].

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 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 1.5×10^8 . The McFarland Standard of 0.5 OD was made by the addition of 9.95 ml of a solution of 1% H₂SO₄ to 0.05 ml of 1.175% of BaCl₂ · 2H₂O

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°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°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°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°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°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°C, 60°C, 80°C, 100°C and 120°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 dH₂O, 5ml of Lowry's Reagent and 0.5 ml of Folin-Ciocalteu 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 dH₂O. 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

3.1. Identification of Producer and Indicator strains

Twenty samples of potential sources of *Lactobacillus spp.* were collected and cultured on MRS agar for 48 hrs. For identification, 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 1. Bacterial Strains used in the Study

Strain	Source	Growth Conditions
KAL 01	Fresh Yogurt	
KAL 03	Packed Yogurt	
KAL 05	Milk	MRS A, 48h, 37°C
KAL 06	Packed Yogurt	
KAL 07	Subculture	

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 10 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.

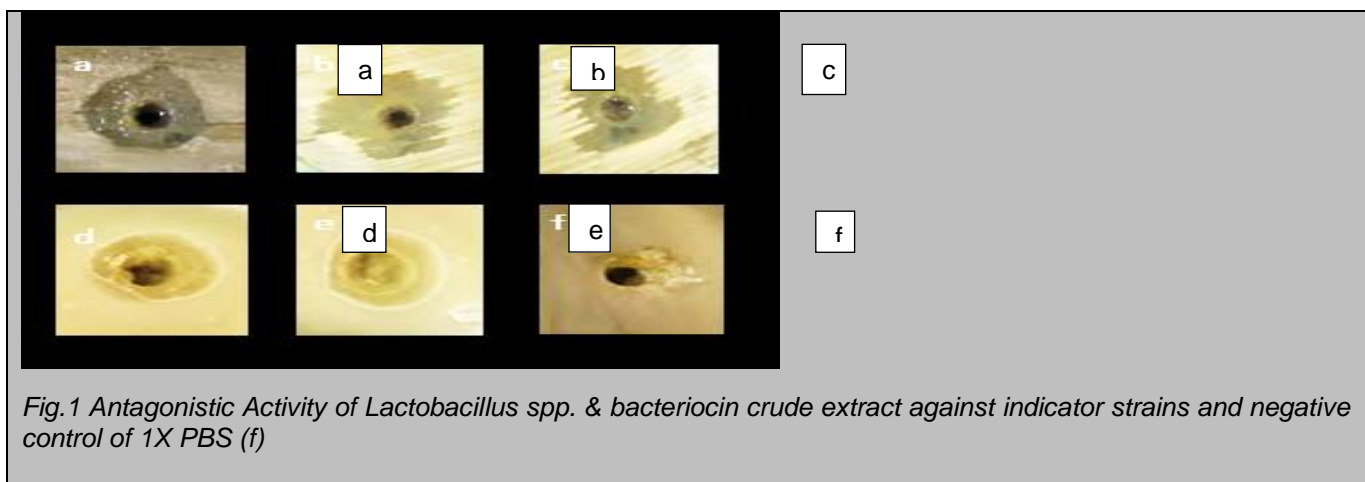


Table 2: Inhibition zones produced by partially purified proteins against indicator strains.

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

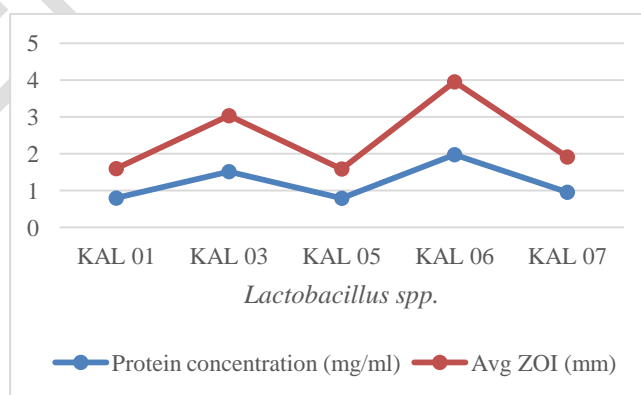
The total concentration of proteins in the different samples following the different purification protocols after its estimation using Lowry's Method are listed in the following chart.

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



4. DISCUSSION

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 were 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 were proven to be thermostable with activity at 100°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.

5. 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.

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