

ORIGINAL ARTICLE

Isolation and Identification of Bacteriocin by Dairy Products

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ABSTRACT

Bacteriocins are antimicrobial substances produced by bacteria, identified by lactic acid bacteria (LAB) which has been used as food bio-preservatives. The study was conducted at "Department of Life sciences, University of Management and Technology," Lahore, Pakistan and was aimed to isolate and identify bacteriocin producing bacteria from milk and yogurt. A total of 30 samples were collected (milk n=15; yogurt n= 14) from different local areas of Lahore. All the samples were serially diluted (10^{-1} to 10^{-7}) and processed on nutrient agar by spread plate method for 24 h at 37°C. All the isolates were identified through colony morphology on agar plates, Gram stain reaction and different biochemical tests such as catalase, oxidase, coagulase and indole respectively. *Bacillus subtilis* and *Escherichia coli* were used as sensitive indicator bacteria towards bacteriocin producing species. Out of 87 isolates, 30 were found as bacteriocin producers. Out of 30 producer species, 45% were from milk and 24% were from yogurt samples. The prominent producer isolates were *Lactobacillus* (40%), *Lactococcus* spp. (17%) and *Enterococcus* spp. (7%) respectively. Good inhibitory activity was observed against indicator species such as *B. subtilis* whereas; it was moderate against *E. coli*. The result indicated that the raw milk as a potential source of bacteriocin producing bacteria. Further research should be done on molecular level of bacterial isolates from dairy products.

Keywords: Bacteriocin, Bio-preservatives, Lactic acid bacteria, Antimicrobial activity

INTRODUCTION

Bacteriocin is the small thermostable, ribosomal synthesized antimicrobial peptide, hydrophobic peptide (20 to 60 amino acid) and extra cellular releasing protein that can inhibit the bacterial growth. It is host defense peptide that are produced by bacterium with antimicrobial properties (1). Lactic acid bacteria (LAB) produce a bactericidal protein, bacteriocin that can serve as a natural bio preservative and utilized as antibiotics. Bacteriocin produced by various Gram positive and Gram-negative bacteria but those produced by LAB that used in medical and food industry. Prestigious bacterium lactic acid bacteria produce almost more than 230 bacteriocins that enhance the antimicrobial properties and one-half of them identify by DNA level (2).

The antimicrobial activity of bacteriocin play important role for the destabilization of function of the bacterial cell components such as cell wall and cell membrane and alter the permeability of bacterial cell. Bacteriocin work as a probiotic, antimicrobial activity and work as immunomodulatory effects that modulate the inflammation (3). Bacteriocin have such activity against pathogenic bacteria such as multi-resistant species without sensitive strains (4). Antibiotics bacteriocin have dual nature that inhibit the cell wall of the bacteria and inhibits the other mechanism such as biosynthesis of DNA and protein synthesis (Kaškonienė et al., 2017). From dairy products such as milk and yogurt for the isolation and identification of Bacteriocin species with antimicrobial effect against such pathogens (6). LAB are producing organic molecules that give aroma and flavor. LAB has some properties such as it has protective effects in milk and yogurt against harmful pathogenic bacteria.

To the best of our knowledge, limited information is available about the identification of bacteriocin spp with antibacterial activity against pathogens. This present research work aimed to identify bacteriocins producing bacteria by isolation of bacteriocins, by characterize bacteria, by morphological and biochemical tests, and by screening production of Bacteriocin by Stab overlay method (7).

MATERIAL AND METHOD

Study design: This cross sectional study was carried out at department of Life sciences, University of Management and Technology, Lahore from November 2020 to March 2021.

Collection of samples: Thirty samples in which, yogurt (15) and milk (15) were collected from local areas of Lahore. The collection

of Milk (M) and Yogurt (Y) samples from different area of Lahore city as shown in Table 1.

Isolation of bacterial strains from milk and yogurt samples:

For the isolation of bacteria, serial dilution method was used for the dilution under aseptic conditions. Serial dilutions were made as 10^{-1} to 10^{-7} in seven test tubes. One mL of sample was taken and dissolved in 100 mL of deionized water. For media preparation, 2.8g of nutrient agar was dissolved in 100 mL of deionized water. Streak plate method was used for get isolated colonies (8). Petri dishes were covered with Parafilm and were then placed in an incubator at 37°C for 24 hours.

Screening of LAB for the production of Bacteriocin: The bacterial isolates were screened for bacteriocin development using "Stab and overlay method" (9). Stab and overlay method was used to screen multiple producer strains against sensitive strains (*B. subtilis*, *E. coli*). Antimicrobial activity against indicator species was suggested by the development of strong inhibition zones around the colonies.

Identification and Colony morphology: The initial observation of the isolates was observed on the basis of microscopy and biochemical identification to get pure colonies. We were determined the colony morphology to identify bacterial cultures on growth media by a number of factors, the size, pigmentation, opacity, margin, and elevation of colonies that appeared on the surface of the growth media. The other way to the identification of Colony morphology by using simple gram staining and microscopic examination. This Gram's staining procedure was followed for all bacterial isolates and were grouped as gram positive and gram negative respectively. All the slides were examined under the oil emulsion objective lens (100X) of optical microscope (10).

Biochemical identification: For further identification of bacterial species, following biochemical tests such as oxidase, catalase, coagulase and indole, test were performed (11).

Oxidase test: The oxidase test was used to see whether the bacteria produced cytochrome C oxidase. Organisms were deemed positive if they developed blue color within 5-10 seconds, and negative if they did not.

Catalase Test: This catalase test was used to check whether the bacteria isolates break down hydrogen peroxide into water and oxygen when it is present. We put out bacteria isolate on this test tube and wait for 15 seconds. If bubbles are produced species is positive otherwise it is negative for catalase.

Coagulase test: We performed coagulase test to determine clotting by the conversion of fibrinogen into fibrin. Blood into anticoagulant tube and centrifuged it at 400rpm. We replaced a drop of plasma on slide and spread bacterial isolates on it with the help of loop. We observed the clumping of the organisms for 10 to 15 seconds and if clot produced in slide it gives positive result and if no clot it means it gives negative result.

Indole test: This test established that the ability of organisms to break down the amino acid tryptophan into indole. We added Kovac's reagent on the bacterial isolates in the test tube that helps differentiate Enterobacteriaceae species after the formation of red or pink ring on the top. A red color ring indicates positive test. While in negative test no red color ring appears.

RESULT

Out of 30 samples, 45 isolates from milk (M) and 42 from yogurt (Y) were identified. The most prominent species were found in milk were *Lactococcus* 5(17%) and *Lactobacillus* spp 12(40%). The gram staining showed the gram positive (figure 1) and gram negative (figure 2) rod shaped bacteria and the macroscopic and microscopic characteristic of bacteria is summarized in table 02.

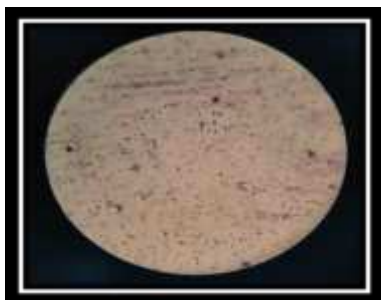


Figure 1: Gram Positive, Rod Shape

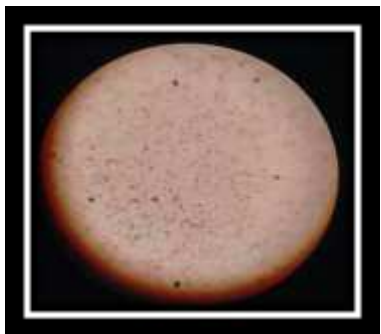


Figure 2: Gram Negative, Rod Shape

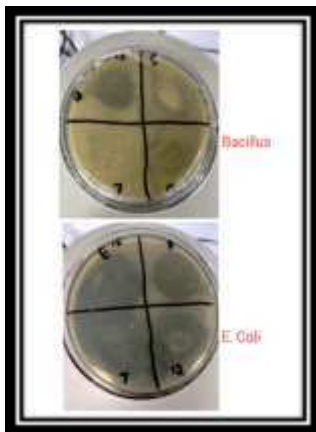


Figure 3: Bacteriocin Production

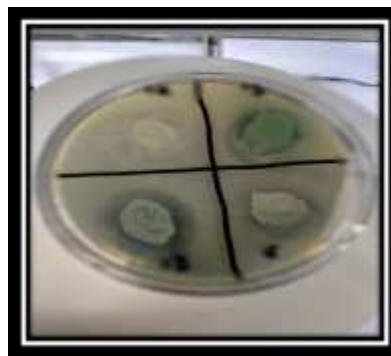


Figure 4: Zone of Inhibition

Table 1: Collection of Milk and Yogurt samples from different area of Lahore city

SR. NO	Milk Samples		Yogurt Samples	
	Area	Sample no.	Area	Sample no.
1	Kashmir block, Lahore	1	Noman milk canal road, Lahore	1
2	Numan milk shop, Johar town, Lahore	2	Babar milk shop, Lahore	8
3	Ali milk shop, Lahore	3	Rehman milk shop, Lahore	4
4	Salman milk shop, Lahore	4	Ali milk shop, Lahore	2
5	Arsalan milk shop, Kasoor	5	Taj milk shop, Lahore	6
6	Butt milk shop, Kasoor	7	Butt milk shop, Kasoor	7
7	Adeen milk shop, Lahore	6	Sherry milk shop, Kasoor	12
8	Hijveri milk center, Lahore	8	Ali milk shop, Lahore	13
9	Sakandaar milk shop	11	Ali hijvery shop, Pakpattan	14
10	Medina milk shop,	12	Sufyan milk shop, Lahore	9
11	Home	14	Sakandar milk shop, Pakpattan	5
12	Home	13	Salman milk shop, Lahore	3
13	Home	9	Adeel milk shop, Lahore	11
14	Bilal milk shop, Lahore	3	Hafeez milk shop, Lahore	10
15	Home	15	Salamat milk shop, Lahore	15
	Total Samples	15		15

Biochemical Test Result: For the further identification of bacterial isolates, biochemical test results shown in figure 6, 7 and 8 and Graph 1. The catalase test and oxidase test were the positive result strain 15M and formed bubbles, also identified as *Pseudomonas* spp (n=2) (Table 2). Similarly, catalase test gave positive result in sample 13M and identified as *E. coli* spp (n=3) (Table 2) and coagulase test gave positive result in sample 12M and it's a *Lactobacillus* spp (n=12). For further detail of identification of Bacteriocin are shown in Table 2.

Screening of bacterial isolates: Out of 30 samples, 87 isolates were isolated in which 30 isolates showed zone of inhibition against sensitive strains (*B. subtilis*, *E. coli*). On the basis of colony features and biochemical tests 12(40%) were *Lactobacillus* spp, organisms were identified as *Pseudomonas* in 2 (7%), *E. coli* in 3 (10%), *Salmonella* sp in 1 (3%), *Lactococcus* spp in 5 (17%), *S. aureus* in 1 (3%), *S. epidermidis* sp in 1 (3%), *Enterococcus* sp in 2 (7%) and *Bacillus* spp in 3 (10%) were observed (Table 2). Mostly isolates were gram positive and in rod shape.

Antibacterial Activity: For identification of antibacterial isolates, antibacterial activity was observed against *B. subtilis* and *E. coli* by

determining their zone of inhibition. In this study, isolates number 2Y, 3M, 4Y, 5M, 6M, 12M, 13M and 15M shown any antibacterial activity against *B. subtilis*. Antibacterial activity of

bacteriocin in isolates is shown in figure 3 and zone of inhibition for antibacterial activity is shown in figure 3 and 4.

Table 2: Identification of bacterial isolates through morphological, Gram reaction and biochemical tests

c	Sample No.	Colony Features	Gram Reaction	Coagulase	Oxidase	Indole Test	Catalase Test	Identified Organisms
1.	15M	Small size, small zone, circular form colony with dark green pigmentation	-ve Rods	-	+	-	+	<i>Pseudomonas</i> sp. n=2
2.	13M	Large zone against indicator <i>B. subtilis</i> , strong producer, large circular colony	-ve/+ve Rods	-	-	-	+	<i>E. coli</i> n=3
3.	12M	White, usually mucoid colonies, vary from small to medium gray colonies	+ve Rods	+	-	-	-	<i>Lactobacillus</i> spn-12
4.	8M	Large, gray white, moist, smooth, convex, entire edge	-ve Rods	-	-	-	+	<i>Salmonella</i> sp. n=1
5.	6M	Large, circular, gray, smooth, with concentric circles	+ve Rods	-	-	+	-	<i>Lactococcus</i> spn=5
6.	5M	Round, smooth, raised, gray to deep golden yellow	+ve Cocci	+	-	-	+	<i>S. aureus</i> n=1
7.	3M	Round, raised, shiny, gray, and have complete edges	+ve Cocci	-	-	-	+	<i>S. epidermidis</i> spn=1
8.	4Y]	Large, irregular pattern, green in colour, large zone	-ve Rods	-	+	-	+	<i>Enterococcus</i> spn=2
9.	2Y	Large, irregular pattern, circular, green in color	+ve Rods	-	-	-	-	<i>Bacillus</i> spn=3



Figure 6: Catalase Result



Figure 7: Coagulase Result



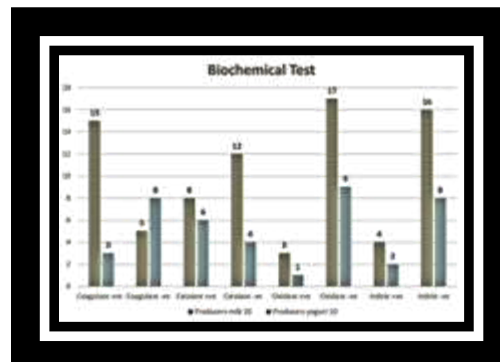
Figure 8: Indole test Result



Figure 1: Sub culturing of isolate



Figure 2: Growth of bacteria on by streak plat method nutrient agar



Graph 1: Result of Biochemical Tests

DISCUSSION

On the current study, the lactic acid bacteria are isolated from different milk and yogurt was *Lactobacillus* spp, five (17%) is *Lactococcus* sp, three (10%) is *Bacillus* sp and three (10%) is *E. coli* spp(12, 13) have also identified such species of LAB such as *Lactobacillus*, *Lactococcus* 20% and *Streptococcus* spp. About 87 isolates were obtained by isolation process and the results indicates that LAB isolates are more prominent in yogurt rather than milk, this is similar result by (14).

Thirty-four percent (34%) isolates were found to be capable of producing BLIS (Bacteriocin-like inhibitory substance), in our findings. Further research revealed that the thirty isolates chosen were more successful at delaying the growth of Gram-negative bacteria than Gram-positive isolates. The results proposed that the selected isolates of BLIS were active against closely related groups of bacteria. The current study was related to previous study and in which he obtained the same result as I isolated the same *Lactococcus* spp from raw milk (15). Out of 87 isolates, 34% isolates showed zones against bacteriocin sensitive inhibitors (*B. subtilis* and *E. coli*) (16).

About 23% isolates as lactic acid bacteria spp were identified on the morphologically characters and biochemical studies some as (17) that proved for the accurate identification of lactic acid bacteria morphological and biochemical. Twelve (40%) isolates/strains of *Lactobacillus* spp, five (17%) is *Lactococcus* sp, three (10%) is *Bacillus* sp and three (10%) is *E. coli*. My result is related to previous study (18). Maximum inhibitory activity was observed against *B. subtilis* and minimum activity was observed against *E. coli* same as in previous study (19). Inhibitory effect have been reported by bacteriocin against several others strains. The results of our present study are in related to the earlier findings of other workers who described highest inhibitory activity of bacteriocin producing *Lactobacillus* LBC against *B. subtilis*.

According to previous study, mostly bacteria obtained from dairy products like milk and yogurt was *Lactobacillus* spp, *Lactococcus* spp or *Streptococcus* spp (20). In microscopic analysis, it observed that mostly bacteria like 80% were gram positive rod shaped. Whereas 20% were gram negative rod shaped. According to previous study, *Bacillus* species are mostly gram-positive rod shaped (21).

According to previous study, after 24 hrs, zone of inhibition were observed strong against *Bacillus subtilis* as compared to *E. coli*. So the results correlate with each other (22).

CONCLUSION

In this current study bacteriocin producing strain were isolated from different milk and yogurt samples were found to be *Lactococcus* spp and *Lactobacillus* spp. Most prominent bacterial isolates identified in milk were *Lactobacillus* spp (40%) and *Lactococcus* spp (17%), whereas, *Enterococcus* spp. (7%) and *Bacillus* spp (10%) were found in yogurt samples. The current finding shows increased number of Gram positive bacteria in both milk and yogurt samples. Indicator bacteria such as *Bacillus subtilis* showed increased sensitivity to producer species as compared to *E. coli*. Thus, bacteriocin can be recommended to be used as a safe, natural, and effective bio preservation.

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