Evaluation of Bacteriocin activity of Lactic Acid Bacteria (LAB) Isolates of Milk and Yogurt

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Abstract

This study was conducted to learn about the potential of bacteriocins, formed by lactic acid bacteria, as inhibitory agent against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* specie. Nine lactic acid bacteria namely *Lactobacillus bulgaricus* (four strains), *Lactobacillus acidophilus* and *Lactobacillus plantarum* (two strains), *Lactobacillus helvaticus* (two strains) *and Lactobacillus acidophilus* were isolated from milk and yogurt. These isolates were identified morphologically and biochemically by standard methods. Bacteriocins were separated from the bacterial cultures, by centrifugation and their antibacterial activity was tested by well diffusion method. Isolated bacteriocin indicated the inhibitory effect against *S. aureus* and *P. aeruginosa* and *Salmonella* specie. The strongest inhibitory activity was shown by the strain M26 against *P. aeruginosa* which formed an inhibition zone of 21 mm. The most sensitive pathogen to the antibacterial activity of all bacteriocin producing lactic acid bacteria was *Salmonella* specie. The bacteriocins extracted from these nine lactic acid bacteria retained their activity after treating with temperatures 37^{o}C and 45^{o}C but decreased at 60^{o}C 80^{o}C and 90^{o}C. The bacteriocins also retained their antibacterial activity after the change in pH value (4,

6, and 8). The result of this study determined that bacteriocins are quite useful against commonly known clinical pathogens and thus can be used against these pathogenic bacteria.

Key words: *Lactobacillus helvaticus*, antibacterial, *Salmonella*, *Lactobacillus acidophilus*, Pathogens

Introduction

Due to fungi and bacteria, which spoil the food, many food industries are facing problems. Mycotoxin produced by microorganism's cause food poising in humans and is harmful to health (Ajmal et al. 2011). Techniques like freezing, sterilization, low or high temperature, thermal pasteurization, fermentation and use of stabilizer has been used from the start of life to control the spoilage of food but plentiful use of these methods may alter the quality of nutrients and can change the taste (Yasmin et al. 2015).

In the day-to-day world the risk of contamination due to pathogens especially food born microorganisms is becoming a great concern. This lowers the quality control of the food products and therefore decreasing its demand by consumers in the market. The pathogens which are originated from food are listed by Food and Drug Administration Authority (FDA) in their Bacteriological Analytical Manual and prioritize them as top (FDA. 2012). Various microorganisms like *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella* and *Salmonella* species are include in the list. *Streptococcus* and *Staphylococcus* species are the pathogens recorded previously in the food industry causing food contamination (Das et al. 2014). Lactic acid bacteria (LAB) made it possible for human to increase the shelf life of food and food products by using antimicrobial activity of LAB without damaging food contents (Yasmin et al. 2015).

Either as starter culture or natural micro flora cultures added under precise conditions, lactic acid bacteria generally recognized as safe (GRAS) microorganisms. Due to organic acid formation such as lactic acid which effect in lowered pH, lactic acid bacteria employed

antibacterial effect (Yang et al. 2012). High molecular weight (HMW) compound like bacteriocins and low molecular weight (LMW) compounds such as hydrogen peroxide, carbon dioxide, acetic acid and lactic acid are the antimicrobial compounds produce by LAB (Erdogrul and Erbilir, 2006). Thus, increasing the food safety and lengthening the life of food stuff by avoiding spoilage and pathogenic microorganisms (Tufail et al. 2011).

Both Gram negative and Gram-positive bacteria produce bacteriocins. Through bacterial cell ribosomes bacteriocins are released in the form of low molecular weight proteins or peptides and on closely related species they have bacteriostatic or bactericidal effect (Muhankumar, 2011). Though bacteriocin can be considered as antibiotics but actually they are not. The antibiotics have a wide activity range and even if their action is limited this does not express any enhanced effect on interrelated species and bacteriocin on the other hand restricted their action to the strain of species related to the bacteriocin producing species and predominantly to the similar species that is the key difference between antibiotics and bacteriocins (Zacharof and Lovitt, 2012). Some important bacteriocins include Plantaricins, Lactacins, Helveticins, Bulgarican, Acidophilin, Diplococcin, and Nisin. Bacteriocins have strong antagonistic activity beside important medical pathogens and are widely used as prospective antimicrobial agents in food additives (Ashokkumar et al. 2011).

1. Materials and methods

2.1 Sample collection

A total of 50 samples of yogurt and 50 milk samples were collected from the local markets of district Mardan, Pakistan in sterilized bottles and were then transferred to the lab. Each sample was diluted up to 4^{th} dilution (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in the sterilized distilled water by serial dilution process.

2.2 Media preparation

MRS (de Man, Rogosa and Sharp) agar and MRS broth (Sigma), a selective media for *Lactobacilli*, was prepared in sterilized condition as described by De Man et al. (1960). 1 ml of each dilution of milk and yogurt samples was then inoculated in tube containing nine ml MRS broth, under sterilized condition. A control without bacterial sample, was also run with each batch of sample. The tubes were then incubated for 24 hours at 37^{o}C. 100µl of broth from each culture tube having bacterial growth, was inoculated to MRS agar plates and allowed to incubate for 24 hours at 37^{o}C to obtain bacterial colonies. Pure cultures of each morphologically different colony were obtained by streak plate method as described by Awan and Rahman (2005).

2.3 Extraction of bacteriocins from cultures of lactic acid bacteria

Culture of each pure colony was grown in MRS broth at 37^{o}C for 24 hrs incubation. 1ml of each culture broth was centrifuged in eppendorf at 6000 rpm for 15 minutes to obtained cell free solution. The supernatant was then filtered through 0.2µm pore size filter paper. The supernatants were then neutralized with 1N NaOH.

2.4 Well diffusion assay

Cell free supernatants were tested for bacteriocin activity against selected pathogenic bacteria (*Pseudomonas aeruginosa, Salmonella sp.* and *S. aureus*) on nutrient agar plates by well diffusion method. For antibacterial activity of isolated bacteriocins, three inoculum sizes i.e., 70 ul, 50µl and 30 µl of supernatants were used. The inoculated plates were incubated aerobically at 37^{o}C for 24 hrs and the diameters of the inhibitory zones were measured.

2.5 Sensitivity to heat

Sensitivity to different temperature was studied on the activity of bacteriocins produced by isolates. 100µl supernatants containing bacteriocins were heated for 10 min at 37^{o}C, 45^{o}C,

60ºC, 70ºC and 90ºC. The agar well diffusion assay test was done to check their activity (Tolinacki et al. 2010).

2.6 Sensitivity to various pH values

To determine the effect of pH on the stability of bacteriocins, it was investigated by adjusting the pH of extracts fluid at 4, 6, and 8. Agar well diffusion assay was performed to test the activity of the bacteriocin containing supernatants (Tolinacki et al. 2010).

2.7 Morphological, biochemical characterization and identification of lactic acid bacteria

The bacterial cultures showed inhibition to the pathogenic bacteria were then examined for identification of bacteriocins producer strains, by using various morphological, cultural and biochemical testing methodologies, i.e., Gram staining, catalase, carbohydrate fermentation and Bacteriocin production tests (Bergey and Holt, 1994).

2.8 Statistical Analysis

Results of each parameter including bacteriocin activity of isolate at different inoculum size, pH and temperature were compared statistically by using Chi square test. P-value <0.05 at 95% CI (confidence interval) was considered significant.

2. Results

A total of 134 isolates, 60 from milk and 74 from yogurt showed growth on selective MRS media indicating presence of lactic acid bacteria (LAB). Out of these lactic acid bacteria 9 (6.7%) isolates were found bacteriocin producers. In 74 lactic acid bacteria isolated from yogurt, five (7%) were bacteriocin producers and in 60 milk lactic acid bacteria, four (6.6%) were bacteriocin producing (Table1).

Table 1: Overall bacteriocin producing bacteria isolates

Samples nature	Total no. of Samples	Bacteriocin producer	%age
Yogurt	74	5	7%
Milk	60	4	6.6%

Total	134	9	6.7%

3.1 Antibacterial activity of bacteriocin from isolated strains against *P. aeruginosa, S. aureus* and *Salmonella* sp

Isolated bacteriocin indicated the inhibitory effect against *S. aureus* and *P. aeruginosa* and *Salmonella* specie. At different concentration in microliter of bacteriocins from isolated strains, inhibitory zones appeared were different for these pathogens. Highest activity was observed by M26 that was 21mm, at an inoculum of 70, 50 and 30µl of bacteriocin. The most sensitive pathogen to the antibacterial activity of bacteriocins was *Salmonella* specie. The lowest inhibitory zones were formed by Y47 that was 10.5 at all inoculum sizes (Fig 1, 2 and 3). Overall there was no significant difference in activity of any isolate at different tested inoculum sizes (P>0.05), indicating no effect of inoculum size.

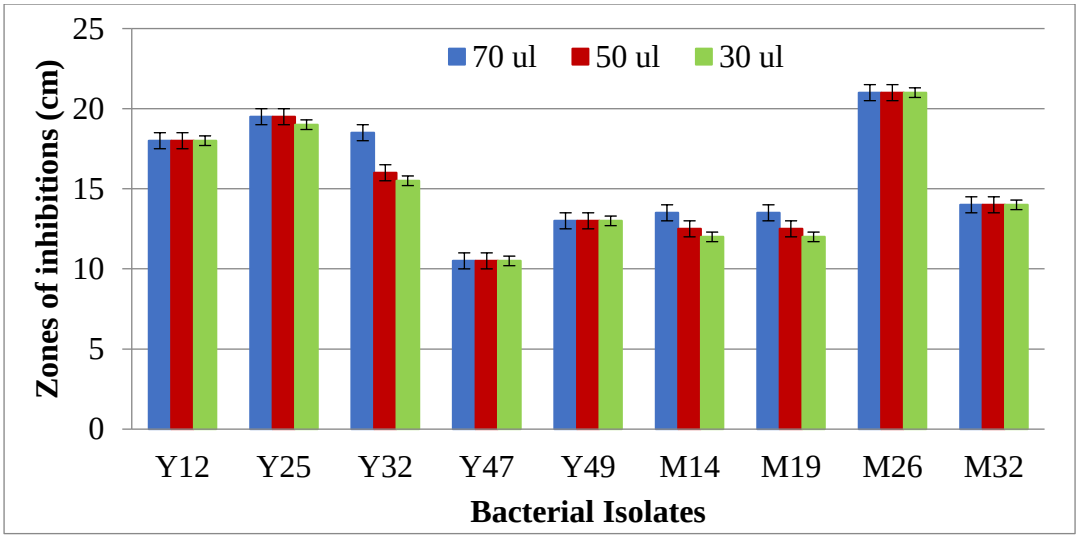

Figure 1: Bacteriocin activity of isolated strians against *P. aeruginosa* at different inoculum size (70, 50 and 30µl).

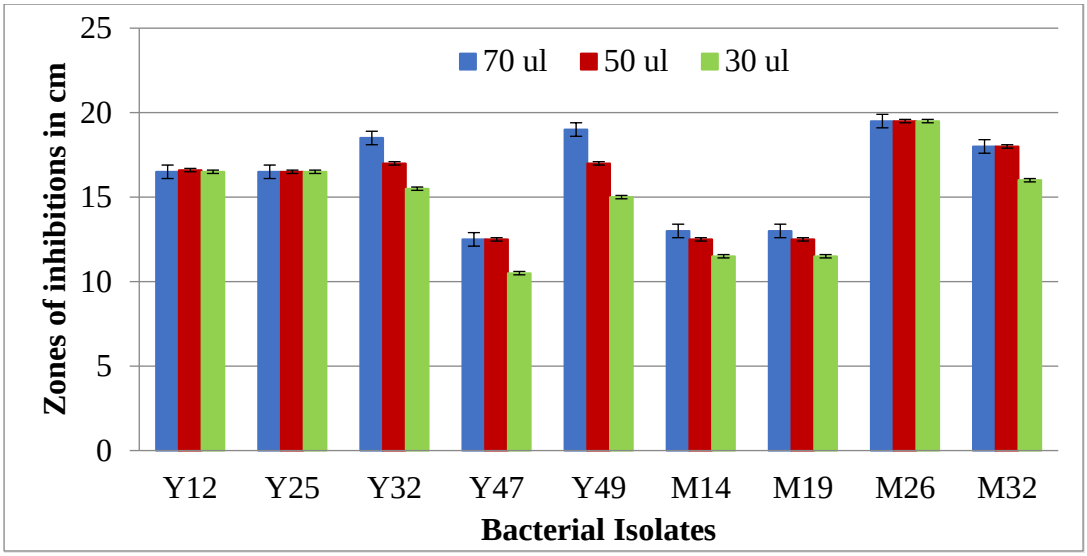

Figure 2: Bacteriocin activity of isolated strains against *S. aureus* at different inoculum size (70, 50 and 30µl).

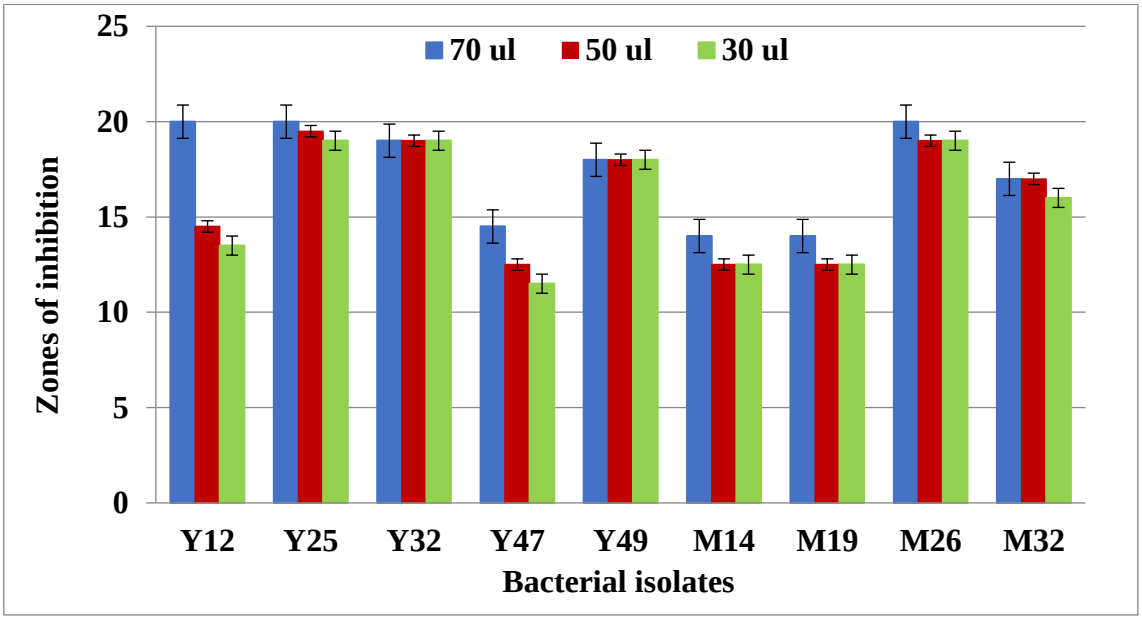

Figure 3: Bacteriocin activity of isolate strains against *Salmonella* specie at different inoculum size (70, 50 and 30µl).

3.2 Sensitivity to heat

Effect of temperature revealed that the bacteriocins were able to maintain their activity after treating with wide range of temperature between 37-90°C. However, they well retained their antibacterial activity at 37°C and 45°C but declined afterwards (Figures 4, 5 and 6). Statistical

analysis revealed there was no significant difference in activity of any isolates at different temperatures (P>0.05).

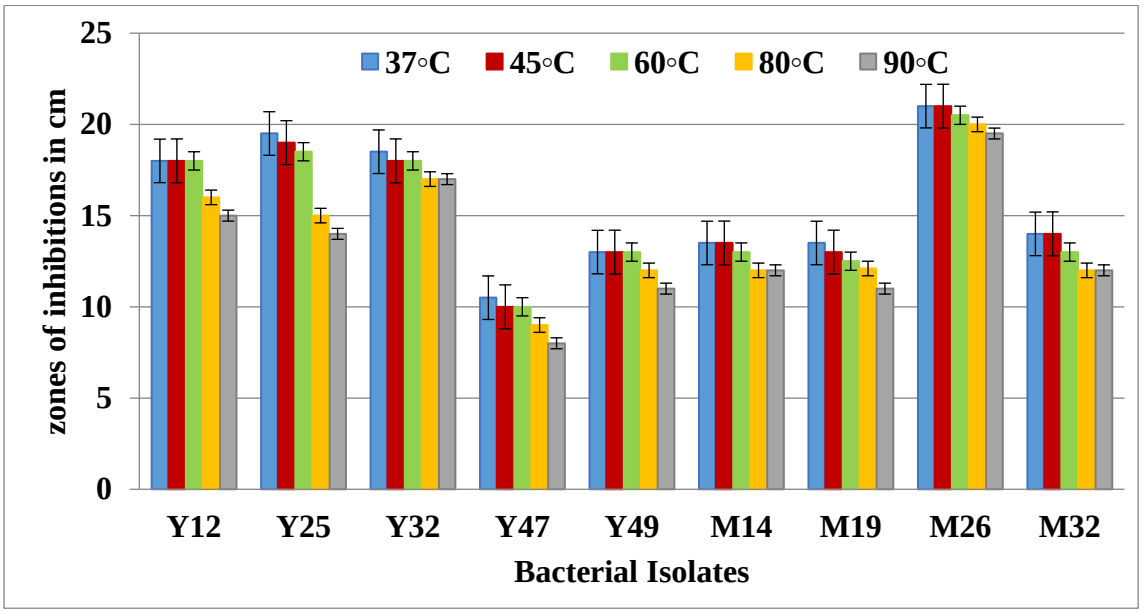

Figure 4: Effect of different temperatures (37-90ºC) on the activity of bacteriocin against *P. aeruginosa*.

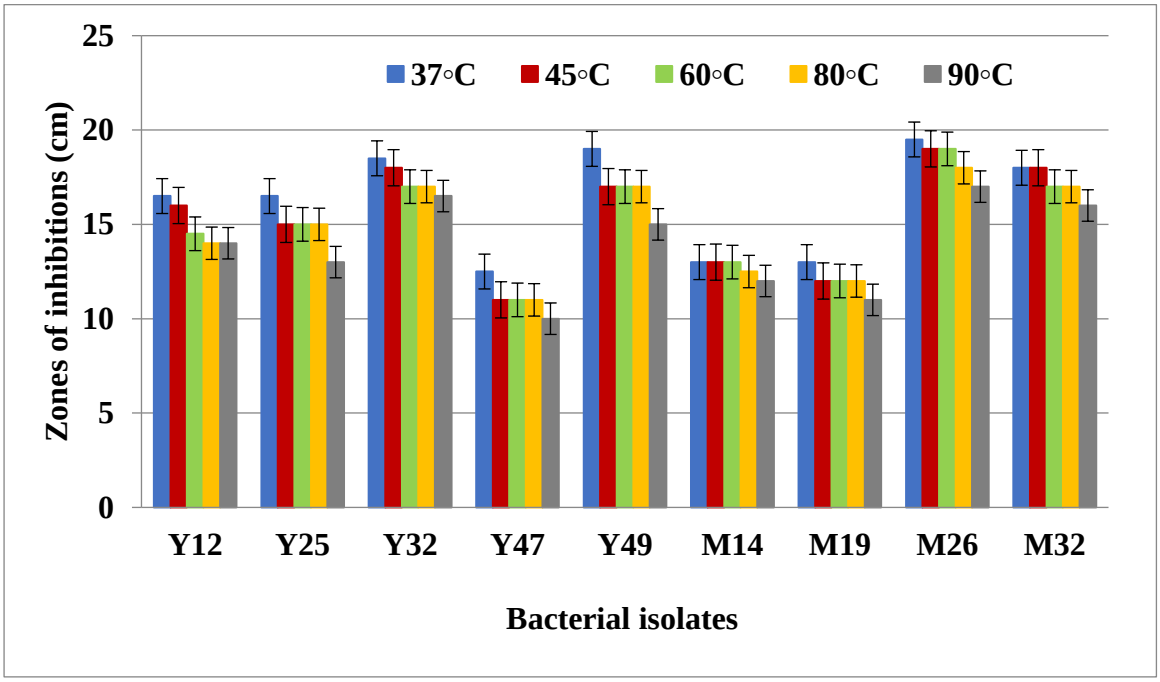

Figure 5: Effect of different temperatures (37-90ºC) on the activity of bacteriocin against *S. aureus*.

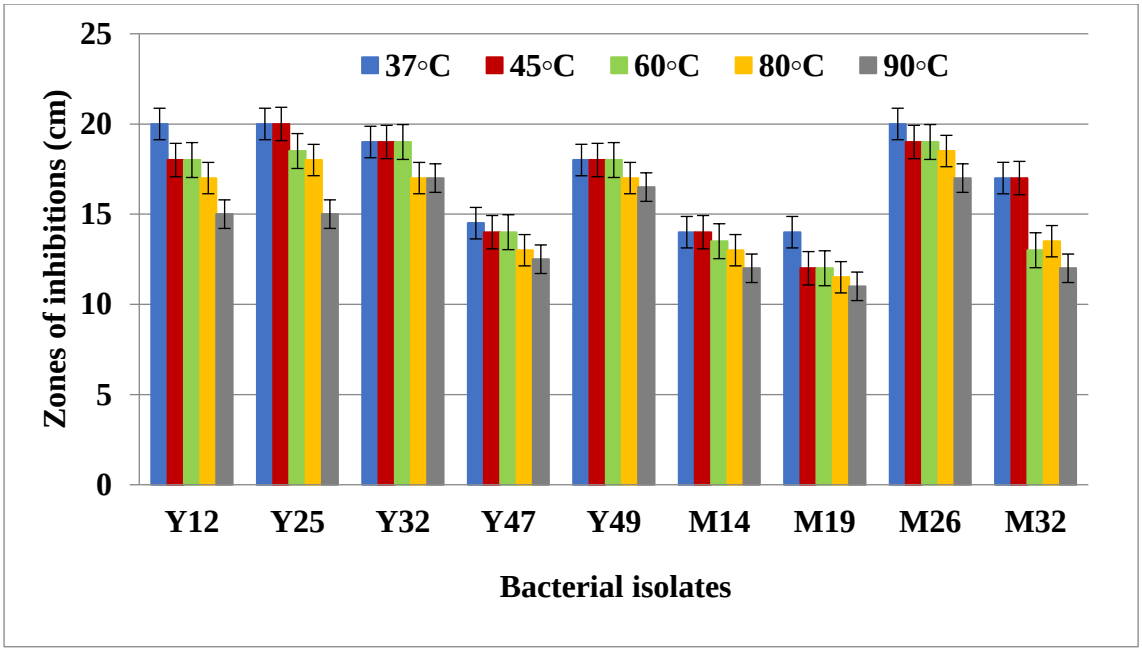

Figure 6: Effect of different temperatures (37-90°C) on the activity of bacteriocin against *Salmonella* species

3.3 Sensitivity to pH

Effect of pH on bacteriocin activity showed that, highest activity was at 6 followed by pH 8 and lowest at pH 4 (Figures 7, 8 and 9). Statistically there was no significant difference in activity of any isolate at different pH (P>0.05).

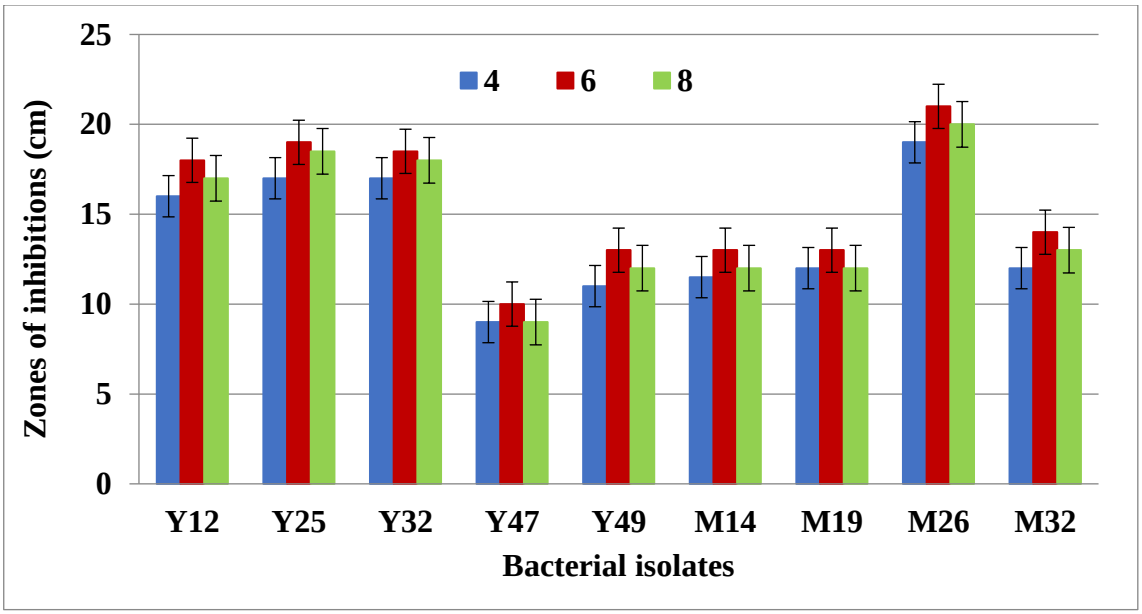

Figure 7: Effect of pH (4, 6 and 8) on the activity of bacteriocin against *P. aeruginosa*

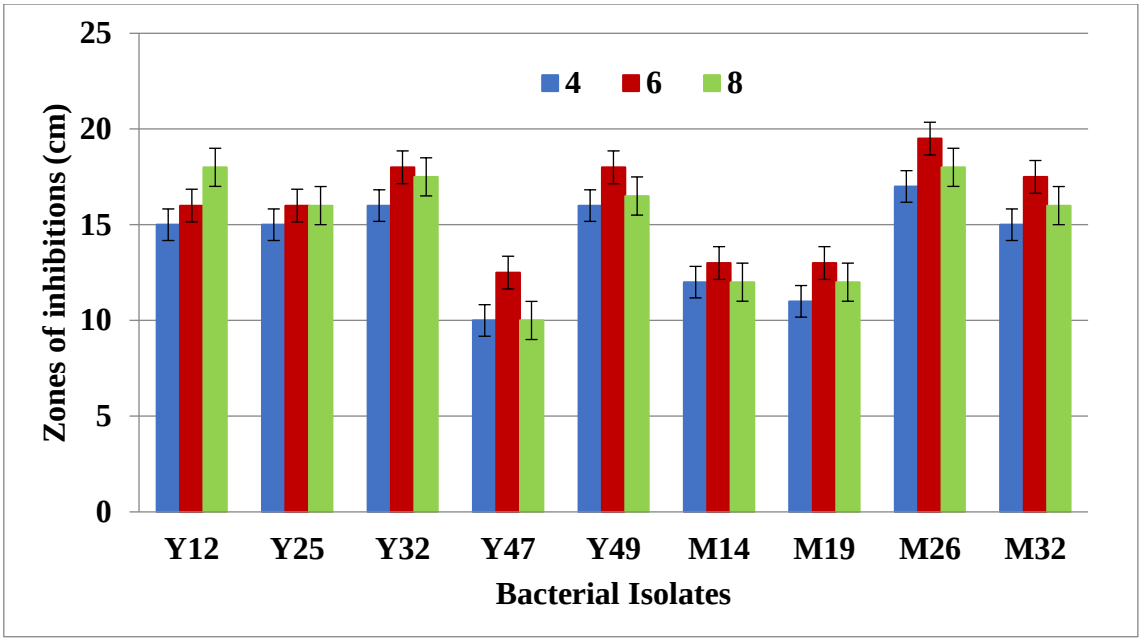

Figure 8: Effect of pH (4, 6 and 8) on the activity of bacteriocin against *S. aureus*

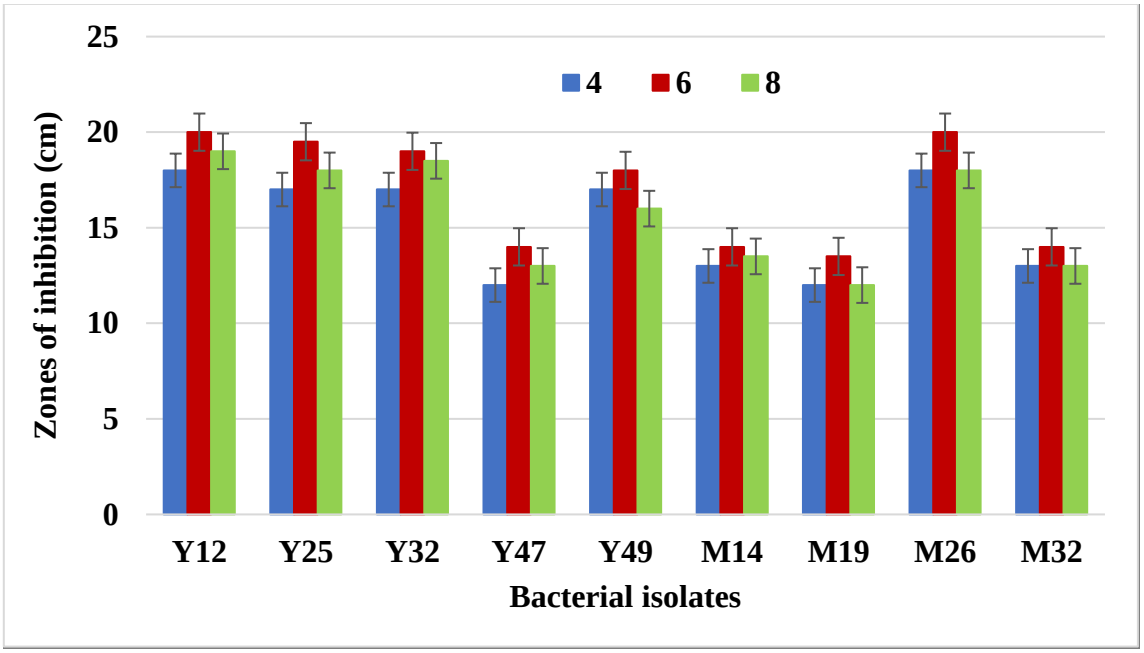

Figure 9: Effect of pH (4, 6 and 8) on the activity of bacteriocin against *Salmonella* sp.

3.4 Biochemical identification of isolated strains

Identification of isolated bacteria were carried out by standard biochemical tests. All the strains showed negative result to catalase test and did not form any bubble with hydrogen peroxide. The recognition of *Lactobascillus* strains were made by the carbohydrate fermentation test. Three types of carbohydrates were used in the carbohydrate fermentation test i.e., sucrose,

glucose and fructose. According to carbohydrate fermentation Y12 ferment glucose, sucrose and fructose. Y25 were able to fermented glucose and fructose but not sucrose. Y32 fermented sucrose fructose and glucose. Y47 did not ferment sucrose but fermented glucose and fructose. M14 did not ferment sucrose and fructose but fermented glucose. Y19 fermented all the three carbohydrates sucrose, fructose and glucose. M26 fermented sucrose glucose and fructose. M32 fermented fructose and glucose but did not ferment sucrose. Based on the biochemical test and colony and cell morphology Y12 was *L.dilbrueckii*, Y25 was *L.bulgaricus*, Y32 was *L. helvaticus*, Y47 was *L. bulgaricus*, Y49 was *L. plantarum*, M14 was *L. helvaticus* M19 was *L. acidophilus*, M26 was *L. plantarum* and M32 was *L. bulgaricus* as identified by Khedid et al. (2009) and Nikita and Himangi, (2012); (Table 2).

Table 2: Catalase test and carbohydrate fermentation test for identification of lactic acid bacteria.

LAB types	Carbohydrate type fermented			Catalase Positive / Negative	Bacteria type*
	Sucrose	Fructose	Glucose		
Y12	$-^{N}$	$+^{p}$	$+^{p}$	$-^{N}$	*Lactobacillus delbruekii*
Y25	-	+	+	-	*Lactobacillus bulgaricus*
Y32	-	-	+	-	*Lactobacillus helvaticus*
Y47	-	+	+	-	*Lactobacillus bulgaricus*
Y49	+	+	+	-	*Lactobacillus plantarum*
M14	-	-	+	-	*Lactobacillus helvaticus*
M19	+	+	+	-	*Lactobacillus acidophilus*
M26	+	+	+	-	*Lactobacillus plantarum*
M32	-	+	+	-	*Lactobacillus bulgaricus*

Positive (+ve) = + Negative (-ve) = -

Bacterial type* = according to Khedid et al. (2009).

3. Discussion

In the present study the focus was on the collection of lactic acid bacteria which were good producers of bacteriocin. From different samples of yogurt and milk 134 bacterial strains were obtained and studied for their strength to produce bacteriocin. 9 strains were able to produce bacteriocin and showed antibacterial action against both Gram-positive and Gram-negative pathogenic bacteria. The study showed resemblance to the study of Ahmed et al. (2013) who isolated *Lactobacillus* species from yogurt that showed a broad range of antimicrobial activity against some spoiling pathogens. There also in the similarity with the findings of Mohankumar and Murugalatha (2011), who found out the antimicrobial activities of bacteriocins which were produced by *Lactobacillus* isolated from the fresh milk of the goat, cow and buffalo. There were 10 strains of the bacteria which were able of producing bacteriocins hence their activity was examined carefully against pathogenic bacteria.

In the present study bacteria isolated from yogurt and fresh milk were identified by using morphological and biochemical tests. In our study 9 strains of bacteria Y12, Y25, Y32, Y47 and Y49, from yogurt and M14, M19, M26 and M32 from milk were screened and identified as *L. delbruekii* sub. sp. *bulgaricus* (4 strains), *L. hevleticus* (2 strains), *L. plantarum* (2 strains) and *L. acedophilus* based on the biochemical tests and morphology. According to the study of Khedid et al, (2009), who from the milk of camel (dromedary) in Morocco isolated 123 different strains of bacteria capable of producing Lactic acid and identified them on the basis of morphological and biochemical tests. Also have similarity to the results of Tufail et al. (2011).

Bacteriocin isolated from the isolated lactic acid bacteria were tested against 3 types of pathogenic bacteria namely *P. aeruginosa*, *S. aureus* and *Salmonella* specie. Bacteriocins showed antimicrobial effect on these bacteria means that they have the ability to stop or slows down the growth of both the Gram-negative and Gram-positive bacteria. The study show

similarity to the work of Jin et al (2004). The study of Sifour et al, (2012) was contradictory with the present work as they found that Gram-positive pathogenic bacteria show more sensitivity to bacteriocin of lactic acid bacteria strain in comparison with Gram negative indicator bacteria. This difference may be due to difference in chemical nature of bacteriocins produced by different isolates.

Maximum zone of inhibition formed by *L. delbruekii* (Y12) was 20 mm against *Salmonella* specie. Significant inhibiting potential has been observed against *Staphylococci* and *P. aeruginosa*. The results also are accordance with previous work carried out by Devi et al, (2013) but on different strains. On the other hand our results are contrary to the findings of Ravaei et al, (2013). They found some evidences that *Lactobacilli* have no effect against Gram-negative bacteria as compared to Gram positive. In our study our isolates showed antibacterial effects on both type of pathogenic bacteria which either Gram negative or Gram positive. These findings were also agreed to Mohammed et al. (2013).

In our study strain M14 identified as *Lactobacillus helveticus,* showed growth inhibition affinity towards tested pathogenic bacteria of *P. aeruginosa*, *S. aureus* and *Salmonella* specie. In a study by Jena et al, (2013), an antibacterial substance (bacteriocin PJ4) manufactured by *Lactobacillus helveticus* which showed its activity against a huge range of both G-negative and Gram-positive bacteria, involved in various ailments. Another researcher, Nikolova et al. (2009) identified a strong inhibitory activity of *Lactobacillus helveticus* on the growth of *P. aeruginosa*, *Bacillus subtilis*, *E. coli* and *Bacillus alvei*.

In current study, highest zone of inhibition formed by *L. plantarum* (strain M26) against *P. aeruginosa* that was 21 mm at a concentration of 70µl of supernatant. Abo-Amer (2007) reported that antibacterial agent excreted by *L. plantarum* AA135 found to be highly active against a wide spectrum of Gram-positive and Gram-negative bacteria. The maximum zone of

inhibition formed by *L. plantarum* AA135 at a concentration of 50µl was 21 mm against *Pseudomonas aeruginosa*.

It was analyzed in current work that the antimicrobial activity of bacteriocins retained at different temperatures. The activity was almost the same when treated at 45^{o}C, 60^{o}C, for 10 minutes of all the bacteriocin producing bacteria. There was no significant difference in the diameters obtained from the zones of inhibition matched to the zones' diameter at 37^{o}C. But at high temperatures (80^{o}C and 90^{o}C) the activities of bacteriocins decreased indicating heat sensitivity of these bacteriocins activity. The results show resemblance to the study of Assefa et al. (2008) and with the study of Kaur et al. (2015) who reported the bacteriocin activity upto 80^{o}C. Further, they reported that at sterilization temperature of 121^{o}C, either their activity decreased minimum or totally stop. While, Xie et al. (2009) reported no change in antimicrobial activity when bacteriocin was heated at 60, 70, 80, 90 and 100°C indicating their antimicrobial activity was heat resistant.

Current results exposed that antibacterial activity of bacteriocin from lactic acid bacteria had not significant effect at different pH (4, 6 and 8). Although highest effect was noted at 6 pH but activity retained at all three tested pH. The results have shown the similarity with the study by Assefa et al. (2008) and Kaur et al. (2015). They reported that bacteriocins retained their activity at wide range of pH i.e., 2 to 10 but maximum activity was reported at 6 pH.

Conclusions

It can be concluded from our findings that yogurt and milk are rich sources of bacteriocin-producing lactic acid bacteria and had good antibacterial activity against the tested pathogenic bacteria i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* specie. LAB naturally present in yogurt and milk may play a role to enhance the microbiological safety of these products. The combined application of antibiotics and bacteriocins may have synergistic

effect to increase the control of both human and animal disease causing organisms. There is need for more research on antibiotic resistance profiles of these bacteria.

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