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Use of Bacteriocin Producing *Lactobacilli* Strains from Fruits and Vegetables in Food Preservation

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ABSTRACT: *Bacteria, that generates bacteriocin plays an important role in the preservation of food. Lactobacilli strains that produced bacteriocin were acquired from various vegetables and fruits from the markets of Multan, Pakistan. Bacterial growth was observed on MRS broth as well as agar, which is a Lactobacilli-specific media, and their metabolic activity as well as antibacterial activity against diverse pathogens were investigated. The endeavor of bacteriocin at different parameters i.e., pH, temperature, and proteolytic enzymes were explored for further validation of bacteriocin generating Lactobacilli. These bacteria's antibiotic activity against ampicillin, ciprofloxacin, streptomycin and tetracycline, was also tested. Ten Lactobacilli strains were identified based on their morphology as well as biochemical characterization and six were further selected to perorm study. Among them, 80% strains exhibited inhibition zone and the significant zones were illustrated by Ct_2 isolate (14 mm) embracing E.coli (9473) and A_3 isolate(14 mm) against Enterobacteria spp. Bacteriocin ability of these designated strains was maximum at 7 pH and 35℃. T_1 strain was sensitive to ciprofloxacin, ampicillin as well as streptomycin although T_2 strain was resistant to streptomycin, ciprofloxacin as well as tetracycline. The most notable strains were identified for the preservation of fruit juice, where Lactobacilli that produce bacteriocin inhibit the growth of other bacteria that may spoil food.*

Keyword: *LAB, MRS media, Lactobacilli, Antibacterial activity, Bacteriocin*

INTRODUCTION

A large number of bacteria that produced lactic acid are present in nature and their industrial uses are also recognized (Dhamale et al., 2015). Lactic Acid Bacteria (LAB) belongs to "Gram-positive" group and have clusters of physiological, morphological and metabolic categorization (Mohankumar

and Murugalatha, 2011). They also showed positive results for the Indole test, Methyl-red and nitrate reduction test while, gives a negative result for Oxidase production test, Catalase test and Voges-prosker test (Dhamale et al., 2015). For the fermentation of feed and food, lactic acid bacteria assumes a critical duty and generally recognized as safe microbe (GRAS) microbe, hence under controlled conditions its use as a starter culture is also well known (Yang et al., 2012). Lactic acid is produced through either homo-fermentation or hetero-fermentation mechanisms. Nature has generously disseminated this bacteria all around and even found in human digestive track (Mohankumar and Murugalatha, 2011).

Lactobacillus and *Leuconostoc* are the members of LAB and they hold industrial importance and utilized extensively in the food industry. *Aerococcus* and *Vagococcus,* are also used in the biopharmaceutical industry. *Carnobacterium* and *Pedicoccus* are used in the production of bacteriocin (Huys et al., 2012). Use of *Tetragenococcus* as a probiotic is also well known (Rodpai et al., 2021). Other LAB bacteria like *Streptococcus* and *Lactococcus* are also frequently used in the dairy industry (Holzapfel et al., 2006). These are the bacteria that are known for their contributions in various industries and productions like, color, taste, texture and smell of several food items after its fermentation. Another bacteria i.e. *Lactobacillus acidophilus* is a part of the natural flora of the intestine. It is actually a non-pathogenic bacterium which is known for its use in medicine, as well as industrial fields. It has been reported that the production of acid causes reduction of yeast and bacteriain the intestine. It also aids in digestion of milk (Ahmed et al., 2010). Bio-preservation bacteria contains metabolites or even the living cells of microbe in humans and animals. They have a queer stimulatory effect on the digestive and immune systems of their hosts (Wang et al., 2010). Through the preservation of food the shelf life of food products is increased and ultimately there is an increase the supply. The food products that hold a lesser shelf life and are at a risk of perishing can be preserved for several days and sometimes upto weeks. Throughout the year, seasonal foods become accessible and results in the increase in variety of food (Zacharof and Lovitt, 2012).

Another use of LAB bacteria is that it is a safer microbe that prevents the food item from developing fungal or even bacterial pathogens. Food-borne

diseases, such as bacterial and fungal infections, infect about 30% of the population. In the year 2000, over ten million individuals died worldwide as a result of a diarrheal sickness (Black et al., 2003)

To restrict or limit the growth of disease-causing food borne pathogens, safe preservation items such as essential oils, surfactants, and bacteriocin derived primarily from lactic acid bacteria are used (Ahmad et al., 2014).

Food preservation is an important requirement of life. To increase the shelf life of the product has now become a necessity. The most crucial compound that is used for preservation of food is bacteriocin. It is an ancient technique to preserve vegetables using the method of lactic acid fermentation. It involves lactic acid bacteria that mainly produce lactic acid and other components like bacteriocin. Bacteriocin has antibacterial activity against a variety of different microorganisms.

LAB is renowned for their ability to commonly create bacteriocin. However, LAB strains and their produced bacteriocin need tostudy in detail to see effective activity against the microbes. Strangely it showed a lesser activity in comparison to the antibiotics. Nowadays much use of antibiotics results in resistant strains against the antibiotics. The present study was designed to isolate bacteriocin from collected samples of fruits and vegetables. The strains were tested at variable temperature, pH proteolytic activity and also used against different pathogens.

MATERIALS AND METHODS

Collection of Samples

Samples of fruits and vegetables were collected from divergent locations throughout Multan, Pakistan, and transported to the department of Microbiology and Molecular Genetics' research facility/laboratory. The samples were kept at 4°C until further processing.

Isolation and characterization of Lactobacilli

The fruits were washed in autoclaved sublime water and grinded with an aseptic mortar and pestle. *Lactobacilli* strains were obtained using MRS broth as well as agar. For the goal of pre-enrichment of *Lactobacillus* spp., squeezed fruits were enumerated in MRS broth. For each sample, inoculum from the broth was spread on MRS agar plates individually. Streaking was performed to isolate a purified colony of *Lactobacilli* strains (Ravi and Subramanyam, 2011). Gram staining and biochemical tests were implemented

as stated by Bergey's Manual of Bacteriology (Holt, 1994).

Pathogens used for antibacterial activity

Pathogenic species i.e. *E.coli* (9473), *Salmonella spp.*, *Klebsiella*, *Enterobacteria spp.*, and *MRSA*, were collected from The Women University Multan, Pakistan.

Bacteriocin production

Bacteriocin producing isolates were incubated at 37ºC for 48 hours in MRS broth and centrifugation was done for 10 minutes at 5000 × g to acquire separation of cells from by-products (Kumar and Kumar, 2015).

Antibacterial activity test

Antibacterial activity was scrutinized by well diffusion method aerobically. 500 µL of indicator microorganisms were inoculated in agar plates after incubate them in a nutrient broth and diluting them adequately. Muller-Hinton (MH) agar was used for this purpose in which 6 mm wells were compelled in plates of MH agar and 150 µL of crude bacteriocin (supernatant) was poured into separate well and the plates were incubated at 37ºCfor 24 hours. Antibacterial activity was deliberated by assessing the inhibition zone diameter (Kumar and Kumar, 2015).

Purifying bacteriocin

Divergent quantity of ammonium sulphate were added in crude bacteriocin and retained by stirring and was preserved overnight at the temperature of 4ºC bereft of disturbing. Then centrifugation was done for 10 minutes at 10000 × g, further precipitates were gathered subsequently and diffused in 20 mM potassium phosphate (KH_2PO_4) buffer (pH 7) (Udhayashree et al., 2012).

Bacteriocin Characterization

a) Heatlabile

5 ml bacteriocin was baited in distinct test tubes and labelled accordingly. Test tubes were subsequently heated at divergent temperatures i.e. 35ºC, 50ºC, 70ºC for 15 minutes and was further checked against pathogen (Kumar and Kumar, 2015).

b) Effect of pH

5ml partially purified bacteriocin was baited in divergent test tubes and their pH was regulated to 2, 7 and 9 separately making use of NaOH or HCl and were placed at room temperature. Their antibacterial activity was scrutinized against divergent pathogenic species (Kumar and Kumar, 2015).

c) Effect of proteolyticenzyme

5 ml partially purified bacteriocin was baited and treated with enzyme papain at 7 pH. Test tubes with and without enzyme (control) were incubated at 37ºC for 2 hours and were further heated for 3 minutes at 100ºC with the aim of denaturing enzyme and were checked for antibacterial activity against variant pathogens (Kumar and Kumar, 2015).

d) Effect of antibiotics

Lactobacilli that was grown overnight, was swabbed on soft agar plates of MRS media and discs of antibiotics were allocated on the surface media. Discs that were used for this purpose includes Ampicillin, streptomycin, tetracycline and ciprofloxacin (Kumar and Kumar, 2015).

e) Efficacy of bacteriocin as bio preservative

The bio-preservation efficacy of the strains was scrutinized by adding 5% bacteriocin in apple juice and was refrigerated. Serial dilution of 10^6 was prepared with the sample and incubated for 3 days at 37°C. The colony count (CFU) was noted and compared with and without bacteriocin control (Udhayashree et al., 2012).

RESULTS

Isolation and characterization of Lactobacilli isolates

Isolated strains were conceded as *Lactobacilli* after biochemical characterization that was carried out in accordance to Bergey's manual. The isolated strains were Gram positive, non-motile and non-spore formers as shown table1.

Antibacterial activity

Screening of *Lactobacilli* isolates was done and results were noted. T_1 and T_3 exhibited significant results abutting every pathogen included in the present study i.e. *E.coli* (9473), *Methicillin-resistant Staphylococcus aureus* (*MRSA*), *Salmonella spp*., *Enterobacteria,* and *Klebsiella.* Nevertheless strain A_2 and A_3 exhibited zone of inhibition abutting all pathogens excluding *MRSA 6* strain. O_2 and Ct_2 manifested the inhibition zone abutting all the pathogens besides *Klebsiella* as shown in table 2. Six strains that showed best results were selected for further test of bacteriocin.

Table 1: Isolated *Lactobacilli* strains and their characterization

Sample	Locality	Origin	No. of purified colonies	Selected strains	Gram reaction
01	Fruit shop Sabzazar Multan	Apple	2	A_1	+ve
02	Fruit shop near Multan Cantonment	Olives	1	O_1	+ve
03	Fruit shop in Muzaffargarh	Olives	1	O_2	+ve
04	Fruit shop near Khanewal Road Multan	Tomatoes	2	T_1	+ve
05	Fruit shop on BZU Road Multan	Apple	2	A_2	+ve
06	Fruit shop near Chowk Shah Abbas	Apple	1	A_3	+ve
07	Vegetable shop near Gulshan market Multan	Carrots	1	Ct_1	+ve
08	Fruit shop near children's Hospital Multan	Tomatoes	1	T_3	+ve
09	Vegetable shop near Chungi No. 06 Multan	Carrots	1	Ct_2	+ve
10	Fruit shop near Kalma Chowk Multan	Olives	1	O_2	+ve

Table 2: Screening of *Lactobacilli* strains against various pathogens.

Strains	**Zone of inhibition**				
	***E.coli* (9473) (mm)**	***MRSA* (mm)**	***Klebsiella* (mm)**	***Salmonella* (mm)**	***Entero bacteria spp* (mm)**
A_1	12	-	-	10	11
A_2	13	-	11	11	12
A_3	13	-	10	11	14
Ct_1	11	-	-	11	11
Ct_2	10	13	-	12	13
O_1	11	-	-	11	11
O_2	11	12	-	13	11
T_1	12	12	12	11	12
T_2	12	12	12	12	11
T_3	11	11	10	10	12

Characterizing bacteriocin

Following parameters were scrutinized for the characterization of bacteriocin:

a. Distinct pH
b. Distinct Temperatures
c. Proteolytic activity

Strains that showed activity at all temperatures, pH levels, and enzyme activity were thought to have the best bacteriocin activity.

Characterizing bacteriocin at distinct temperatures

Bacteriocin were examined at distinct temperatures including 35ºC, 50ºC and 70ºC to inspect that at which temperature bacteriocin exhibit best performance. At 35°C, strains A_1, Ct_1, O_1 and O_2 did not show any anti-*Klebsiella* activity and A_1, Ct_1, O_1 did not show any activity abutting *MRSA*. Apart from all

pathogens chosen for this study, strains T_1 and T_2 demonstrated bacteriocin activity at 50°C, and each strain showed activity abutting E.coli (9473). Apart from pathogen, most strains were ineffective at 70°C and showed no zone of inhibition. Conversely, T_2 strain was efficacious against all the pathogens selected for this study and the table 3 summarized the findings.

Characterizing bacteriocin at distinct pH

Strains were scrutinized against pathogens at distinct levels of pH including 2, 7 and 9. Apart from the pathogens (in this study), strains Ct_1, O_1, and T_2 showed activity under acidic condition i.e. pH 2. At pH 7, T_1 and T_2 strains exhibited bacteriocin activity against all pathogens under study however, strain T_1 and T_2 exhibited bacteriocin activity at pH 9. Results were recorded in table 4.

Table 3: *Lactobacilli* strains' bacteriocin activity at diverse temperatures

Test Strains	Zone of inhibition				
	***E.coli* (9473) (mm)**	***Salmonella*spp. (mm)**	***Klebsiella* (mm)**	***MRSA* (mm)**	***Enterobacteria* (mm)**
			At 35°C		
A_1	12	10	-	-	11
Ct_1	11	11	-	-	11
O_1	11	11	-	-	11
O_2	12	13	-	12	11
T_1	12	11	12	12	12
T_2	12	12	12	12	11
			At 50°C		
A_1	11	-	-	-	10
Ct_1	10	-	-	-	9
O_1	12	10	-	-	11
O_2	11	-	-	11	-
T_1	10	10	10	10	10
T_2	11	11	11	11	12
			At 70°C		
A_1	9	-	-	-	11
Ct_1	-	-	-	-	-
O_1	-	-	-	-	-
O_2	10	-	-	-	-
T_1	-	-	10	9	-
T_2	10	9	7	7	10

Table 4: Lactobacilli strains' bacteriocin activity at diversepH

Test Strains	Zone of inhibition				
	***E.coli* (9473) (mm)**	***Salmonella spp.* (mm)**	***Klebsiella*spp. (mm)**	***MRSA* (mm)**	***Enterobacteria* (mm)**
			pH2		
A_1	-	-	-	-	-
Ct_1	10	-	12	11	10
O_1	11	11	10	12	-
O_2	12	9	-	-	9
T_1	-	-	-	-	10
T_2	11	10	9	10	-
			pH 7		
A_1	12	10	-	-	11
Ct_1	11	11	-	-	11
O_1	11	11	-	-	11
O_2	11	13	-	12	11
T_1	12	11	12	12	12
T_2	12	12	12	12	11
			pH 9		
A_1	9	10	9	11	-
Ct_1	10	9	-	-	10
O_1	9	-	-	-	-
O_2	11		11	-	-
T_1	8	11	11	10	12
T_2	9	10	9	11	10

Characterizing bacteriocin by proteolysis

O_1 and O_2 strains showed bacteriocin activity in the presence of papain enzyme abutting every pathogen included in this study. Results were recorded in table 5.

Antibiotic resistance test

Resistance of strains was scrutinized besides various antibiotics

such as ampicillin, ciprofloxacin, streptomycin and tetracycline. Effects of antibiotics were noticed after 24 hours of incubation. Strain A_1 was found to be resistant to ampicillin and streptomycin; Ct_1 was resistant streptomycin; O_1 was resistant to ciprofloxacin and all the findings were summarized in Table 6.

Efficacy of bacteriocin in preservation of food

Each strain exhibited bacteriocin was compared with the control plate after incubation and was noticed that A_1 showed better prevention from spoilage as compared to Ct_1, T_1, T_2 and O_1 whereas O_2 showed the best protection against food rotting (Fig. 1).

Table 5: Proteolytic activity of Lactobacilli strains' bacteriocin

Test Strains	Zone of inhibition			
	***E.coli* (9473) (mm)**	***Salmonellaspp* (mm)**	***Klebsiella spp* (mm)**	***MRSA* (mm)**
A1	-	-	9	-
Ct1	11	13	12	-
O1	10	12	9	10
O2	12	11	9	9
T1	-	-	9	11
T2	-	10	10	-

Table 6: *Lactobacilli* strains with antibiotic resistance

Strains	Ampicillin	Ciprofloxacin	Streptomycin	Tetracycline
A_1	R	S	R	S
Ct_1	S	S	R	I
O_1	S	R	S	R
O_2	R	I	S	S
T_1	S	S	S	R
T_2	S	R	R	R

S= sensitive, R= resistant, I= intermediate

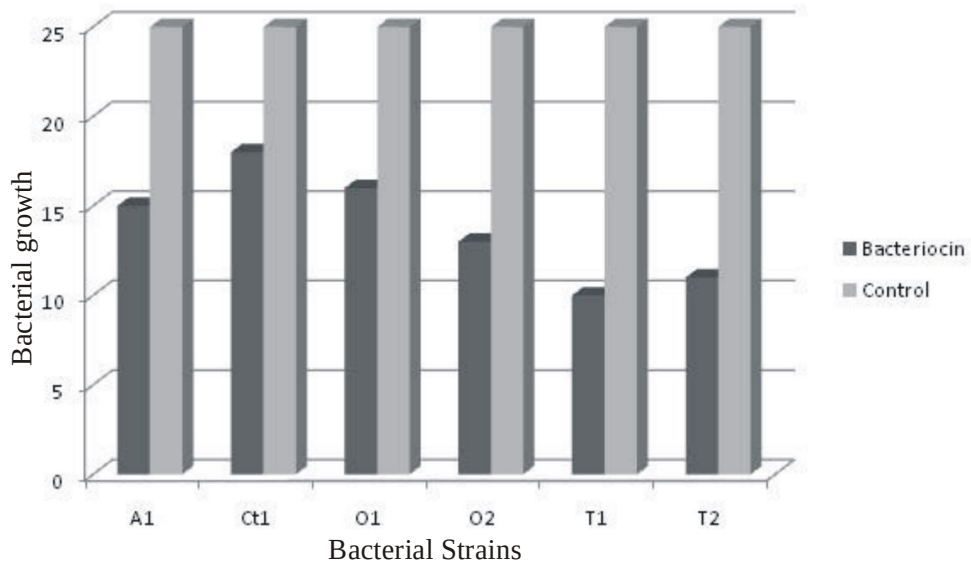

Fig. 1. Variation in bacterial growth amid with and without bacteriocin

DISCUSSION

Fruits and vegetables were selected because a large number of people in Multan love to take fruits and vegetables in their diet. Scrutinizing the probable activities of fruits and vegetables is the second purpose of the study. MRS media is differential medium for *Lactobacilli* growth used in this study. Strains that were isolated previously were normal flora of chicken and intestine and were *Bacillus* in shape and some were cocci nevertheless, *Pedicococcus, Tetragenococcus halophilus* (Udhayashree et al., 2012). In our study isolated strains were *Lactococcus* and some were *Lactobacillus* which was further tested by biochemical tests. The isolated strains were designated as *Lactobacillus* based on various morphological, biochemical and physiological attributes. In accordance with the results of our study, strains were gram positive rods. The chief motive for selection of fruits mainly apples is that it contains ample quantity of lactic acid bacteria which are beneficial in delivering immunity and probiotic potential to the host's body. Fruits and vegetables containing LAB are wide ranging but in our study carrot, grapes, tomatoes, olives, apple were used to check the presence of lactic acid bacteria and its utilization in preservation of food.

There is a wide range of fruits with varying degrees of microbial loads. Overall, concealed by agar well dispersal of two pathogenic organisms, *Klebsiella* and *MRSA* (*Methicillin-*

resistant Staphylococcus aureus) were immune to 50% of the selected isolates since *MRSA* is immune to many other drugs, but in this study, T_1, T_2, T_3,Ct_2, and O_2 were effective against *MRSA*, but *Klebsiella* was sensitive to T_2, T_3, A_2, A_3. In summary, our broad screening indicates that *Lactobacillus* species can produce anti-microbial compounds such as bacteriocin. Following a general screening, the six best strains for bacteriocin representation were chosen. Bacteriocin was characterized by testing it at various temperatures, pH levels, and with a protein degrading enzyme. Bacteriocin organization was nearly identical at 50°C to that of bacteriocin activity at 35°C, however bacteriocin was unproductive at 70°C because it is a protein, which degrades at very high temperatures. After the formation of bacteriocin was disrupted, its efficacy was also changed at the same level at pH 2 (acidic), and its effectiveness was the same with little changes of unevenness, whereas at pH 7, it has its normal effectiveness, and at pH 9, its activity against lab pathogens (*Klebsiella*, *Salmonella*, *MRSA*, *P. aeruginosa*, *E.coli*, and others) was influenced due to disordering of peptide bonds. The findings revealed that isolated bacteriocin was a proteinaceous substance (Udhayashree et al., 2012).

For food preservation, the best-characterized bacteriocin-producing strains were chosen.

CONCLUSION

The study's major goal was to explore the antibacterial activity of *Lactobacillus* spp. in order to inhibit the growth of bacteria (*E.coli*, *Klebsiella*, *Salmonella*, *P. aeruginosa*, *MRSA*). It was concluded that extracted chemical has antibacterial activity against the bacteria that have been infected. The current study also focused at the crushing of spoilage bacteria by counting CFU (Colony forming unit) prior to and following adding bacteriocin as a preventive agent and compared it to power (without bacteriocin). The activity of the extracted compound was further observed in the presence of various anatomical and enzymatic variables. Augmented or lowered that condition may lead to decreased activity. Low temperature and around the pH reveals its activity, decreased or increased that condition may guide to reduced activity. Finally, due to the proteinaceous nature of antimicrobial compounds, proteolytic enzymes such as amylase, lipase, chymotrypsin, and trypsin were not produced. It's probable that the lactic

acid bacteria growth during fortification chocked the growth of other damaged microorganisms, however needs to be investigated further. This method of preservation was one of the most natural process to keep food away from spoiling.

CONFLICT OF INTEREST

There is no potential for a conflict of interest.

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