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Research Article

Determination of Antibacterial Activity of Bacteriocins of Lactic Acid Producing Bacteria

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ABSTRACT

This study was conducted to explore the potential of bacteriocins, produced by lactic acid producing bacteria, against *Staphylococcus aureus*, *Eneterococcus fecalis* and *Escherichia coli*. Different lactic acid producing bacteria viz. *Lactobacillus fermentum*, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were isolated from milk, milk products of dairy animals and intestinal contents of healthy broiler chicken. These isolates were identified morphologically and biochemically by conventional methods. Bacteriocins were obtained from these bacteria, by precipitation method. Antibacterial activity of bacteriocins was determined by disk diffusion method. Isolated bacteriocin showed the inhibitory activity against *Staphylococcus aureus* and *Enterococcus feacalis* but showed very poor inhibitory activity (2mm) against *Eschrechia coli*. The result of this study showed that bacteriocins are quite useful against commonly known food pathogens and thus can be used for the preservation of food and food products.

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INTRODUCTION

Fermentation is the oldest known technique in food processing technology that has been used for preserving milk, meat, and vegetables. The lactic acid bacteria as natural or controlled micro flora enabled man to extend the shelf life of many foods and food products by antimicrobial activity of their antibacterial products (Fox, 1993; Stiles, 1996). Strong antagonistic activities have been shown by lactic acid bacteria against many pathogenic and food spoiling microorganisms. Some strains have been used for food preservation because of their potential to produce bacteriocins (Brink *et al*., 1998).While lactic acid fermentation; lactic acid bacteria have potential to produce different compounds of lactose such as organic acids, diacetyl, hydrogen peroxide, and proteins (Brink *et al*., 1998; Ouwehand, 1998; Zhennai, 2000; Oyetayo *et al*., 2003).

Bacteriocins are produced by both Gram positive and negative bacteria. These are released extra-cellular by the bacterial cell ribosome in the form of low molecular weight peptides or proteins that have bactericidal or bacteriostatic effect on other closely related species (Tagg *et al*., 1976; Clevelan *et al*., 2001; Chen and Hoover 2003; Cotter *et al*., 2005). Due to the stable antimicrobial activity at high temperature and different pH of many foods, there is a great interest in bacteriocins as food bio-preservatives. Nisin and Pediocin PA-1 are classical examples of bacteriocins produced from lactic acid bacteria that have practical application as food preservative (Montville and Chen, *et al*., 1998; Galvez *et al*., 2007).

There are speculations that many commensal and lactic acid bacteria have antibiotic resistance genes similar to the other human pathogenic and food spoiling bacteria (Ammor *et al*., 2007). Due to the resistance of many bacteria to antibiotics and increase demand for less processed, safe and having less chemical additives in food have provoked the interest in replacing these products by naturally safe products such as bacteriocins (Parada *et al*., 1980; Chopra *et al*., 1998; Rao, 1998). This study, therefore, has been conducted to ascertain the antibacterial activity of bacteriocins against commonly known food pathogens, and to investigate whether the use of bacteriocins is beneficial for the preservation of food and food products.

MATERIALS AND METHODS

Isolation and Identification of Lactic Acid Bacteria

Lactic acid bacteria were isolated from 15 different samples of raw milk from household cow, yogurt collected from different commercial sites, and intestinal contents of broiler chicken from five different farms (n = 5 each) from Faisalabad, Pakistan. All samples were stored at +4 °C for further processing. Yogurt, milk and supernatant of intestinal contents were diluted (1:10) separately in phosphate buffer saline (PBS). Lactic acid bacteria were isolated using deMan'sRegosa and Sharpe (MRS) agar plates procedure as described before (De Man Rogosa *et al*., 1960; Tufail, *et al*., 2011). Cultures were purified by streak plate method and lactobacilli species were identified on the basis of morphology and biochemical characteristics by conventional methods (Kalalou *et al*., 2004; Adesokan *et al*., 2008). Three species *Lactobacillus fermentum*, *Lactobacillus rhmanosis*and *Lactobacillus acidophilus* were isolated and identified and were stored at -80 °C in MRS broth medium containing 25ml glycerol/L (De Man Rogosa *et al*., 1960).

Cell Free Culture for Isolation of Bacteriocins

The isolated lactic acid bacteria were propagated in 500 ml of MRS broth at pH 7.0, incubated at 37 °C for 48 hours. For

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extraction of bacteriocin, a cell-free solution was obtained by centrifugation (10,000 rpm) for 30 min at 4°C. The extracted bacteriocin solution was precipitated by the addition of ammonium sulphate and phosphate buffer saline (PBS) was used to inhibit the effect of organic acids. These precipitates were suspended in 50 ml of 0.1 M potassium phosphate buffer (pH 7.0). Precipitates were collected and used in disc diffusion assay as described before (Savadogo *et al*., 2004).

Determination of Antibacterial Activity of Bacteriocins

Antibacterial activity of bacteriocins was evaluated against known food spoiling bacteria such as *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* (obtained from Institute of Microbiology UAF Faisalabad).A disc diffusion assay procedure was followed (Savadogo *et al*., 2004; Tagg and McGiven, 1971) where 50 µg from each extract was placed on each disc. All the tests were performed on Muller-Hinton agar. Muller Hinton plates were inoculated by pathogenic bacteria (5×10^7 CFU/ml) and were left undisturbed for 2 h. Then the inoculated plates were incubated at 37°C for 24 h. The zones of inhibition were measured as recommended (Tagg and McGiven, 1971).

RESULTS AND DISCUSSION

The morphological characteristics of all the isolated bacteria were determined, which collectively had shown that these are common features for the lactic acid bacteria (Galvez *et al*., 2007; Krieg, 1984) (outlined in Table 1). Isolates A were identified as *Lactobacillus fermentum*, B as *Lactobacillus acidophilus* whereas C was identified as *Lactobacillus rhmanosus* on the basis of morphological and biochemical properties. In a study by Yang *et. al*., a very little amount of bacteriocins was isolated due to adsorption on to the cell surface. So an improved method was used by the use of ammonium sulphate for precipitation of protein, this method has several limitations. The results showed that the lactic acid bacteria were heterogeneous in both fermented product and in intestine. The inhibitory spectrum of bacteriocin, determined by disc diffusion assay, was mediated against Gram negative and Gram positive pathogenic bacteria. The extracts showed zones of inhibition against different known organism such as pathogenic strains of diverse bacteria (*Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus*). The varied range of inhibition zones were observed, which were measured in millimeters (inhibition diameter, mm) against indicator strains (Table 2). This inhibition indicates that these have tendency to contribute in the hygienic quality of foods and food products.

Inhibitory activity was checked by disk diffusion method and zones of inhibition were measured by Vernier's caliper. The diameters of inhibition were ranged from 2 to 11 mm; the largest diameter of 11 mm was shown by *Lactobacillus fermentum* on the indicator strain *Enterococcus faecalis* which indicates the variability and sensitivity of indicator strain against isolated bacteriocins. The smallest zone of inhibition was observed by the *Lactobacillus rhamnosus* bacteriocins on the indicator strain of *E. coli*. Most inhibited strain were *Staphylococcus aureus* and *Enterococcus faecalis* while only a single strain of *E. coli* was inhibited by the bacteriocin but at very low level. It showed that Gram positive bacteria were much more sensitive to bacteriocin of our lactic acid bacteria as compared to that of Gram negative ones.

Maximum inhibition was shown by the isolate A (11 mm) and minimum was by the isolate C (2 mm) as shown in the figure. These results are partially in accordance to the study performed by Aslam*et.al*. (2011) where they have isolated bacteriocin from *Streptococcus thermophilus* and tested against Gram positive *Staphylococcus aureus* and *Bacillus subtilus and* Gram negative bacteria like *Escherichia coli* and *Pasteurella multocida*. Their results showed poor activity against Gram negative bacteria than presented in this study.

Our results are also in compliance of the previous work performed by Savadago*et al*.(2004). However, in contrast, they have isolated eight strains of lactic acid bacteria from fermented milk to produce bacteriocins. Isolated bacteriocin showed inhibitory activity against *Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. Gram-positive indicator bacteria were most inhibited. These results indicate that the isolated strains of lactic acid bacteria are able to synthesize inhibitory substances against pathogenic bacteria. These inhibitory substances act differently on pathogenic reference indicator bacteria. A recent study conducted by Adesokan *et al*. (2008) have observed less inhibitory activity against Gram negative bacteria as compared to Gram positive bacteria, by studying the effect of bacteriocin on *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Escherichia coli*. It has been observed that the inhibiting substances produced by lactic acid bacteria are generally protein in nature (Klaenhammer, 1993; Vandenberg, 1993). However inhibitory spectrum varies from each other, which depends on the variations in the strains used.

Table 1: Morphological and Biochemical Characteristics of Isolated LAB

Parameters	Isolate A	Isolate B	Isolate C
Colony	Circular, Irregular, whitish white, 1-3 mm diameter	Small, entire margins 1-3 mm diameter	Small, Sharpe edges 2-5 mm in diameter
Gram's reaction	Positive	Positive	Positive
Gas from glucose	Positive	Negative	Negative
Catalase	Negative	Negative	Negative
Cell morphology	Rod shape non spore forming single cell	Rods, varying length, in pairs or chains Non spore forming	Short rods in chain Non spore forming

Table 2: Antibacterial activity of isolated bacteriocins (mm)

Lactic acid bacteria	Indicator strain	Zone of inhibition(mm)
Isolate A	*Enterococcus faecalis, Staphylococcus aureus, Escherichia coli*	11, 9, 5
Isolate B	*Enterococcus faecalis, Staphylococcus aureus, Escherichia coli*	9, 7, 3
Isolate C	*Enterococcus faecalis, Staphylococcus aureus, Escherichia coli*	4, 3, 2

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Gram positive pathogenic bacteria can be most sensitive to bacteriocin produced by the lactic acid bacteria, as compared to selected Gram negative bacteria which have no suppressive effect on growth. This resistance showed by Gram negative bacteria was due to the meticulous nature of their cellular envelope, as bacteriocins effect by phenomena of adsorption. This may be due to the variation of the bacterial cell wall (presence or absence of peptidoglycan) against this bacteriocin.

Lactic acid bacteria are mostly found in the fermented products of milk and vegetables.LAB occurs naturally in several raw materials such as milk, meat and flour used to produce foods (Garrity, 1984; Rodriguez *et al*., 2000). Bacteriocins are the compounds produced by the lactic acid bacteria. The ubiquitous nature of LAB can be beneficial for the natural preservation of food and food products.

Bacteriocin, the ribosomal synthesized protein is biologically active complex protein with antimicrobial action against other bacteria, principally closely related bacterial species. They are not termed as antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can elicit allergic reactions in humans and other animals. While in case of bacteriocin there is no side effect known yet. They are easily digested in digestive tract by the enzymatic action; therefore most of the LAB have safe status (GRAS) and are known as probiotics.

Different diameter of zone of inhibition revealed that the pathogenic bacteria have different rang of sensitivity to bacteriocin except the *Escherichia coli*, which showed resistance against bacteriocins, but the exact cause is not known yet. Several studies have analyzed the level of inhibition in different bacteria of varied pathogenecity; the results are comparable with the previous studies (Kalalou *et al*., 2004; Schillinger and Lucke, 1989).

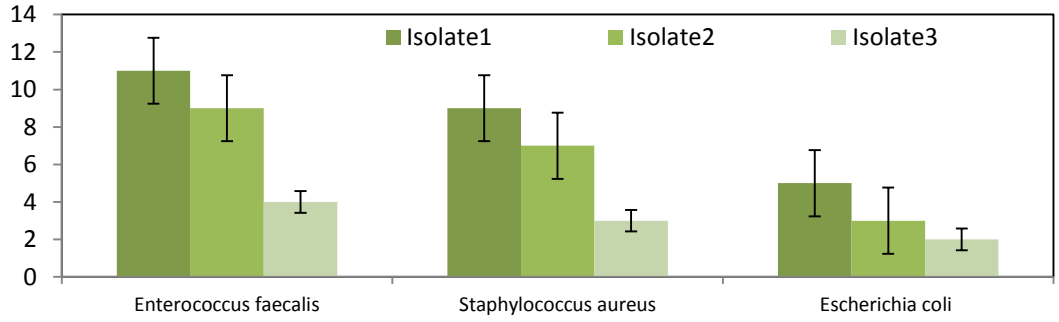

Figure: Effect of bacteriocin on the growth of three different pathogenic bacteria.

CONCLUSIONS

The Bacteriocin of LAB has the potential to be used as antimicrobial and bio-preservatives in foods and food products and have no side effect or toxicity to the consumers. Lactic acid bacteria such as *Lactobacillus fermentum, Lactobacillus acidophilus* and *Lactobacillus rhamnosus* have great potential to produce bacteriocin in anaerobic conditions. Bacteriocins, antimicrobial compounds of LAB have provided with a competitive benefit over the other microbiota. Further studies should be focused on the genetical modification and characterization of amino acids and nucleotides sequence of these antibacterial compounds. There is also need to evaluate the compatibility of these products with other food preservatives or additives that are used to enhance the food quality and texture.

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