Antimicrobial Activity and Characterization of Lactobacillus Reuteri Isolated From Human Milk

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ABSTRACT
Breast milk is the primary food for an infant. Chief ingredients of breast milk include lactobacillus strains. Lactobacillus strains isolated from breast milk were investigated for their antimicrobial activity against pathogenic microorganisms. The antibacterial activity is attributed to bacteriocin secreted by Lactobacillus. Bacteriocin present in cell free supernatants of Lactobacillus reuteri exhibited inhibitory activity against microorganisms such as Escherichia coli, Shigella dysentriae, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Proteus spp. and Vibrio cholera by agar well diffusion method. Optimization of bacteriocin production and characterization of extra cellular bacteriocin were studied. The bacteriocin produced by Lactobacillus reuteri was stable at 60°C for 10 minutes, but inactivated by heating at 80°C for 10 minutes. It was stable at pH 4.0 to 7.0 but sensitive to pH 9.0. 1% and 2% sodium chloride enhanced the production of bacteriocin from 25% to 75% in MRS broth. Molecular weight of bacteriocin purified by ammonium sulphate precipitation method was 2.5 kDa. Thus, bacteriocin of Lactobacillus reuteri strains isolated from human milk exhibited antibacterial activity against common clinical pathogens. It also exhibited properties of a good probiotic.

Key words : Bacteriocin, Antimicrobial activity, Lactobacillus reuteri, Human health

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positive therapeutic effect. Studies on this biological fluid indicate that human milk is a challenging source for potential probiotic bacteria (Gismondo, et al., 1999; Çakir 2003).

Although many researchers have thoroughly studied the antibacterial activity of lactobacillus strains of human milk, very few studies have focused on bacteriocin of lactobacillus. This study was aimed to isolate lactobacillus from human milk, identify the bacteriocin-producers, study the physiochemical properties of bacteriocin and to study the antimicrobial activities of bacteriocin against the common human pathogens including Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus spp., E.coli, Klebsiella pneumoniae, Proteus spp., Pseudomonas aeruginosa, Salmonella typhi, Shigella dysentriae, Vibrio cholera and MRSA (Methicillin Resistant Staphylococcus Aureus).

**MATERIALS AND METHODS**

Human milk samples were collected aseptically from lactating women visiting the Department of Pediatrics, SRM Medical College Hospital and Research Centre, over a period of one year from Feb. 2008 to Jun. 2009 and samples were transported in ice and stored in refrigerator until processing.

**Isolation and Identification of lactobacillus:**

Lactobacillus strains were isolated using the standard method described by Todorov and Dicks (2004). Plating was done within 3-6 hrs of arrival of the samples in the laboratory. Enumeration of lactobacilli were done by spread plating (10⁴ and 10⁵) on MRS agar (HiMEDIA) and incubated in Mc Intosh-Fildes jar (5%CO₂ at 37°C) for 48-72 hrs. Individual colonies with characteristics, typical of Lactobacilli, such as pure white, small (2-3 mm in dia.) with entire margin were picked out from each plate, subcultured, purified and transferred to MRS broth. The lactobacilli strains were identified (Michael 1981) based on growth in MRS agar (pH 7.2), cell morphology, Grams reaction, catalase activity, oxidase activity, indole and nitrate reduction tests. Further identification of the species of Lactobacilli was performed according to sugar fermentation patterns and esculin hydrolysis as described in Bergey’s Manual of Systematic Bacteriology. The glycerol stores of the typed Lactobacilli were stored at -80°C.

**Indicator bacterial strains:**

A total of 11 clinical isolates were obtained from Department of microbiology, SRM Medical College Hospital and Research Centre. Out of these twelve, four were gram positive (Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus). Streptococcus pneumoniae and Enterococcus spp) and seven were gram negative (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus spp., Salmonella typhi, Shigella dysentriae and Vibrio cholera) (Todorov and Dicks, 2004).

**Preparation of culture supernatants (Bacteriocin production):**

Well isolated, bacteriocin producing strains were transferred to 5 ml of sterile MRS broth (pH 5.5) and incubated at 35°C for 18-24 hrs. The lactobacillus culture was centrifuged at 10,000 rpm for 20 mins at 4°C. The supernatant was adjusted to pH 6.5 to 7.0 with 1N NaOH to exclude antimicrobial effect of organic acid. Un-inoculated MRS broth served as control. (Savadogo et al., 2004; Reinheimer et al., 1990).

**Determination of bacteriocin activity:**

Antimicrobial activity was confirmed by using the agar well diffusion method as described by Takahiro et al., 1991. 5mm-dia. wells were made on pathogen spread MHA agar plates and then aliquots of 50µl of cell free supernatants were added to the wells. After drying, the dishes were kept for 2 hrs in a refrigerator to facilitate diffusion of bacteriocin in agar. The inoculated plates were then incubated for 24 hrs at 37°C and the diameter of the zone of inhibition in millimeters was measured and recorded.

**Purification and Physiochemical Characterization of Bacteriocin:**

**Ammonium Sulphate Precipitation:**

The crude bacteriocin was treated with different concentration of ammonium sulphate; 10, 20, 30, 40, 50, 60, 70 and 80% (Yang et al., 1992). After 3 hrs, the suspension was centrifuged at 10,000 rpm for 30 mins. The supernatant from each concentration was dialysed (Cut off 1000) against demonized water with four changes over 3 days and tested for antimicrobial activity.

**Effect of heat, pH, NaCl on bacteriocin:**

To test heat sensitivity, aliquots of 100µl of culture supernatant were heated for 10 mins at 60°C, 70°C, 80°C, 90°C and 100°C and their antimicrobial sensitivity was tested by agar well diffusion method. The pH of culture supernatant was adjusted to 3.0, 4.0, 5.0, 6.0 and 9.0 and then kept at room temperature for 4 hrs. The antimicrobial activity of bacteriocin in each of these aliquots was

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measured by agar well diffusion method. Larsen et al. (1993); Brink et al. (1994). L. reuteri was grown under different NaCl concentration from 0.1 to 1.0% and the supernatant was tested for antibacterial activity. Todorov and Dicks (2004).

Effect of enzyme and protease inhibitor:
Culture supernatants were treated with proteinase K, pronase E and trypsin each at a final concentration of 0.1 mg/ml. The samples, with and without proteases were incubated at 30°C for 1 h and residual antibacterial activity was determined (Wanda et al., 1991).

SDS PAGE analysis:
To estimate the molecular weight of the bacteriocin SDS PAGE was carried out, according to the method of Schagger and Von Jagow (1987). Samples in a ratio of 1:2 were diluted with samples buffer (MEDOX biochemical’s) containing 2-mercaptoethanol and denatured by boiling for 2-5 min. Poly acrylamide concentrations in the stacking gel and separating gel were 9.6% and 16.0%, respectively. Electrophoresis was conducted at the constant voltage of 30V for 18 hrs. After the run, the gel was washed during 5 hrs with sterile water that was replaced every hour. Protein bands were visualized UV transilluminator according to the manufacturer’s manual. The gel was stained with Coomassie Brilliant Blue and the molecular weight of the Bacteriocin was determined in comparison with marker protein standards (low molecular weight MEDOX marker protein standards).

RESULTS AND DISCUSSION
Breast milk samples were obtained from 150 lactating women aged 19 to 36 years old. The samples were collected 6 to 32 days after delivery.

Gram positive, catalase negative, cocci, cocobacilli or rod shaped, non branching, non capsulated, non sporing isolates with characteristic cell arrangement when grown on MRS growth media were identified as lactic acid bacteria. 130 bacterial strains were isolated from 150 samples of breast milk, of these 85 belonged to Lactobacillus spp. (Fig. 1). 13 belonged to Lactococcus, 13 belong to Leuconostoc, 11 belong to Micrococcus and 8 belong to Streptococci spp. 85 lactobacillus were identified to the species level according to Bergey’s manual of determinative Bacteriology (Table 1). 42 of these isolates were identified as L.acidophilus, 31 as L.gasseri, 38 as L.fermentum, 34 of these isolates were identified as L.reuteri.

The antimicrobial activity of bacteriocin of Lactobacillus reuteri and degree of inhibition is shown in Fig. 2. No considerable zone of inhibition was observed

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<th>Table 2: Effect of heat, pH, NaCl and proteolytic activity on bacteriocin:</th>
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S= Sensitive, R=Resistant, P-K=Proteinase K, P-E=Pronase E, Trp=Trypsin.

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against Streptococcus pneumoniae.

Culture supernatant from Lactobacillus reuteri were concentrated by subjecting them to four different concentrations (0-20, 20-40, 40-60 and 60-80%) of ammonium sulphate. The fraction precipitated with 20-40% ammonium sulphate showed the highest bacteriocin activity. The activity measured as zone on inhibition of growth around the agar well containing bacteriocin increased from 11mm to 26 (2.36 fold increase compared to crude bacteriocin). Fig. 3.

Bacteriocins are antimicrobial agents produced by bacteria which are active against closely related bacteria (Klaenhammer, 1993). They are active against many other bacteria including pathogens (Flythe and Russell, 2004). Hence they may be used as probiotic or as medicine especially in human beings.

Molecular weight of bacteriocin produced by Lactobacilli reuteri has been reported to be 2.7 kDa by Toshihide kabaki (1997). In the present study also SDS-PAGE analysis of ammonium sulphate precipitated cell free supernatant fluids showed that the Bacteriocin of L.reuteri was a peptide with low molecular weight of 2.5 kDa. Bacteriocin production was strongly dependent on pH, nutrient source and incubation temperature as claimed by Todorov and Dicks (2004). Various physiochemical factors seemed to affect bacteriocin production as well as its activity. Being resistant to low pH and temperature is one of the major selection criteria for probiotic strains (Quwehand, et al., 1999, Çakir, 2003). In our study the minimum activity was noted at pH 4, maximum activity was noted at temperature 80°C and 2% NaCl, so L.reuteri may act as a probiotic.

The bacteriocin produced by Lactobacilli reuteri were heat stable at 121°C/60 mins; Stable in acidic to neutral pH (ie) from 4 to 7, but inactivated at alkaline range. Culture of L.reuteri in the presence of Sodium chloride above 1% enhanced the production of bacteriocin from 25% to 75%. (Table 2). By SDS P AGE analysis of purified bacteriocin ,the molecular weight of the protein was found to be 2.5kDa.

The present study highlights the probiotic and antibacterial activity of bacteriocin produced by lactobacillus reuteri isolated from breast milk. It was tested against 11 different bacterial pathogens isolated from clinical specimen. The bacteria selected were Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus), Streptococcus pneumoniae and Enterococcus spp, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus spp, Salmonella typhi, Shigella dysentriae, and Vibrio cholera. (Todorov and Dicks 2004). The result indicated the present strains seemed to have antimicrobial activity against 10 in the order of Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus). Enterococcus spp, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus spp, Salmonella typhi, Shigella dysentriae, and Vibrio cholera. The study had proved the possibility of using this strains as a probiotics or medicine.
Fig. 2: The antimicrobial activity of bacteriocin of *Lactobacillus reuteri* and degree of inhibition
potential biotherapeutic agent in view of inhibition of enteric pathogens.

Modulation of micro biota by probiotic bacteria has been shown to regulate the immune function and to enhance defence against pathogens. Thus the addition of suitable breast milk probiotics to infant formula could be a new alternative to mimic some of the functional effects of human milk in children, who are not breastfed.

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