PRELIMINARY DETECTION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCES PRODUCED BY *ENTEROCOCCUS SPECIES*ISOLATED FROM DIFFERENT SOURCES

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ABSTRACT

Some lactic acid bacteria and particularly species belonging to the genus Enetrococcus are known to produce bacteriocin like inhibitory substance (BLIS). Usually they are small cationic peptide with bactericidal activity. The antimicrobial peptide produced by bacteria that deserve considerable interest for their use as natural and non-toxic food preservatives. The use of bacteriocin is among the new approaches as it has major potential in preservatives. Broad spectrum activities against prominent pathogens make it an issue of medical interest. The ability to produce such a biocompound may play role in providing an ecological advantage on non-bacteriocin producer species. 34 strains of Enterococci were isolated from different sources. These strains were identified to species: *E. faecalis* and *E. faecium*. Direct antimicrobial activity against indicator strain *S. aureus* was detected in 34 of the tested isolates. From these, only 7 displayed strong inhibitory activity against this indicator strain. The antimicrobial activity was altered after treatment with trypsin, α-chymotrypsin, papain which confirms the proteinaceous nature of the inhibition. This fact suggests that bacteriocin-like substance produced by Enterococcus strains may find application as biopreservatives in food products. Hence, the focus here is put on bacteriocin like substance screened by *Enterococcus species* isolated from different sources

Key words: Antimicrobial activity, Bacteriocin like substance, Enterococci, Enterocin.

INTRODUCTION

Antimicrobial peptides present new possibilities for combacting infectious diseases. They inhibit the growth of pathogenic microorganisms, without affecting the host or the animals and plants that produce them, have a broad spectrum of antimicrobial activity. It is well known that bacteria, induced by stress, produce bacteriocins that may cure infectious diseases (Bianchi *et al*., 1999). There is increasing concern about the resistance of microorganisms to various drugs and the perspective of continuous use of antibiotics is not yet well defined. Therefore, many measures to solve this problem need to be adopted e.g., the controlled use of antibiotics, expansion of research for the better understanding of resistance mechanisms, and continuing attempts to develop new synthetic and natural drugs (Coutinho *et al*., 2004). Peptides, we believe, constitute a novel potential therapeutic agent against diseases caused by pathogenic organisms. Bacteriocins are antibacterial peptides or proteins with spectra of inhibition usually confined to strains closely related to the producing strain. However, a number of bacteriocins from Gram positive bacteria have fairly broad inhibitory spectra (Jack *et al.*, 1995) and bacteriocins from lactic acid bacteria (LAB) are attractive as antimicrobial agents.

Antagonistic properties of lactic acid bacteria (LAB) allied to their safe history of use in traditional food fermented products make them very attractive to be used as bio preservatives (Parada, 1984; Caplice and Fitzgerald, 1999). Antibiotics are at present restricted for use in foods and feeds, and bacteriocins are an interesting group of biomolecules with antimicrobial properties that may represent a good alternative (Jack *et al*., 1995). The increasing interest in these compounds has stimulated the isolation of LAB producers and the characterization of many novel peptides (Deraz *et al*., 2005).

Enterococci are among the dominant lactic acid bacteria (LAB) in the intestinal microbiota of mammals and other animals (Nes *et al*., 2007). They are Gram-positive, nonspore forming, catalase-negative, oxidase-negative, and facultative anaerobic bacteria that occur singly, in pairs Orin chains (Moreno *et al*., 2006; Ogier and Serror, 2008).

Many of them are generally recognized as safe non pathogenic probiotic strain and synthesize bacteriocins. These peptides are often cationic and amphiphilic or hydrophobic, and many of them kill bacteria by permeabilising the target cell membrane (Venema *et al.*, 1995). In recent years bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatment of human and animal diseases. A consequence, multiple resistance strains appeared and spread causing difficulties and the restricted use of antibiotics as growth promoters. So, the continued development of new classes of antimicrobial agents has become of increasing importance for medicine. In order to control their abusive use in food and feed products, one plausible alternative is the application of some bacterial peptides as alternative substances in place of antibiotics of human application. Amongst those, enterocins produced by Enterococci have attracted increasing attention, since they are active in a nano molar range and have no toxicity.

MATERIALS AND METHODS

Bacterial Strain and Media: Thirty four strains of enterococci were isolated from various sources. Samples viz. urine, curd, milk were collected. Collected samples were inoculated in MRS broth for enrichment at 37^{o}C for 24 hours. Enriched culture was surface plated on Enterococcal selective medium (Bile Esculin Agar and Enterococcus Confirmatory Agar). Plates were incubated at 30 ^{o} C for 24 hours.

Extraction of Enterocins:

Isolated enterococcal strains were propagated in MRS broth, for extraction of enterocin, a cell free solution was obtained by centrifuging (10,000 rpm for 20 min, at 4^{o}C) the culture and was adjusted to pH 7.0 by means of 1M NaOH to exclude antimicrobial effect to organic acid.

Determination of Enterocin Activity:

The antimicrobial activity of enterocin was tested by agar well-diffusion assay against *S. aureus* as target strain. The antimicrobial activity was scored positive in presence of a detectable clearing zone around the well. The supernatant fluids of bacteria with antimicrobial activity were studied to determine the nature of inhibitor. In order to rule out acid inhibition and the hydrogen peroxide, the CFS was adjusted to pH6.8 with 1N NaOH and by the addition of catalasein another sample. The sensitivity to proteolytic enzymes of neutralized CFS was realized by the addition of trypsin, α-chemotrypsin, and papain at final concentration1mg.ml. In all the cases, a positive control sample was tested in parallel. The inhibitory activity of every sample was determined following the agar well-diffusion assay.

RESULTS AND DISCUSSION

Present investigation indicated that 34 strains of enterococci were isolated from various sources as depicted in (Table 1). The most frequent sources of enterococci were found to be urine (41.17%) followed by milk (35.29 %) and cheese (23.52%). Prevalence of enterococci in clinical specimens can thus be attributed to their ability to grow and survive due to selective pressure of antimicrobial agents. Enterococci isolated from various clinical specimens in this study do not reflect the true incidence of infection caused by these organisms, but definitely suggest the increased frequency of their isolation from various clinical materials.

The prevalence of *E. faecium* and *E. durans* in milk raw milk and raw milk cheese is common (Freitas *et al*., 1999; Rodríguez *et al*., 1995). The species of enterococci are found in dairy products but *E. faecalis* and *E. faecium* remain the prevailing ones. These strains are capable of producing a variety of enterocins with inhibitory activities against *L. monocytogenes*, *S. aureus* and *Clostridium* spp. (Floriano *et al.,* 1998; Franz *et al.,* 1999; Gelsomino *et al*., 2001).

Table 1: Source of Sample

Source of Sample	No. of isolates n=34
Milk	12
Curd	08
Urine	14

Table2: Identification of Enteroccocus Isolates Producing Antimicrobial Compound

Characteristic	Isolates	
	E. faecalis	*E. faecium*
Cell morphology	Cocci	Cocci
Gram reaction	Gram positive	Gram positive
Catalase	Negative	Negative
Haemolysin production	Negative	Negative
CO_2 Production from glucose	Positive	Positive
Hydrolysis of Esculin	Positive	Positive
Growth at 10°C 40° C 45°C pH 9.6	 Positive Positive Positive Positive	 Positive Positive Positive Positive
Growth in presence of NaCl 4% 6.5%	 Positive Positive	 Positive Positive
Growth on Enterococcus Confirmatory Agar Bile Esculin Agar	 Yellow colony Black colony	 Yellow colony Black colony
Utilization of Carbohydrate L-arabinose Ribose Sucrose Mannitol D-raffinose Lactose Sorbitol	 Negative Positive Positive Positive Positive Positive Positive	 Positive Positive Positive Positive Negative Positive Negative

Table 3: Frequency Distribution of Enterococci in Different Sources

Identified strains	No. of strains	Urine	Milk	Cheese
E. faecalis	21	09	08	02
E. faecium	13	05	04	06
Total	34	14	12	08

Table4: Frequency of Enterocin Production Screened By Agar Well Diffusion Method.

Identified strains	No. of producer strains/ No. of tested strains	Frequency Percentage (%)
E. faecalis	13/21	61.90
E. faecium	7/13	53.84
Total	20/34	58.82

Table 5: Efficient Enterocin Producing Enterococcal Strains Selected For Assessment of Antimicrobial Activity of Enterocin.

Specimen	Isolate identified as	Isolate No.	Inhibitory substance
U	*E.faecalis*	EU4	Enterocin EU4
U	*E. faecium*	EU2	Enterocin EU2
C	*E.faecalis*	EC 7	Enterocin EC 7
M	*E. faecium*	EM 5	Enterocin EM 5
M	*E.faecalis*	EM5	Enterocin EM5
C	*E.faecalis*	EC12	Enterocin EC12
U	*E.faecalis*	EU 10	Enterocin EU 10

Key: U= Urine, M=Milk, C=Cheese

Table 6: Effect of Enzymes on Enterocin Activity

Enzymes	Residual Activity of enterocin
Trypsin	+/-
α-chemotrypsin	-
Papain	+
catalase	+
lipase	+
protease	+

(+) activity retained, (-/+) activity lost partially, (-) activity lost.

Phenotypic and biochemical identification of isolates was carried out according to the characteristics shown in Table 2. All isolates were cocci, Gram positive, catalase negative and were found to be negative for haemolysin. *Enterococcus* isolates were identified on the basis of their growth on Enterococcus Confirmatory Agar showing typical yellow colonies and by forming black colonies on Bile Esculin Agar containing 40% bile.

Sugar fermentation patterns are considered to be reliable methods of distinguishing *Enterococcus* sps. (Mundt, 1986; Klein, 2003). From the sugar fermentation profiles and arginine catabolism (Table 2), the isolates were identified; *Enterococcus faecalis* raffinose (+) arabinose (-), mannitol (+), sorbitol(+) and arginine (+), *Enterococcus faecium* raffinose (-), arabinose(+), mannitol (+) , sorbitol (-) and arginine (+).

Table 3 displays the distribution and species identification of entercoccal isolates according to the source. The majority of the isolates were *E. faecalis* 61.76% while *E. faecium* accounted for 38.23%. *E. faecalis* accounted for greater percentage of isolates from urine followed by milk and cheese. After screening of 34 Enterococcal strains against indicator strain, 20 strains (58.82%) exhibited antimicrobial activity. Inhibitory spectra of those isolates and frequency of enterocin production are presented in (Table 4). The strains isolated from urine revealed a strong inhibitory activity towards indicator bacteria followed by milk. The antimicrobial activity of these isolates have been evaluated by measuring the diameter, seven selected strains, exhibiting strong antagonistic activities, were characterized for their antimicrobial compounds (Table 5). NaOH and /or catalase were added to avoid acid and hydrogen peroxide inhibitions. All isolates exhibited inhibition zones.

The effects of various enzymes on the inhibitory agent were investigated (Table 6). The antimicrobial compounds from all strains were completely inactivated by treatment with α-chymotrypsin. Trypsin partially affected the activity of cell-free supernatants of all strains. However, catalase had no effect, indicating that hydrogen peroxide did not account for the observed inhibition.

The antimicrobial activities expressed by these strains were sensitive to proteolytic enzymes, indicating that the active compounds are of proteinaceous nature, a general characteristic of bacteriocin. Such proteinaceous compounds that inhibit closely related bacteria can be included in the category of bacteriocins (Tagg *et al*., 1976; Jack *et al*., 1995). Because molecular characterization of the compounds has not yet been done, they will be referred to as bacteriocin-like substances.

The activity of the supernatants was lost after treatment with proteolytic enzymes (trypsin, α-chymotrypsin, protease or papain) which indicates that the active compounds secreted were extracellularly and proteinaceous. Furthermore, the inhibitory activity was not altered by neutralization of the supernatant. Treatment with lipase did not cause any loss of activity, probably because of the absence of lipid moiety in the compounds.

CONCLUSION

The cell-free supernatants from enterococcal strains exhibited antimicrobial activity. The potential application of enterocins as consumer friendly bio preservatives either the form of protective cultures are as additives is significant besides being less potentially toxic than current antimicrobial agents, enterococci and their by products have been shown to be more effective and flexible in several applications. Non-identified bacteriocin like substances produced by enterococcal strains shown large inhibitory spectrum. The characteristics offer useful protection against pathogenic or spoilage microorganisms. Thus, further investigations are needed to determine their molecular properties as novel bacteriocins.

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