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BACTERIOCIN PRODUCTION AND PROBIOTIC PROPERTIES OF *ENTEROCOCCUS* SPP. ISOLATED FROM RAW MILK

AHMED MOUSTAFA HAMMAD
Laboratory of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt.

Email: hammad@vet.usc.edu.eg Assiut University web-site: www.aun.edu.eg

ABSTRACT

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Little is known about using *Enterococcus* strains as probiotics in the dairy industry in the Middle East. The aim of this study was to analyze the probiotic properties of *Enterococcus* spp. isolated from 75 raw milk samples. Overall, 65 milk samples (86.66%, 65/75) analyzed were contaminated with enterococci. 70 presumptive enterococcal isolates were recovered, and 60 isolates were confirmed by PCR as *Enterococcus* spp. *E. faecium* and *E. faecalis* were detected in 28 (28/60) and 12 (12/60) of isolates, respectively. Fifteen isolates showed negative results in safety relevant characteristics including antibiotic susceptibility and virulence gene profile. Seven strains showed antimicrobial activities towards *Staphylococcus aureus* and two strains showed antimicrobial activities against *Listeria monocytogenes* and 6 strains showed antimicrobial activities towards both pathogens. None of the isolates showed antimicrobial activities towards Gram negative bacteria tested in this study. Some strains showed survival in gastric juice after 2 hours and all isolates showed survival in different degree in bile after 24 hours. PCR analysis revealed that six strains carried both enterocins A and B. Neither enterocin L nor enterocin P genes were detected in isolated strains. In conclusion, the results obtained in this study showed that six *E. faecium* strains have great potential to be used as probiotic organisms in human food and/or animal feed.

Key words: *Bacteriocin, Probiotic, Enterococcus spp., Raw milk*

INTRODUCTION

The increasing number of foodborne illnesses caused by microbial contamination, and the increasing consumer conscience of the risks resulting from chemical preservatives made the discovery of natural inhibitory substances active against foodborne pathogens an important topic in the last few years. Enterococci mainly occur as non-starter lactic acid bacteria in different types of cheeses produced worldwide. Some strains have different applications in the dairy industry. As starters or adjunct cultures, these LAB play an important role in improving flavour and quality of different types of cheeses such as Cheddar, Feta, Mozzarella, and Hispanico (Malek *et al.,* 2012). The species *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus durans* have been characterized in some of these studies (Moreno *et al.,* 2006; Sarantinopoulos *et al.,* 2001). For example, *E. faecium* K77D was approved by the British "Advisory Committee on Novel Foods and Processes" (ACNFP, 1996) as starter culture for manufacturing of fermented dairy products and *E. faecium* PR88 was used as a probiotic in Cheddar cheese (Giraffa, 2003).

Numerous strains of enterococci associated with food systems, mainly *E. faecium* and *E. faecalis*, are capable of producing a variety of bacteriocin called entericin with broad spectrum activity. Bacteriocins are defined as ribosomal antimicrobial peptides that are produced by some bacteria to inhibit the growth of similar, closely related or non-related bacteria. There are several approaches have been taken to categorize bacteriocins including producing strain, common resistance mechanisms, and mechanism of killing (Zouhir *et al.,* 2010). One, which is used to classify the bacteriocins into 3 classes; Class I peptides, which undergo post-translational modification (lantibiotics). Class II peptides, which are heat-stable and largely unmodified and further sub-divided into 3 classes; namely, class IIa (*Listeria* active peptides), class IIb (2-peptide bacteriocins), and class IIc (cyclic peptides). Class III bacteriocins comprises high-molecular-weight and heat unstable bacteriocins (Yi *et al.,* 2010). Bacteriocins become one of the weapons against bacteria due to the specific characteristics of large diversity of structure and function, natural resource, and being stable to heat (Goh and Philip, 2015). There have been numerous published reports on bacteriocin producing bacteria, primarily among strains of lactic acid

bacteria (LAB) associated with food systems. Currently, the production of bacteriocin by enterococci is extensively studied due to their Generally Recognized As Safe (GRAS) status, since they prevent the growth of many food-borne pathogens and spoilage bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas* spp., *Bacillus* spp. and *Clostridium* spp. (Aran *et al.*, 2015). They can also be used in different food products in order to enhance their shelf life.

The World Health Organization (WHO) defines probiotics as, "Live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (Dobson *et al.*, 2012). The characteristics of probiotics should include: a group of strains beneficial to the host animal that can stably survive and have metabolic activity in the intestinal environment, and being non-pathogenic and non-toxic, remain stable and viable for long periods of storage and harsh conditions (Vandenplas *et al.*, 2015). However, even though enterococci are now being used as probiotics but they are also among the most common nosocomial pathogens associated with many human infections. They are known to cause endocarditis, bacteremia, and urinary tract, central nervous system, intraabdominal, and pelvic infections, and can cause multiple antibiotic resistance (Fisher and Phillip, 2009). Most of the pathogenic strains are multi-antibiotic resistant and produce adhesins, invasins, pili, and hemolysin (Rehaiem *et al.*, 2014). Therefore, the objective of this study was to characterize the properties of *Enterococcus* spp. isolated from raw milk, in order to determine their abilities to produce bacteriocins and theirprobiotic properties.

MATERIALS and METHODS

Isolation and identification of *Enterococcus* spp.

Samples (n = 75) of raw milk were collected from different supermarkets, retail and dairy shops in El-Menofia province, Egypt, over a period of 5 months (from December 2014 to April 2015). Samples were immediately placed on ice for transportation. In the laboratory, 25 ml of raw milk was mixed with 225 ml of buffered peptone water (BD Diagnostic Systems, Sparks, MD, USA) in sterile wide-mouth jars. The homogenate (0.1 ml) was transferred to kanamycin aesculinazide (KAA) agar (Merck, Darmstadt, Germany). Following 24 h incubation at 35°C, three morphologically different colonies from the KAA were inoculated onto Columbia sheep's blood agar (Merck) as described previously (Domig et al., 2003). Presumptive identification of enterococci was made based on colony morphology, catalase test, pyrrolidonyl arylamidase test (Oxoid, Basingstoke, England), and growth in brain-heart infusion broth with 6.5% NaCl.

DNA extraction and species identification

Total genomic DNA was prepared using a DNeasy Tissue kit (Qiagen,). The genus *Enterococcus* was confirmed using specific primers E1 and E2 as described by Deasy *et al.* (2000). Multiplex PCR was used to identify four common species: *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* (Kariyama *et al.*, 2000).

Detection of safety-relevant characteristic

Antibiotic susceptibility

All isolates were characterized by antimicrobial susceptibility testing for 17 antibiotics by a disc diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2011). The 17 antibiotics tested comprised aminoglycosides (kanamycin [30 µg], gentamicin [10 µg], and streptomycin [10 µg]), cephalosporins (cefotaxime [30 µg] and cefoperazone [75 µg]), a fluoroquinolone (ciprofloxacin [5 µg]), glycopeptides (teicoplanin [30 µg] and vancomycin [30 µg]), a lincosamide (clindamycin [2 µg]), macrolides (azithromycin [15 µg], clarithromycin [15 µg], and erythromycin [15 µg]), penicillins (ampicillin [10 µg], amoxicillin/clavulanic acid [20 µg/10 µg], and oxacillin [1 µg]), tetracycline (30 µg), and chloramphenicol (30 µg).

Screening of *E.faecalis* and *E. faecium* for virulence genes

All E.faecalis and *E. faecium* isolates were screened for five putative virulence determinants, including *asa1* (aggregation substance), *cylA* (cytolysin), *esp* (*Enterococcus* surfaceprotein), *gel E* (gelatinase), and *hyl* (hyaluronidase), using multiplex PCR as described previously (Vankerckhoven *et al.*, 2004).

Gastric Juice and Bile Tolerance

For tolerance to the gastric juice, 3 mg/mL pepsin was added into phosphate buffered saline adjusted to pH 2.5 (Lim and Im, 2009). To test bile tolerance, 0.3% bile salt was added to the MRS broth. Selected isolates were adjusted to OD = 0.4 at 600 nm and 1% of each isolate was inoculated into the artificial gastric juice and bile solution. The inoculated solutions were incubated at 37°C and viable bacterial cells were counted at 0 and 2 h for the gastric juice tolerance and 0 and 24 h for the bile tolerance.

Antibacterial spectrum

An antibacterial spectrum was performed, by agar well diffusion assays as previously described by García-Cano *et al.* (2014). Fifteen milliliters of 1 g/100 ml (brain heart infusion) BHI agar were poured in petri dishes and let solidify then 10 ml of agar (0.8g/ 100 ml) containing 107CFU/ml of tested bacteria were added. The microorganisms tested were obtained from our laboratory including: *S. aureus* SRM-47, *Listeria monocytogenes* LRM-

15, *Salmonella enterica* typhimurium SSRM-45, *E. coli* ERM-18, and Aeromonas hydrophila ARM-95.

PCR detection of enterocin genes

PCR amplification of enterocin genes coding for enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*), and enterocin L50 (*ent L50 A* and *ent L50B*) were carried out as previously described (DuToit *et al.*, 2000). PCR was performed on a DNA thermal cycler in a final volume of 50 ml containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each of the four dNTPs, 0.5 mM of each primer and 1.25 units of Taq DNA polymerase. The PCR conditions were 35 cycles of denaturation at 95 °C for 5 min, annealing at different annealing temperatures, and 72 °C for 30 s. The PCR products were resolved by electrophoresis in 1.5% agarose gels.

RESULTS

Characteristics of isolated strains

Overall, 65 milk samples (86.66%, 65/75) analyzed were contaminated with enterococci. The current study recovered 70 presumptive enterococcal isolates, and 60 isolates were confirmed by PCR as *Enterococcus* spp. *E. faecium* and *E. faecalis* were detected in 28 (28/60) and 12 (12/60) of isolates, respectively.

Safety evaluation

Forty five isolates showed resistance to one or more antibiotic tested and some carried virulence genes. Twenty five isolates showed negative results in safety relevant characteristics including antibiotic susceptibility and virulence gene profile. These isolates were further analyzed.

Antibacterial spectrum

As shown in Table 1, seven strains showed antibacterial activities towards S.aureus and two strains showed antibacterial activities against L.monocytogenes and 6 strains showed antibacterial activities towards both pathogens. None of the isolates showed antimicrobial activities towards Gram negative bacteria.

Incidence of enterocin genes

As shown in Fig. 1 and 2, both enterocins A and B genes were detected in sixstrains (ERM-5, ERM-9, ERM-10, ERM-12, ERM-22, and ERM-30). Neither enterocin L nor enterocin P genes were detected in isolated strains.

Gastric juice and bile tolerance

As shown in Table2, six strains (ERM-5, ERM-9, ERM-10, ERM-12, ERM-22, and ERM-30) showed survival in large number in gastric juice after 2 hours and all isolates survived in bile with different degrees after 24 hours.

Table 1: Antibacterial activity of *Enterococcus* spp. against indicator strains.

Strains	Indicator strains				
	L. monocytogenes LRM-15	*S. aureus* SRM-47	*Salmonella* typhimurium SSRM-45	*E. coli O157* ERM-18	*A. hydrophila* ARM-95
ERM-3	+	-	-	-	-
ERM-5	+	+	-	-	-
ERM-6	+	-	-	-	-
ERM-9	+	+	-	-	-
ERM-10	+	+	-	-	-
ERM-12	+	+	-	-	-
ERM-17	+	-	-	-	-
ERM-21	+	-	-	-	-
ERM-22	+	+	-	-	-
ERM-25	+	-	-	-	-
ERM-26	-	+	-	-	-
ERM-28	+	-	-	-	-
ERM-30	+	+	-	-	-
ERM-31	-	+	-	-	-
ERM-32	+	-	-	-	-

Table 2: Tolerance of selected Enterococcus strains to artificial gastric juice at 0 and 2 h.

Strains	Species	Tolerance to gastric juice (log cfu/mL)	
		Initial population (0 h)	After 2 h
ERM-3	*E. faecalis*	6.63 ± 0.06	3.77 ± 0.1
ERM-5	*E. faecium*	6.88 ± 0.19	6.58 ± 0.18
ERM-6	*E. faecalis*	6.49 ± 0.09	–
ERM-9	*E. faecium*	6.68 ± 0.25	6.88 ± 0.75
ERM-10	*E. faecium*	6.63 ± 0.06	6.83 ± 0.76
ERM-12	*E. faecium*	6.51 ± 0.00	6.91 ± 0.08
ERM-17	*Enterococcus* spp.	6.68 ± 0.31	1.77 ± 0.1
ERM-21	*E. faecium*	6.69 ± 0.03	–
ERM-22	*E. faecium*	6.63 ± 0.06	6.63 ± 0.25
ERM-25	*E. faecalis*	6.88 ± 0.19	2.59 ± 0.05
ERM-26	*Enterococcus* spp.	6.49 ± 0.09	–
ERM-28	*E. faecalis*	6.93 ± 0.06	2.59 ± 0.05
ERM-30	*E. faecium*	6.73 ± 0.08	6.69 ± 0.03
ERM-31	*Enterococcus* spp.	6.28 ± 0.11	5.69 ± 0.34
ERM-32	*Enterococcus* spp.	6.59 ± 0.08	3.45 ± 0.21

Values are means ± standard deviation.–, none detected.

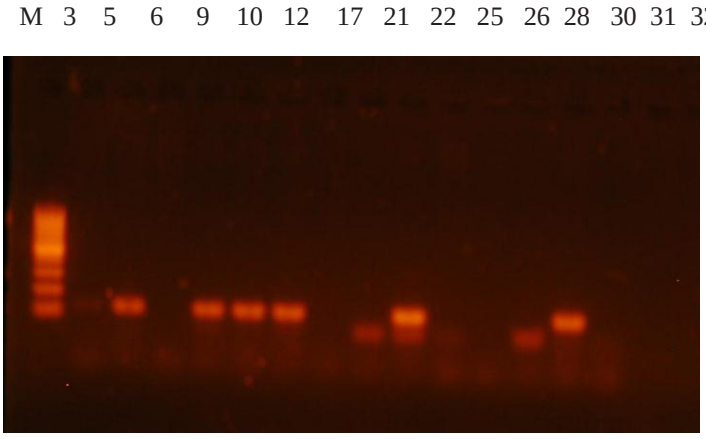

Fig. 1: Enterocin A gene screening. Electrophoresis analysis using 1.5% agarose of PCR products.
M: 100 bp ladder EnterocinA gene: 126 bp
Positive strains: 5, 9, 10, 12, 22, 30
Negative strains: 3, 6, 17, 21, 25, 26, 28,31, 32

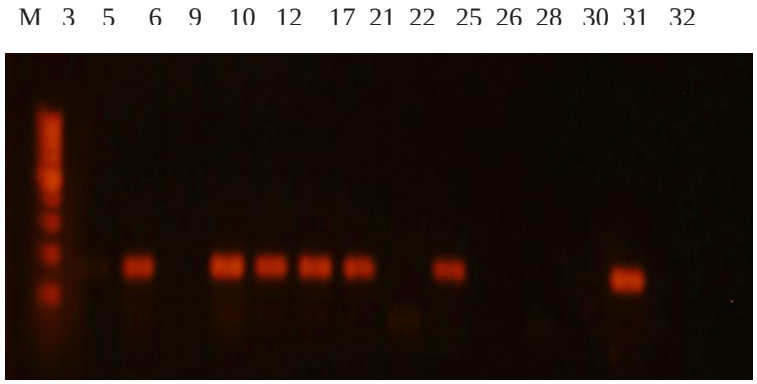

Fig. 2: Enterocin B gene screening. Electrophoresis analysis using 1.5% agarose of PCR products.
M: 100 bp ladder Enterocin B gene: 162 bp
Positive strains: 5, 9, 10, 12, 17, 22, 30
Negative strains: 3, 6, 21, 25, 26, 28, 31, 32

DISCUSSION

The risks resulting from chemical preservatives used to control foodborne pathogens has led to continued drive to find novel bacteriocins that can control food pathogens more effectively. Food strains of enterococci were found to harbour antibiotic resistance and virulence genes normally located in conjugative plasmids, increasing the risk of genetic transfer (Eaton and Gasson, 2001). The presence and expression of virulence determinants and antibiotic resistance in enterococci isolates raises concerns on their applications in foods. Therefore, the potential pathogenicity and antibiotic resistance of bacteriocin producers was evaluated in this study by investigating the presence of virulence factors and antibiotic resistance patterns. Remarkably, 25 *Enterococcus* spp. tested negative for all the virulence genes assayed and showed susceptibility to all tested antibiotics. These results confirm the safety of using these strains for manufacture of fermented dairy products.

The antibacterial activity of *Enterococcus* spp against food borne pathogens included Gram positive and negative bacteria. It has also been reported that bacteriocin-producing strains of *E. faecium* is of great potential in dairy technology (Banwo *et al.,* 2013). In this study, 15 strains have antibacterial activities. The strain's spectrum of antimicrobial activity includes Gram-positive bacteria, *L. monocytogenes* and S.aureus. Notably, *L. monocytogenes* is a widely distributed food borne pathogen with characteristics such as growth temperatures from 1 to 45°C, high tolerance to salt and low pH, which make it difficult to control in food. Additionally, *S. aureus*, is among the most common foodborne pathogens worldwide. However, little is known about the antimicrobial effect of *Enterococcus* spp. on these pathogens. In the present study, six strains showed antimicrobial activities against both pathogens (Table 1) indicating their significance potential for biocontrol of both pathogens in dairy products.

The purified DNA of isolated strains was subjected to PCR amplification to determine the existence of structural genes encoding the described enterocins (*entA*, *entB*, *entP*, *entL50A*/*entL50B*), all known to be readily spread among enterococci (Du Toit *et al.*, 2000). The PCR results (Fig. 1 and 2) indicated that enterocins A and B are highly prevalent in the isolated strains. According to the classification scheme of Franz *et al.* (2007), enterocins A is grouped in the class IIa bacteriocins (pediocin-like bacteriocins), which have a very effective anti-listerial activity, and enterocin B is grouped in the class II.3 bacteriocins. Casaus *et al.* (1997) reported that enterocin B exhibited synergistic activity with enterocin A. Fortunately, six strains isolated in this study carried both enterocins A and B genes (ERM-5, ERM-9, ERM-10, ERM-12, ERM-22, ERM-30). Interestingly, production of multiple bacteriocins is not unusual and seems to be a common feature of enterococci. It has been reported that many enterococci produce multiple bacteriocins, such as *E. faecium* WHE81 *E. faecium* KV-B5, *E. faecium* NKR-5-3A, *E. faecium* DAC2 and *E. faecium* JCM 5804T. According to several studies, the multiple enterocins-producing isolates are likely more efficient and might show a broader range of inhibition in preventing the growth of undesirable bacteria than a simple bacteriocin producer (Ishibashi *et al.,* 2012). According to Sanchez *et al.* (2007), the coproduction of enterocin A and enterocin P by *E. faecium* DAC2 produces a higher antagonistic activity than those of the controls *E. faecium* P13 (producer of enterocin P) and *E. faecium* T136 (producer of enterocin A). In this study, the co-production and variability of enterocin genes in enterococcal strains detected in our study is probably facilitated by the horizontal gene transfer among enterococcal strains and by the well-known ability of enterococci to incorporate DNA material. The acquisition of multiple bacteriocins by *E. faecium* may not only be useful to antagonize various microbial pathogenic and spoilage bacteria, but also to confer a selective advantage and enhance intra specific competition of the strain against other competitors for the microbiological niches (Rehaiem *et al.,* 2014). Moreover, in populations where the dominant bacteriocinogenic strain produces multiple bacteriocins, the development of resistant bacteria could be slowed down in comparison with the populations in which the dominant producer synthesizes a single bacteriocin (Tessema *et al.,* 2009). Of note, the isolated strains carried both enterocins A and B showed antimicrobial effect against both *S. aureus* and *L. monocytogenes* (Table 1).

When selecting a probiotic strain a number of aspects should be considered and the theoretical basis for selection should involve functional as well as desirable technological properties (FAO/WHO, 2002). Examples of desirable characteristics for a probiotic strain include the ability to survive and retain viability at conditions (acid and bile concentrations) mimicking the harsh environment of a healthy human gastrointestinal tract (Charteris *et al.,* 1998), safety criteria such as the absence of acquired antibiotic resistance genes, as well as the ability for producing antimicrobial substances (FAO/WHO, 2002; Ogier and Serror, 2008; Rehaiem *et al.,* 2014). Therefore, we investigated the stability of isolated strains for gastric juice and bile salts. Interestingly, 6 strains showed tolerance to both condition indicating their significance to be used as probiotic strains (Table 2). However, probiotic strains are likely to be buffered by food or

other carrier matrix molecules after consumption and are not likely to be exposed to the extremes of pH in the stomach (Prasad *et al.*, 1998).

In conclusion, the results obtained in this study showed that six *E. faecium* strains have great potential to be used as probiotic organisms in human food and/or animal feed. PCR analysis of these strains revealed the genes coding for two enterocins (A and B), whose concomitant production and synergistic activity could be likely more efficient in preventing the growth of undesirable bacteria than a single bacteriocin.

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انتاج البكتريوسين وخصائص المعززات الحيوية فى بكتريا الانتيروكوكاى المعزولة من اللبن الخام

أحمد مصطفى حماد

Email: hammad@vet.usc.edu.eg Assiut University web-site: www.aun.edu.eg

هناك قلة فى المعلومات عن استخدام عترات الانتيروكوكاى فى صناعة الألبان فى الشرق الأوسط. هدف هذه الدراسة هو معرفة خصائص عترات الانتيروكوكاى المعزولة من ٧٥ عينة من اللبن الخام. ٦٥ عينة كانت ملوثة بميكروب الانتيروكوكاى. تم عزل ٦٠ عترة وتم التأكد منها باستخدام (PCR) اختبار تفاعل البلمرة المتسلسل أنها انتيروكوكاى. تم عزل ٢٨و ١٢ عترة من الانتيروكوكاى فيكم وفيكاليز على التوالى. خمسة عشر عترة كانت سالبة بالنسبة للمواصفات الآمنة للمعززات الحيوية مثل الحساسية للمضادات الحيوية وخلوها من جينات الضراوة. سبع عترات أظهرت تأثير قوى على ميكروب العنقود الذهبى وعترتين أثرت على ميكروب اللستريا مونوسيتوجين و٦ عترات أثرت على الميكروبين. لم يؤثر أى من العترات على البكتريا سالبة الصبغة. بعض العترات قاومت الحموضة بعد ساعتين وكل العترات قاومت عصارة الصفراء بنسب مختلفة بعد ٢٤ ساعة. فحص هذه العترات باستخدام PCR أوضح أن ستة عترات تحتوى على البكتريوسينات A و B. فى الملخص النتائج التى تم الحصول عليها فى هذه الدراسة توضح أن ستة عترات من الانتيروكوكس فيكم يمكن استخدامها كمعززات حيوية فى غذاء الانسان والحيوان.