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Antimicrobial activity of *Enterococcus faecium* NM_2 Isolated from Urine: Purification, Characterization and Bactericidal Action of Enterocin NM_2

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ABSTRACT

Cell Free Supernatants (CFS) containing bacteriocin of *Enterococcus faecium* NM_2 (*E. faecium* NM_2) isolated from urine inhibited many gram-positive and gram-negative pathogenic bacteria. It also inhibited the *Candida albicans* M_2 fungus. The antibiotic sensitivity test of the indicator bacteria showed that these strains were resistant to 60-75% of the antibiotics used. The *E. faecium* NM_2 bacteriocin was purified by ammonium sulphate precipitation and gel filtration and a 3600 fold-increase in specific activity of bacteriocin was obtained. The purified bacteriocin showed an apparent molecular mass of Ca, 5 KDa. Amino acid analysis showed that the *E. faecium* NM_2 bacteriocin consists of 16 amino acids with high content of glycine, alanine, glutamic acid and asparagine. The *E. faecium* NM_2 bacteriocin was designated enterocin NM_2 and showed a bactericidal action on sensitive bacterial strains used. Enterocin NM_2 could be classified as a novel variant within class IIc bacteriocins.

Key words: Antimicrobial activity, *Enterococcus faecium* NM_2, bacteriocin, enterocin NM_2, purification

INTRODUCTION

Enterococcus is an important genus with Generally Regarded As Safe (GRAS) status lactic acid bacteria involved in food fermentation and preservation (Badarinath and Halami, 2011). Some species of this genus particularly *Enterococcus faecium* and *Enterococcus faecalis* are typical probiotics since they were used to suppress the carcinogenesis, reduce cholesterol level by their cholesterol oxidase activity and prevent bacteria-associated diarrhoea by their antimicrobial activities related to bacteriocins (Agerholm-Larsen *et al*., 2000; Dunne *et al*., 2001; Turgis *et al*., 2013; Enan *et al*., 2002, 2013a, 2014; Abdel-Shafi *et al*., 2014).

Bacteriocins are antimicrobial proteins produced by bacteria and active against gram positive and gram negative bacterial pathogens (Klaenhammer, 1988; Ouda *et al*., 2014). Enterocins are a wide group of bacteriocins produced by species of enterococci and showed a bactericidal activity

against bacterial pathogens (Floriano *et al*., 1998; Enan, 2000, 2006a, b, c; Enan and Al-Amri, 2006; Enan *et al*., 2012) and recently against pathogenic fungi including *Candida* species and *Aspergillus* spp. (Smaoui *et al*., 2010). The selection and identification of a bacteriocin produced by *Enterococcus* strains isolated from urine is of interest, because it can be used as probiotic bacterium to inhibit other bacterial pathogens. In this regard, *Enterococcus faecium* NM_2 isolated from urine of healthy man produced inhibitory substance which was characterized as a bacteriocin (Enan *et al*., 2014). This bacteriocin, in latter study, inhibited some bacterial pathogens of our culture collection including *Enterococcus faecalis* (*E. faecalis*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*), *Listeria monocytogenes* (*L. monocytogenes*), *Bacillus cereus* (*B. cereus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Burkholderia cepacia* (*B. cepacia*) (Enan *et al*., 2014).

The prime objectives of this study was to (1) Study and evaluate the antibacterial and antifungal activities of bacteriocin produced by *E. faecium* NM_2 against some bacterial and fungal pathogens isolated from urine of urinogenital patients which were identified in a previous study (Enan *et al*., 2014), (2) Purify *E. faecium* NM_2 bacteriocin and (3) Characterize this bacteriocin by elucidation of its molecular mass its amino acid composition and its quantitative effect on the more sensitive bacteria.

MATERIALS AND METHODS

Bacterial strains and culture media: *E. faecium* NM_2 was isolated from urine of healthy man. It was characterized and identified previously (Enan *et al*., 2014). It inhibited other lactic acid bacteria and some food-borne pathogens of our culture collection including *B. cepacia*, *S. pyogens*, *S. aureus*, *L. monocytogenes*, *B. cereus* and *P. aeruginosa* (Enan *et al*., 2014). This NM_2 strain was sucultured in brain heart infusion broth (BHI, Oxoid) and was stored at -20°C in BHI broth plus 20% glycerol (Joerger and Klaenhammer, 1986; Ismaiel *et al*., 2014; Enan *et al*., 2013a, b; Abdel-Shafi *et al*., 2013).

The indicator organisms are listed in Table 1. These strains were isolated from Taisear International Hospital of Cairo and Zagazig cities, Egypt, from urine of patients suffering from urinogenital infections. They were characterized and identified in preivous study (Enan *et al*., 2014). The *Candida albicans* M_2 strain was provided from MIRCEN Culture Collection, Faculty of Agriculture, Ain Shams University, Egypt. The bacterial strains, fungal isolate used were subcultured in BHI broth (Oxoid); Sabaroud broth, respectively. They were maintained as frozen stocks at -20°C in glass beads (Oxoid) (Joerger and Klaenhammer, 1986).

Antimicorbial activity of *E. faecium* NM_2 against sensitive organisms: The inhibitory activity of the *E. faecium* NM_2 was studied previously against some food-borne pathogens of our culture collection and was due to bacteriocin (Enan *et al*., 2013a, b; Zakaria, 2013). The inhibitory spectrum of bacteriocin produced by *E. faecium* NM_2 was studied in this study against additional organisms listed in Table 1 by the well diffusion assay (Jack *et al*., 1995; Enan, 2000). Briefly, cell free supernatants were collected after growth of *E. faecium* NM_2 (2×10^3 CFU mL^{-1}) in MRS broth (De Man *et al*., 1960) for 16 h at 30°C by centrifuging the culture (10000×g for 15 min at 4°C). The cell free supernatants were neutralized by 1 M NaOH (pH 7.0) filtered by millipore filtration (0.45 Millipor, Amicon) and this pH-adjusted filtere sterilized cell free supernatants were designated CFS and were used for further experiments. Aliquots of CFS, each containing 100 μL CFS, were inoculated into wells of agar plates seeded with indicator lawns. After incubation for 24-48 h at

30°C, zones of inhibition were recorded. In another experiment, the quantitative estimation of the antibacterial titres of both CFS and partially purified bacteriocin obtained by ammonium sulphate precipitation (PPE) were performed as described previously (Pucci *et al*., 1988; Enan *et al*., 2013a, b; Ouda *et al*., 2014). One arbitrary unit (AU mL^{-1}) of crude bacteriocin preparation was defined as 5 μL of the highest dilution of either CFS or PPE yielding a definite zone of inhibition of growth in the lawn of indicator organism. The highest dilution was multiplied by 200 μL (1 mL/5 μL) to obtain the arbitrary units per milliliter (AU mL^{-1}). The proteolytic treated PPE was assayed also as described in study on the bacteriocin employed herein (Enan *et al*., 2014).

Antibiotic sensitivity test: Antibiotic sensitivity test was carried out using the bacteriocin sensitive bacteria listed in Table 1. The antibiotics used were listed in Table 2. The disc diffusion assay was followed (Bauer *et al*., 1966). A 1% (v/v) cell suspension of each bacterial strain used was spreaded onto surface of BHI agar plates. Then antibiotic discs (Oxoid), 2 cm in diameter, were placed onto lawns of bacteria used. Plates were then incubated at 30°C for 24-48 h. Results were taken according to NCCLS (1999), Ehinmidu (2003) and Enan *et al*. (2013c).

Purification of bacteriocin and molecular weight determination: CFS from *E. faecium* NM_2, were collected as described above, were treated with solid ammonium sulphate upto 40% saturation, were stirred for 12 h at 4°C and centrifuged at 20000×g for 1 h at 4°C (Daba *et al*., 1993). The precipitates (surface pellicels and pellets) were recovered in 10 mM potassium phosphate buffer, pH 6.5 and dialysed against the same buffer for 24 h in Visking Dialysis Tubing (Alex, Pharm. Co., Egypt). This partially purified bacteriocin was sterilized by filtration through cellulose membrane filters (0.45 μm, Millipore, Amicon) and was titrated against *E. faecalis* TW_5 as it was the more sensitive organism. It was designated PPE and was used for further purification steps.

PPE was applied to a 200 mL column (4 cm interior diameter) of Sephadex G200-50 (Sigma) equilibrated with 1 M potassium phosphate buffer, pH 6.5, at room temperature. Elution was started with the same buffer and 5 mL fractions were collected and were monitored for A 280 (absorbance at 280 nm) and bacteriocin activity (AU mL^{-1}) using *E. faecalis* TW_5 as the indicator organism. The 10 mL of fraction No. 5 containing the highest bacteriocin activity were pooled from the column and were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously by (Laemmli, 1970). In another round of the experiment, the unstained SDS-PAGE was overlaid with BHI (Oxoid) soft agar containing *E. faecalis* TW_5 and the indicator organism was incubated for 24-48 h at 30°C. The inhibition obtained in lawn *E. faecalis* TW_5 was evaluated (Smaoui *et al*., 2010).

Amino acid composition: Amino acids were determined using the method described previously (Csomos and Simon-Sarkadi, 2002). The 200 μL of purified bacteriocin obtained after gel filtration was hydrolysed with 6N HCl in sealed tube, heated in an oven at 100°C for 24 h to evaporate HCl. The residue was then dissolved in diluting citrate buffer (pH 6.5). Chromatography was performed with an AAA 400 amino acid analyser (Ingos Ltd., Czech Republic) equipped with an Ostion LG ANB ion exchange column. Free amino acids were separated by stepwise elution using Na/K-citric buffer system (Ingos Ltd., Czech Republic). Post-column derivatization with minhydrin reagent and spectrophotometric measurement were used for determination of amino acids and biogenic amines.

Effect of enterocin NM_2 on the more sensitive bacteria: Effect of enterocin NM_2 produced by *E. faecium* NM_2 was studied and evaluated by employing *E. faecalis* TW_5 and *B. cepacia* TCH_4 as

indicators. This is because, both of them were the more sensitive bacteria. A concentration of about 12000 and 12000 AU mL^{-1} of PPE NM_2 were added to Erlenmeyer flasks containing 50 mL aliquots of BHI broth and inoculated with 6.8×10^8 CFU mL^{-1} of *E. faecalis* TW_5; 2.8×10^5 CFU mL^{-1} of *B. cepacia* TCH_4. The indicator cells were actively growing bacteria and were obtained by centrifugation (10000×g for 15 min). Every 6 h, 1 mL portions of BHI broth treated with the bacteriocin enterocin NM_2 and inoculated with the above sensitive bacteria, were removed and analysed for viable counts (CFU mL^{-1}) (Enan, 2006a, b; Enan and Al-Amri, 2006).

RESULTS

To confirm the proteinaceous nature of *E. faecium* NM_2 bacteriocin, PPE was treated with proteinase K, α-chemotrypsin, trypsin and was then assayed against *E. faecalis* TW_5. No bacteriocin activity was obtained from proteolytic treated PPE; indicating on proteinaceous nature of *E. faecium* NM_2 bacteriocin.

Antimicrobial activity of bacteriocin produced by *E. faecium* NM_2 in either CFS or PPE was studied against sensitive bacterial and the *C. albicans* M_2 strain by both agar well diffusion and critical dilution assays. Results are given in Table 1; *E. faecalis* TW_5, *E. faecalis* TW_{18}, *S. pyogens* TW_{12} and *B. cepacia* TCH_4 were the more sensitive organisms. They showed inhibition zones of about 20-28 mm in diameter and by titration of CFS; PPE, about 2000-2280 and 43600-48000 AU mL^{-1} were obtained, respectively. This showed that the titres of *E. faecium* NM_2 bacteriocin were more in PPE than in CFS by 20-24 times. *E. coli*, *Proteus mirabilis* bacteria showed the lowest sensitivity. They showed inhibition zones of about 11-15 mm in diameter and arbitrary units per millilitre of about 220-800 and 5280-17800 AU mL^{-1} were obtained in CFS; PPE, respectively (Table 1). It is of interest to find herein inhibition of fungal organism by bacteriocin. A novel inhibition of *Candida albicans* M_2 was observed and inhibition zone around the well was about 18 mm and by titration of CFS; PPE, an arbitrary units of about 1800 and 32400 AU mL^{-1} were obtained, respectively (Table 1). Since, *E. faecalis* TW_5 was the more sensitive organism, it was used as the indicator organism for further experiments. Minimum inhibitory activity against this organism was about 2000 AU mL^{-1} and the minimum bactericidal activity was 2400 AU mL^{-1}.

Table 1: Antimicrobial activity of *E. faecium* NM_2 against some sensitive clinical microbes as determined by the agar well diffusion and critical dilution assays

		Antimicrobial titers permilliter (AU mL^{-1})	
Sensitive microbe and its designation	Diameter of inhibition zone (mm) as obtained by CFS	CFS	PEE
Escherichia coli TCH_2	23	480	12000
Escherichia coli TCH_3	14	220	5280
Escherichia coli TM_7	10	1600	35200
Escherichia coli TM_9	13	1400	48000
Burkholderia cepacia TCH_4	20	2200	44000
Enterococcus faecalis TW_5	28	2000	48000
Enterococcus faecalis TW_{18}	26	2000	44000
Streptococcus pyogenes TW_{12}	24	2180	43600
Proteus mirabilis TW_{17}	11	800	17800
Proteus mirabilis TW_{21}	15	400	9600
Candida albicans M_2	21	1800	32400

TCH: Isolates obtained from children, TM: Isolates obtained form men, TW: Isolates obtained form women, T: Taisear international hospital in Egypt, M: Micron culture collection, Ain Shams University, Egypt

It is of interest to inhibit antibiotic resistant bacteria causing urinogenital infections by either probiotic bacteria or their antimicrobial agents like bacteriocins. Therefore, antibiotic sensitivity test was carried out using the bacteriocin sensitive bacteria appeared herein. Twenty types of antibiotics were choosen as they cover different modes of action against gram-positive and gram-negative bacteria (Table 2). The antibiotic sensitivity profiles showed variability in sensitivity of bacteria used (Table 2). There was no organism either completely sensitive or completely resistant to antibiotics used. The indicator bacteria used were resistant to 12-15 antibiotics used but were sensitive to 5-8 antibiotics used. *Enterococcus faecalis* TW_5 and *E. faecalis* TW_{18} were vancomycin resistant bacteria. Such results attracted to do further study on bacteriocin produced by *E. faecium* NM_2. This is to purify and characterize such bacteriocin to use this bacteriocin as prebiotic or food additive and to use its producer strain as probiotic bacterium or protective culture in food industry with inhibition of the antibiotic resistant bacterial pathogens.

The purification scheme of *E. faecium* NM_2 bacteriocin is shown in Table 3. The bacteriocin activity was increased from 2400 AU mL^{-1} in CFS to 48000 AU mL^{-1} in PPE indicating in 160 fold increase in its specific activity. Application of ion exchange chromatography of PPE on sephadex G 200-50 column, resulted in a large peak of bacteriocin acivity reaching 432000 AU mL^{-1} and indicating in 3600 fold increase in specific activity. This was also corresponding to the largest absorbance peak (No. 5) in the elution profile (Fig. 1a, b). SDS-PAGE analysis showed an electrophoretically pure protein with an apparent molecular size of ca. 5 KDa (Fig. 2). To ensure that the purified protein band was bacteriocin, antibiogram was carried out (Fig. 2). No growth in lawn of *E. faecalis* TW_5 indicator organism was observed and a wide clear area was obtained.

Table 2: Antibiotic sensitivity of clinical bacteria isolated from urine of urinogenital tract patients

	Bacterial strains used									
Antibiotic used	TCH_2	TCH_3	TCH_4	TM_7	TM_9	TW_5	TW_{12}	TW_{17}	TW_{18}	TW_{21}
Ciprofloxacin	-	-	-	-	-	+	-	+	+	+
Amikacin	+	+	+	+	+	-	-	+	+	+
Cefoperazone	-	-	+	-	-	-	-	+	+	-
Nitrofurantoin	+	+	+	+	-	-	-	+	+	-
Azithromycin	+	-	-	+	+	-	-	-	-	-
Augmentin	-	-	-	+	-	-	+	-	-	+
Ampicillin	-	-	-	+	-	+	-	-	-	-
Ceftazidime	-	-	-	-	-	-	-	-	-	-
Cefotaxime	+	+	+	-	+	-	-	-	-	-
Cefuroxime	-	-	+	-	+	-	-	-	-	-
Tetracycline	-	-	-	-	-	-	-	-	-	-
Cefamandole	-	-	-	-	-	-	+	-	+	-
Cefaclor	-	-	-	-	-	-	-	+	-	-
Erythromycin	-	-	-	-	-	+	+	-	+	-
Cefopime	-	-	-	-	-	-	-	+	+	-
Gentamycine	-	+	+	-	+	-	-	-	-	-
Norfloxacine	-	-	-	-	-	+	-	+	+	+
Vancomycin	+	-	-	+	-	-	-	-	-	-
Meropenem	-	+	-	-	+	+	-	+	-	-
Sulbactam+amicillin	+	-	+	-	-	+	-	-	-	+

+: Sensitive, -: Resistance

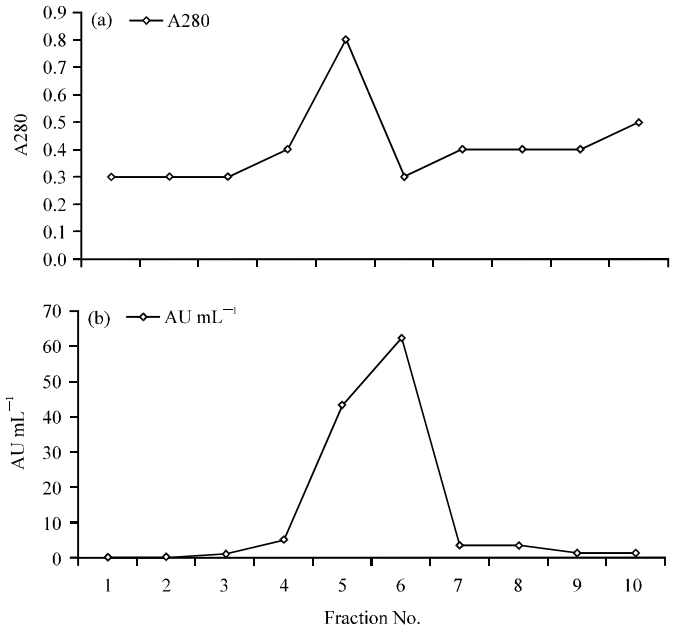

Fig. 1(a-b): Elution profile of PPE on sephadex G200-50, (a) Absorbance at 280 nm and (b) Enterocin NM2 titre (AU mL^{-1})

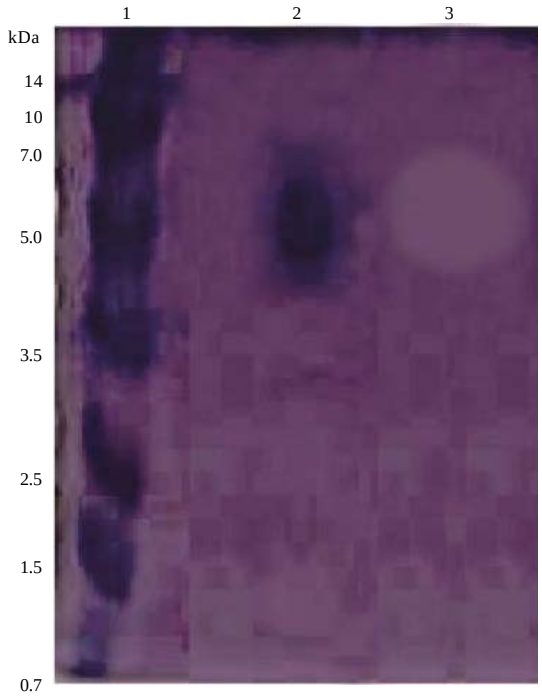

Fig. 2: SDS-PAGE of purified fraction of enterocin NM2 throughout ion exchange chromatography using sephadex G200-50, Lane 1: Molecular weight standard protein, Lane 2: Purified enterocin NM2, Lane 3: Antibiogram of the pure protein band of enterocin NM_2 showing inhibition in lawn of *E. faecalis* NM_2

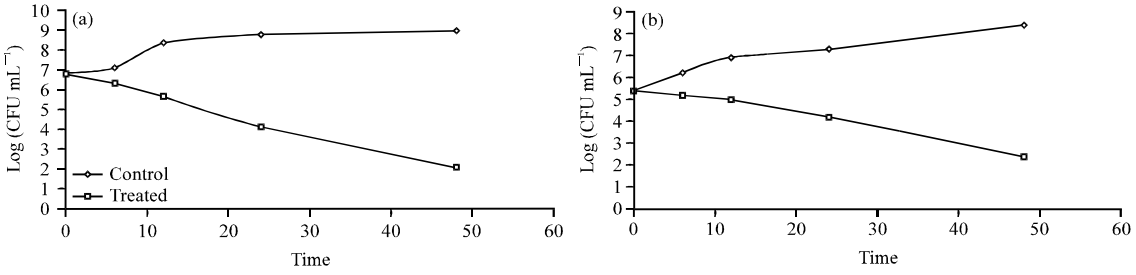

Fig. 3(a-b): Growth of *E. faecalis* TW5 (a) and *B. cepacia* TCH4 (b) in brain heart infusion broth with or without partially purified enterocin NM2, control without enterocin NM2; in the presence of enterocin NM2

Table 3: Purifcation scheme of enterocin NM_2 isolated from urine

Sample	Enterocin NM_2 activity (AU mL^{-1})	Total protein (mg mL^{-1})	Specific enterocin NM_2 activity (Au mg^{-1} protein)	Purification fold (increase in specific activity)
Cell Free Supernatant (CFS)	2400	4.80	500	4
Ammonium sulphate precipitate	4800	0.60	80000	160
Eluted fraction number 5 through sephedex G200-50	432000	0.24	1800000	3600

Table 4: Amino acid composition of enterocin NM2 produced by *E. faecium* NM_2

		Amount	
Amino acid	Percentage	(mg mL^{-1})	(g/100 mL)
Asparagine	2.05	5.5	0.55
Threonine	0.29	0.7	0.07
Serine	0.95	2.5	0.25
Glutamic acid	3.48	9.3	0.93
Proline	0.29	0.8	0.08
Glycine	6.36	17.1	1.71
Alanine	4.31	11.6	1.16
Valine	1.32	3.5	0.35
Methionine	0.82	2.2	0.22
Isoleucine	0.56	1.5	0.15
Leucine	1.75	4.7	0.47
Tyrosine	0.39	1.0	0.10
Phenyl alanine	0.59	1.5	0.15
Histidine	0.44	1.1	0.11
Lysine	1.36	3.6	0.36
Argnine	0.61	1.6	0.16

The amino acid composition of the purified bacteriocin of *E. faecium* NM_2 which was pooled from ion exchange chromatography is shown in Table 4. Sixteen amino acids were obtained with different values in both amino acid percentage and amino acid amount. The amino acids obtained can be arranged in the following descending order according to percentage of amino acid; glycine (5.36%)>alanine (4.31%)>glutamic acid (3.48%)>almost similar values of valine, leucine and lysine (1.32-1.75%)>almost comparable values (0.29-0.55%) of thoreonine, serine, proline, methionine,

isoleucine, tyrosine, phenyl alanine and histidine. Therefore, the antimicrobial compound produced by *E. faecium* NM_2 was proved to consists of antimicrobial protein (bacteriocin) and designated enterocin NM_2.

The quantitative effect of partially purified enterocin NM_2, obtained by ammonium sulphate precipitation against the more sensitive bacteria *B. cepacia* TCH_4 and *E. facealis* TW_5 was studied in BHI broth (Fig. 3). In control experiment, *E. faecalis* TW_5 cells increased from 6.8×10^6 CFU mL^{-1} at 0 time to 1.1×10^9 CFU mL^{-1} after 48 h. However, in sample treated with PPE, viable cell counts of *E. faecalis* TW_5 decreased by 4 log cycles after 48 h of incubation. *B. cepacia* TCH_4 cells alone increased 3 log cycles within 48 h but their viable count in sample treated with enterocin NM_2 decreased 3 log cycles. No regrowth was observed by further incubation, indicating on bactericidal effect of enterocin NM_2.

DISCUSSION

Enterococci normally colonies the intestinal tract of humans and animals, although they are known to be opportunistic pathogens responsible for a wide variety of infections such as endocardities, urinary and genital tract infections, meningities and septicemia (Murray, 1990). Therefore, the inhibition of such bacteria by natural probiotic is of interest and needs further research. In this regard, in previous study on this topic (Enan *et al*., 2014), *E. faecium* NM_2 isolated from urine of healthy man inhibited other lactic acid bacteria and many food-borne pathogens of our culture collection (Hadji-Sfaxi *et al*., 2011; Enan, 2006d; Enan *et al*., 2014). The inhibitory substance was heat resistant and was proved to be protein and characterized as a bacteriocin (Enan *et al*., 2014; Zakaria, 2013). Like all bacteriocins produced by bacteria, PPE appeared herein lost its activity after its treatment with proteases; indicating on the proteinaceous nature of *E. faecium* NM_2 bacteriocin (Klaenhammer, 1993).

In this study, further study was done on *E. faecium* NM_2 bacteriocin. Either CFS or PPE of *E. faecium* NM_2 inhibited other pathogenic gram positive and gram negative bacteria including *E. faecalis* TW_5, *S. pyogens* TW_{12}, *Proteus mirabiois* TW_{17}, *E. coli* and *B. cepacia* TCH_4. Also, the *C. albicans* M_2 fungus was inhibited by *E. faecium* NM_2 bacteriocin. Variable *E. faecium* NM_2 bacteriocin activities were obtained against four strains of *E. coli* used as indicators. This supported previous results on bacteriocin activity against sensitive bacterial species within the same genus (Kang and Lee, 2005). Different spectra of inhibitory action may be obtained depending on the bacteriocin producing strain, the indicator strain and also the method used for bacteriocin detection (Drider *et al*., 2006). The accepted mode of bacteriocin action on both gram-positive and gram-negative bacteria is the adsorption of bacteriocin on cell surface, inducing pore formation. This is resulted in leakage of cell electrolytes which is ended by cell death (Klaenhammer, 1988; Enan *et al*., 1996; Alvarez-Cisneros *et al*., 2011).

To our knowledge, this is the first time to inhibit strains of *B. cepacia, Candida albicans* and gram negative pathogens by bacteriocin produced by *E. faecium* isolated from urine of healthy people. This is very promising result since the bacteriocin producer strain *E. faecium* NM_2 could be used as a probiotic culture to stimulate the immune system of the host by inhibition of other pathogenic bacteria. Most known bacteriocins of enterococci are active against gram positive bacteria (Badarinath and Halami, 2011) but there are some studies reported activity of enterocins of enterococci against gram negative bacteria (Alvarez-Cisneros *et al*., 2011) and against fungi (Hadji-Sfaxi *et al*., 2011; Smaoui *et al*., 2010; Svetoch *et al*., 2011). The antibacterial and antifungal activities of *E. faecium* NM_2 bacteriocin could be exploited as probiotic capability of this

strain in human to control urinogenital infections caused by the indicator pathogenic bacteria used in this study. These indicator bacteria were isolated from urine of urinogenital patients and were characterized and identified previously. In addition to previous study (Enan *et al.*, 2014), the inhibitory activity of PPE was lost after their treatment by proteases. This indicated on the proteinaceous nature of *E. faecium* NM_2 bacteriocin. This is in conform with latter study (Ouda *et al.*, 2014).

There is increasing concern about the resistance of microorganisms to various drugs and many antibiotic resistant bacteria were identified in this study. These antibiotic resistant bacteria include *S. aureus*, *E. coli*, *S.pyogens*, *E. faecium*, *E. faecalis* and *Proteus* species (Valenzuela *et al.*, 2010; Unakal *et al.*, 2012; Enan *et al.*, 2013a, b). This clearly showed that there is a need to continue research to find out new therapeutic agents and to find natural probiotics to suppress such antibiotic research bacteria. Because the indicator strains, used herein, were isolated from urine of patients suffering from urinogenital infections (Enan *et al.*, 2014), their antibiotic resistance ability was studied herein. It was interesting to find variable antibiotic resistance profile. Almost *E. coli*, *B. cepacia*, *S. pyogens*, *Proteus* spp. were resistant to 70-75% of antibiotics used. Similar antibiotic resistance profiles were reported previously (Farzana and Hameed, 2006; Khan *et al.*, 2008; Arjunan *et al.*, 2010; Unakal *et al.*, 2012; Abdel-Shafi *et al.*, 2013). Also *E. faecalis* TW_5 and *E. faecalis* TW_{18} were vancomycin resistant. This is similar to previous results in this respect (Valenzuela *et al.*, 2010). This makes further interest to purify *E. faecium* NM_2 bacteriocin which was active against the antibiotic resistant bacteria which were used as indicator strains in this study.

Purification of *E. faecium* NM_2 bacteriocin was accomplished with the protocol described for other bacteriocins of lactic acid bacteria (Stoffels *et al.*, 1992; Enan *et al.*, 1996; Enan, 2000, 2006a, b). As has been reported for other bacteriocins (Aymerich *et al.*, 1996; Worobo *et al.*, 1994; Floriano *et al.*, 1998; Kumar *et al.*, 2010), a marked increase of about 3600 fold in specific activity of *E. faecium* bacteriocin was occurred. This indicated on presence of pure molecule. This was judged by appearance of clear protein band by SDS-PAGE of molecular mass of about 5 KDa. This is similar to many bacteriocins of lactic acid bacteria which consists of one polypeptide (Badarinath and Halami, 2011). Antibiogram of unstained protein band of SDS-PAGE confirmed that the pure protein band was due to bacteriocin as lawn of *E. faecalis* TW_5 the indicator strain was inhibited vigorously. This is in conform with Kang and Lee (2005).

The amino acid composition of *E. faecium* NM_2 bacteriocin suggests the appearance of 16 amino acids. Glycine, alanine, valine, glutamic acid and asparagine recorded the higher percentage and amount. It was proved that *E. faecium* NM_2 bacteriocin consists of one polypeptide and hence, this bacteriocin fits with criteria applied for bacteriocin characteristics (Tagg *et al.*, 1976; Klaenhammer, 1988) and designated enterocin NM_2.

Franz *et al.* (2007) described a special classification for enterocins according to special characteristics. Four classes of enterocins were proposed. Lanthibiotic enterocins (class I), non-lanthibiotic enterocins (class II), cyclic enterocins (class III) and a large thermolabile bacteriocins (class IV). Enterocin NM_2 is different from class I bacteriocins which contain lanthionine amino acid and differed from class III bacteriocins which contain cyclic polypeptides of bacteriolysins behaviour (Cotter *et al.*, 2005) and differed from class IV bacteriocins which are protein complexes and carbohydrate or lipid moieties (Heng and Tagg, 2006). Biological and physicochemical characteristics of enterocin NM_2 appeared herein are,therefore similar in molecular mass and antimicrobial spectrum to class II bacteriocins (non-lanthibiotic enterocins), However,

class II bacteroiocins have not been reported to inhibit fungi. Class II bacteriocins were subdivided (Franz *et al*., 2007) to class IIa which are prediocin-like bacteriocins that contain cysteine residues, class IIb which contain two polypeptides and class IIc which are peptides with thiol group and leader peptide GG (Klaenhammer, 1993). Therefore, enterocin NM_2 could be classified as a novel variant within class IIc. This is because enterocin NM_2 showed some characteristics regarding its activity against many gram-negative bacteria and fungi and contained high amount of glycine (6.3%) and 0.82% methionine (thiol group) and showed a bactericidal mode of action on the more sensitive indicators and this was reported for class IIc bacteriocins (Badarinath and Halami, 2011).

As reported by Alvarez-Cisneros *et al*. (2011), class IIc contained five fully characterized bacteriocins with known amino acid sequences viz., enterocin B produced by *E. faecium* T136, enterocin L_{50} produced by *E. faecium* L_{50}, enterocin 1071 produced by *E. faecalis* BFE 1071, enterocin RJ11 produced by *E. faecalis* RJ11 and enetrocin EJ97 produced by *E. faecalis* EJ97. Enterocin NM_2 employed herein possessed some properties didn't apply on other enterocins within class IIc bacteriocins. Thus, the enterocin NM_2 producer organism was isolated from urine and the enetrocin molecule contained only 16 amino acids and was active on the *Candida albicans* M_2 fungus (Enan *et al*., 2014). Also some biological and biochemical properties of enterocins within class IIc didn't apply on enterocin NM_2. For instance, enterocins, 1071, RJ11 and EJ97 were produced by strains belonging to *Enterococcus faecalis* (Alvarez-Cisneros *et al*., 2011). Enterocin B produced by *E. faecium* T136 was not active against gram negative bacteria (Casaus *et al*., 1999). Enterocin L_{50} produced by *E. faecium* L_{50} has a molecular mass of about 6.3 KDa and contained 54 amino acids (Cintas *et al*., 1998). Scientifically, comparison of different enterocins based upon spectra of activity with the aim to differentiate them is highly speculative, as it is strongly dependent on the variability of strains used as indicators. Such comparison could only be done using the same indicators. This is in agreement with many authors working on bacteriocins (Enan *et al*., 1996; Cintas *et al*., 2001; Ouda *et al*., 2014). Anyhow, further study will be necessary to determine the amino acid sequence of enterocin NM_2 as well as the gene encoding its production, this will be important to see wheather enterocin NM_2 is a variant of fully characterized enterocin or a novel one within class IIc bacteriocins.

CONCLUSION

Biological and physicochemical characteristics of enterocin NM_2 appeared herein are similar to class II bacteriocins regarding molecular mass and antimicrobial spectrum. However, class II bacteriocins have not been reported to inhibit fungi. Therefore, enterocin NM_2 could be classified as a novel variant within class IIc. This is because enterocin NM_2 showed characteristics didn't apply on enterocins within class IIc bacteriocin such as high glycine (6.3%) and methionine (0.82%) content; activity on gram negative bacteria and fungi. Enterocin NM_2 contained only 16 amino acids but enetrocins produced by *E. faecium* of class IIc contained more than 50 amino acids and possessed molecular mass>6 KDa. In addition, enterocin NM_2 showed a bacteriocidal action on sensitive bacteria.

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