ISSN 1996-3343

Asian Journal of Applied Sciences

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Asian Journal of Applied Sciences 7 (2): 66-78, 2014
ISSN 1996-3343 / DOI: 10.3923/ajaps.2014.66.78

Novel Antibacterial Activity of *Enterococcus faecium* NM_2 Isolated from Urine of Healthy People

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ABSTRACT

The present study was an endeavour to select a probiotic bacteria inhibitory to human pathogenic bacteria. Characterization of bacteria by methods based on phenotypic, biochemical and molecular characters were followed herein and bacteriocin activity was a scope of the present study. Out of 20 isolates of lactic acid bacteria isolated from urine of healthy people, one isolate only: the NM_2 strain inhibited other bacterial isolates isolated from patients with urinogenital infections and other bacterial pathogens of our strain collection. Based on biochemical, phenotypic characteristics and sequences of 16S rRNA gene the NM_2 strain was identified as belonging to *Enterococcus faecium* (*E. faecium* NM_2) and its two sensitive strains TW_5; TCH_4 were identified as *Enterococcus faecalis* (*E. faecalis* TW_5); *Burkholderia cepacia* (*B. cepacia* TCH_4). The inhibitory activity of cell free supernatant (CFS) of the NM_2 strain was lost by proteolytic enzymes, heat resistant and was not affected by organic solvents, lipase and amylase. Consequently the active substance in CFS was proved to be of proteinaceous nature and was, therefore, characterized as a bacteriocin. This bacteriocin was active at acidic pH levels (pH 2.0-6.5) and its activity was lost at neutral and alkaline pH values; indicating on wider application of this bacteriocin.

Key words: Lactic acid bacteria, *Enterococci*, *B. cepacia*, bacterial pathogens, bacteriocins

INTRODUCTION

Lactic acid bacteria are Gram positive, catalase negative and have commonly been used in dairy, meat, bakery and dairy fermentations and are found in the healthy gut of all humans (Campos *et al*., 2006). They possessed certain interest, due to their use as probiotic bacteria since they improve the nutritional benefits for health as they have been reported to antagonise pathogenic bacteria in human gut by their antimicrobial metabolites such as bacteriocins, diacetyl, hydrogen peroxide, acetaldehyde, ethanol, organic acids and carbon dioxide (Vaughan *et al*., 1992).

Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive Gram positive and Gram negative bacteria (Enan, 2006a, b, c; Sawa *et al*., 2009). Among lactic acid bacteria, *Enterococcus* was widely distributed and generally associated with food substrates.Enterococci are part of the normal intestinal microflora and may become opportunistic pathogens in individuals with serious diseases whose immune systems are compromised (Jamaly *et al*., 2010). Common diseases caused by enterococcal infections include endocarditis, abdominal abscesses, bacterimi and urinary tract

infections. On the other hand, *Enterococcus faecium* and *Enterococcus faecalis* are known to produce bacteriocins. Their bacteriocins are called enterocins with heat stability and antilisterial activity (Wilaipun *et al*., 2004). The selection and identification of a bacteriocin produced by *Enterococcus* strain isolated from urine is of interest, because it can be used as probiotic bacterium to inhibit other bacterial pathogens. Nothing is mentioned in literature about inhibition of many bacterial pathogens including *Burkholderia cepacia* complex by a bacteriocin produced by *Enterococcus faecium*. In an attempt to fill this gap, this study described the identification of *Enterococcus faecium* NM_2 isolated from urine; and identification of two indicator strains isolated from urine (*B. cepacia* TCH_4 and *E. faecalis* TW_5). The inhibitory activity of the *E. faecium* NM_2 strain and preliminary characterization of the active bacteriocin was studied.

MATERIALS AND METHODS

Bacterial strains and culture media: Samples of urine were withdrawn from Egyptian population at random conditions. This was carried out to show the reasons of infections in patients and the reasons of resistance of some people to infections under the same conditions. Also sub-sectional analysis was done regarding age,gender and housing (Zakaria, 2013).

***E. faecium* NM_2:** The producer of the inhibitory activity against other pathogenic bacteria, was isolated from urine of normal healthy man. It was isolated on brain heart infusion (BHI) agar (Oxoid) and was subcultured in BHI broth. This NM_2 strain was identified in this study and was stored in BHI broth plus 20% glycerol at -20°C (Pucci *et al*., 1988). *E. faecalis* TW_5 and *B. cepacia* TCH_4 were isolated from urine of patients suffering from cystitis on BHI agar and were subcutured in BHI broth. The TW_5 and TCH_4 strains were used as an indicator organisms in this study and were stored at -20°C in BHI broth plus 20% glycerol (Klaenhammer,1984; Enan *et al*., 2012). Lactic acid bacteria used in this study were maintained as frozen stocks at -20°C in BHI broth plus 20% glycerol (Pucci *et al*., 1988; Enan and Al-Amri, 2006) and were propagated in the same medium. All other indicator bacterial strains were maintained as frozen stocks at -20°C in glass beads (Oxoid) and were propagated in BHI broth (Klaenhammer,1984; Enan, 2006a, b, c).

Bioassay of inhibitory activity produced by *E. faecium* NM_2 against pathogenic bacteria: The antibacterial spectrum of *E. faecium* NM_2 against different pathogenic bacteria was studied by the agar disc diffusion assay (Enan, 2000; Bello *et al*., 2012). A 1% v/v suspension of log phae cells of the NM_2 strain was inoculated onto MRS agar (De Man *et al*., 1960). After solidification, agar plates were incubated for 48 h at 35°C; then, agar discs were made with sterile 7 mm cork porer and transferred onto the surface of the soft agar top layer inoculated with lawns of the indicator organisms. Plates were incubated for 4 h at 4°C to allow diffusion of inhibitory substance(s). The plates were then incubated at 30°C. This is because incubation at 30°C was suitable for growth of all indicator bacteria. Plates were then examined for appearance of inhibition zones after 24 h of incubation as the indicator bacterial growth were recorded.

Phenotypic and biochemical characterization: The NM_2 strain which produced inhibitory activity against other pathogenic bacteria and both TW_5 and TCH_4 strains which were inhibited by the NM_2 strain; were subjected to phenotypic and biochemical characterization as previously published by Garvier and Muriana (1993) and Enan *et al*. (2013). Biochemical characteristics of the TCH_4 strain were examined using API20 strep following the manufacturer's instructions (Biomerieux, Montalieu-Vercieu, France).

Molecular identification of the NM_2, TW_5 and TCH_4 strain: The three strains were identified using 16S rRNA cataloging analysis. Total DNA was extracted from exponentially growing cells (Sambrook and Russell, 2001). The gene encoding 16S rRNA was amplified for each isolate by polymerase chain reaction (PCR) using specific primers viz. 5'-AGAGT TTGATCCTGG CTCAG-3' as a foreward primer and 3'-TTC AGCA TTGTTCCATTGG-5' as a reverse primer (Turner *et al*., 1999; Chenbey *et al*., 2000). PCR products were electrophoresed by running via 1% agarose gels. Then PCR products were cleaned up using Gene Purification kit (Fermentas). Amplified DNA fragments were partially sequenced at GATC Biotech AG (Konstanz, Germany) using ABI373 OXI DNA Sequencer. Sequences analysis and their comparison to the deposited data sequences in Gene Bank was made using Local Alignment Search Tool (BLAST) program at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul *et al*., 1997).

Preparation of cell free supernatant (CFS): *E. faecium* NM_2 was grown in MRS broth (De Man *et al*., 1960) for 16 h at 30°C. This is because the inhibitory substance showed maximum activity at this time of temperature of incubation (Zakaria, 2013). CFS was obtained by centrifuging the culture (10000x g for 15 min at 4°C). The pH of the supernatant was adjusted to pH 6.5 with 1 M NaOH and was sterilized by filtration (Amicon, 0.45 μm, Milipore). This pH-adjusted, filter sterilized cell free supernatant was further designated CFS and was used immediately in the experiments (Jack *et al*., 1995; Enan, 2000).

Estimation of the antibacterial titre of CFS preparation: The quantitative estimation of the antibacterial titre of CFS preparation was performed as described previously (Pucci *et al*., 1988). One arbitirary unit (AU mL^{-1}) was defined as 5 μL of the highest dilution of CFS yielding a definite zone of the growth inhibition on the lawn of indicator organism. The highest dilution was multiplied by 200 (1 mL 5 μL^{-1}) to obtain the arbitrary units per mL (Au mL^{-1}).

Sensitivity of inhibitory activity to enzymes, heat, organic solvents and pH values: To test for heat sensitivity, aliquots of CFS (2400 Au mL^{-1}) were heated for 60, 70, 80, 90, 100°C). Every 5 min, 1 mL samples from heat treatments were taken and tested for residual antibacterial activity.

To test for enzyme sensitivity, samples of CFS preparation (2400 AU mL^{-1}) were treated with some filter sterilized enzymes listed in Table 4 at 1 mg mL^{-1} final concentration in 10 mM potassium phosphate buffer (pH 6.5). Controls were buffers, CFS without enzymes. Samples and controls were incubated at 37°C for 1h and tested for remaining antibacterial activity (Pucci *et al*., 1988; Enan, 2006a, b).

To test for possible effect of organic solvents, aliquots of CFS (2400 AU mL^{-1}) were mixed with 10% (v/v) of the organic solvents listed in Table 4. Controls were CFS without organic solvents and 10 mM potassium phosphate buffer (pH 6.5) mixed with organic solvents. Samples and controls were incubated overnight at 60°C to allow solvents to evaporate and tested for remaining antibacterial activity (Kumari *et al*., 2009).

To test the effect of pH values on the stability of the inhibitory activity, aliquots of CFS (2400 AU mL^{-1}) and samples of fresh MRS broth (controls) were adjusted to different pH values listed in Table 4 and incubated for 24 h at 25°C. After setting pH to 6.5 with 10 mM potassium phosphate buffer, samples and controls were tested for remaining antibacterial activity (Enan *et al*., 1996).

RESULTS

Twenty isolates of lactic acid bacteria were isolated from urine samples of healthy people among Egyptian population. They were surveyed for production of inhibitory activity against other pathogenic bacteria. Results are given in Fig. 1. Only the NM_2 strain isolated from urine inhibited

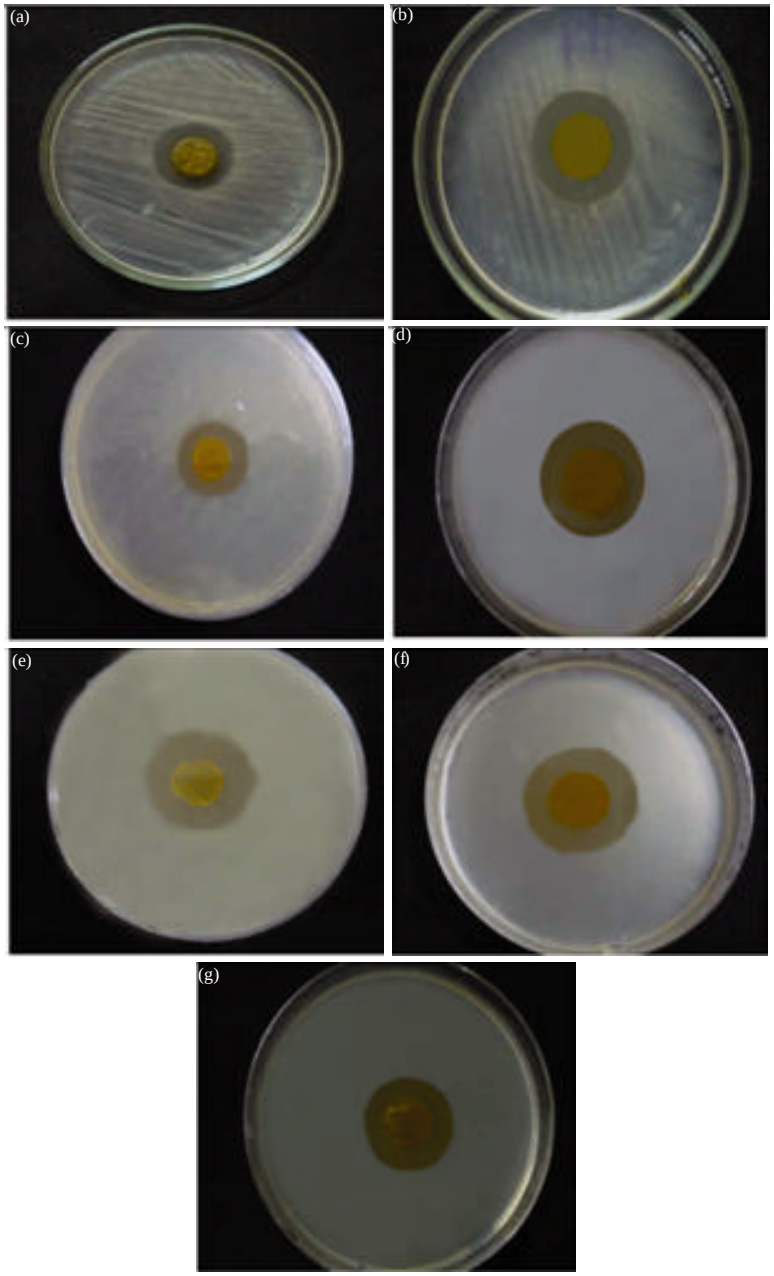

Fig. 1(a-g): Antibacterial activity of the NM_2 strain against (a) TW_5 strain, (b) TCH_4 strain, (c) *Streptococcus pyogens*, (d) *Staphylococcus aureus*, (e) *Listeria monocytogenes*, (f) *Bacillus cereus* and (g) *Pseudomonas aeruginosa* as shown by agar disc diffusion assay onto anti nutrient agar media

Table 1: Biochemical characteristics of the NM_2 and TW_5 strains isolated from urine

	Isolate No.	
Reactions and cultural features	NM_2	TW_5
Gram stain	Positive	Positive
Cell morphology	Cocci	Cocci
Ammonia from arginine	+	+
Reduction of tetrazolium	-	+
Reduction of potassium telurite	-	+
Tyrosin decarboxylase	-	+
Acid from L-arabinose	-	-
Acid from arbutin	+	-
Acid from sorbitol	-	+
Acid from melezitose	-	+
Growth in 0.1% methelene blue milk	+	+
Growth in 6.5% Nacl	+	+
Growth at pH 9.6	+	+
Growth at 10 and 45°C	+	+

other pathogenic bacteria including *Streptococcus pyogens*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* and *Pseudomonas aeruginosa*. Also this NM_2 strain inhibited both TW_5 and TCH_4 strains isolated from urine of patients suffering with cystitis.

It was necessary to identify the NM_2 strain producing obvious inhibitory spectrum against human bacterial pathogens; and the TW_5 and TCH_4 strains which were isolated from urine and were suppressed by the NM_2 strain. Both NM_2 and TW_5 strains were Gram positive, catalase negative and showed coccoid cells under light microscope (Table 1). Both strains produced, ammonia from arginine and were able to grow at 10 and 45°C and grew in 0.1% methelene blue milk and in 6.5% NaCl and grew in BHI broth adjusted at pH 9.6. The TW_5 strain showed positive results with regard to reduction of tetrazolium, reduction of potassium telurite, production of tyrosine decarboxylase and production of acids from both sorbitol and melezitose and showed negative results regarding other tests listed in Table 1. The NM_2 strain produced acid from arbutin, but showed negative results with regard to other tests listed in Table 1. Consequently NM_2 strain; TW_5 strain were identified following (Krieg and Holt, 1984) as belonging to *E. faecium* NM_2; *E. faecalis* TW_5, respectively. To identify TCH_4 strain, their morphological, cultural and biochemical characteristics were studied. Results are given in Table 2. The TCH_4 strain was Gram negative, oxidase positive and showed rod cells under microscope. Results of API 20 of Gram negative bacteria were taken following manufacturer's instructions. The TCH_4 strain produced β-galactosidase, gelatinase and fermented glucose, inositol, sorbitol and sucrose; but showed negative results regarding other tests listed in Table 2. Following diagnostic key of Krieg and Holt (1984) the TCH_4 strain was identified as belonging to *B. cepacia* TCH_4.

To complete identification of the experimental bacteria, DNA was isolated from *E. faecium* NM_2, *E. faecalis* TW_5, *B.Cepacia* TCH_4 and 16S rRNA genes of those DNA samples were amplified by PCR. The amplified PCR products were electrophoresed via agarose gel (Fig. 2). The 16S rRNA genes of the three strains were amplified successfully as sharp bands ≥1500 bp were observed (Fig. 2). The amplified DNA was extracted from agarose gel by Gene Clean Kit (Promega). The 16S

Table 2: Cultural characteristics and API20 profile of isolate No. TCH_4 as described by the manufacturer's instructions

Reaction	Result
Oxidase	+
Beta-galactosidase	+
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Utilization of citrate	-
H_2S production	-
Urea production	-
Deaminase	-
Indole production	-
Acetoin production	-
Gelatinase	+
Fermentation/oxidation of glucose	+
Fermentation/oxidation of mannitol	-
Fermentation/oxidation of inositol	-
Fermentation/oxidation of sorbitol	-
Fermentation/oxidation of rhamnose	-
Fermentation/oxidation of sucrose	-
Fermentation/oxidation of melibiose	-
Fermentation/oxidation of amygdalin	-
Fermentation/oxidation of arabinose	-

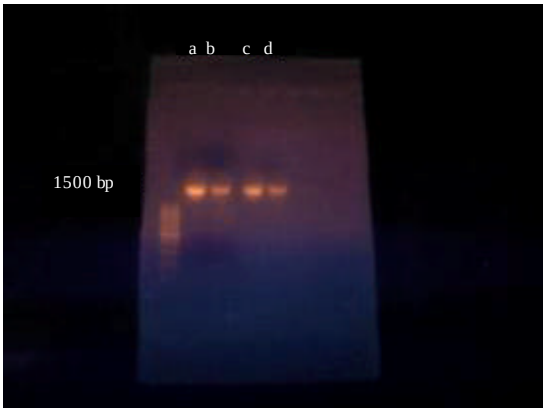

Fig. 2: Agarose gel electrophoresis of PCR products of the amplified 16S rRNA genes of (a) *E. facecium* NM_2, (b) *E. faecalis* TW_5 (C) *B. cepacia* TCH_4 and (d) Positive control

rRNA genes from *E. faecium* NM_2; *E. faecalis* TW_5; *B. Cepacia* TCH_4 were sequenced and sequence results are shown in Fig. 3, 4 and 5, respectively. Sequences were submitted to GeneBank under accession numbers IT 1606206KC660153; IT 606196KC660151; IT 606202KC660152, respectively and were compared to published sequences using Local Alignment Search Tool and showed similarities >97% to *E. faecium*; *E. faecalis*; *B. cepacia*, respectively.

The antibacterial titre of CFS preparation from *E. faecium* NM_2 against different test organisms was studied. Results are given in Table 3. The antibacterial activity showed distinctive titre(s)

CTTAGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGG
TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACGCGGCGTGCTGATCCGCGAT
TACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGAAGC
TTTAAGAGATTAGCTTAGCCTCGCGACTTCGCAACTCGTTGTACTTCCCATTGTAGCACG
TGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTT
TGTCACCGGCAGTCTTGCTAGAGTGCCCAACTGAATGATGGCAACTAACAATAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTCACTTTGCCCCCGAAGGGGAAGCTCTATCTCTAGAGTGGTCAAAGGATGTCAAGA
CCTGGTAAGGTTCTTCGTGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAAT
GCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCATCGTTTACGG
CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAG
TTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCATATATCTACGCATTTCACC
GCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCC
TCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAACCGCCTGCGCTCGCTTTACG
CCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTT
AGCCGTGGCTTTCTGGTTAGASTACCGTCAAGGGATGAACAGTTACTCTCATCCTTGTTC
TTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGG
TCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGAGTTTGGGCCGT
GTCTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTGCGGCTATGCATCGTGGCCTTGGT
GAGCCGTTACCTCACCAACTAGCTAATGCACC

Fig. 3: 16S rRNA cataloging analysis giving the sequence of 16S gene of isolate No. NM_2 and showing a similarity to *Enterococcus faecium* category of about 98.6%

CTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACA
CCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAG
GGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACC
GCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGAT
AGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTA
GTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCG
CTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCA
ACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG
GTGAGGTAACCTTTTTGGAGCCAGCCGCCTAAG

Fig. 4: 16S rRNA cataloging analysis giving the sequence of 16S gene of isolate No. TW_5 are showing a similarity to *Enterococcus faecalis* category of about 99.2%

against many Gram positive bacteria. Also *Pseudomonas aeruginosa* and *B. cepacia* TCH_4 were also inhibited and titre(s) of inhibition were lower than that obtained against Gram positive bacteria.

CTACCGTGGTGACCGTCCTCCTTGCGGTTAGACTAGCCACTTCTGGTAAAACCCACTCCC
ATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCC
GCGATTACTAGCGATTCCAGCTTCATGCACTCGAGTTGCAGAGTGCAATCCGGACTACGA
TCGGTTTTCTGGGATTAGCTCCCCCTCGCGGGTTGGCAACCCTCTGTTCCGACCATTGTA
TGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTC
CGGTTTGTCACCGGCAGTCTCCTTAGAGTGCTCTTGCGTAGCAACTAAGGACAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCA
CCTGTGTATCGGTTCTCTTTCGAGCACTCCCACCTCTCAGCAGGATTCCGACCATGTCAA
GGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGG
GTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTC
CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAG
TATTGGCCCAGGGGGCTGCCTTCGCCATCGGTATTCCTCCACATCTCTACGCATTTCACT
GCTACACGTGGAATTCTACCCCCCTCTGCCATACTCTAGCCTGCCAGTCACCAATGCAGT
TCCCAGGTTGAGCCCGGGGATTTCACATCGGTCTTAGCAAACCGCCTGCGCACGCTTTAC
GCCCAGTAATTCCGATTAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGGTGCTTATTCTTCCGGTACCGTCATCCCCCGGCTGTATTAGAACCAAGGATTTC
TTTCCGGACAAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGA
ACAGGCTTTCGCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCG
TGTCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACCAGCTACTGATCGTC

Fig. 5: 16S rRNA cataloging analysis giving the sequence of 16S gene of isolate No. TCH_4 and showing a similarity to *Burkholderia cepacia* category of about 99.8%

Table 3: Indicator strains and their sensitivity to cell free supernatants (CFS) from the Enterococcus faecium (NM_2) strain isolated from urine of normal healthy man

Indicator strain	Strain origin and No.	Activity units per millilitre (AU mL^{-1})
Enterococcus faecalis	TW_5	2400
Burkholderia cepacia	TCH_4	1450
Streptococcus pyogens	Our strain collection	1300
Staphylococcus aureus	Our strain collection	1600
Listeria monocytogenes	Our strain collection	2100
Bacillus subtilis	Our strain collection	1800
Bacillus cereus	Our strain collection	1650
Escherichia coli	Our strain collection	0
Pseudomonas aeruginosa	Our strain collection	1200
Lactobacillus plantarm	Our strain collection	0
Lactococcus lactis ATCC11454	Our strain collection	0
Leuconostoc mesenteroides	Our strain collection	0

The detection of inhibitory activity from one strain of lactic acid bacteria isolated from urine of healthy person is of interest since this strain could be used as probiotic culture to stimulate the immune system of the host and to inhibit other pathogenic bacteria. Consequently, the characterization of the inhibitory activity produced by *E. faecium* NM_2 was mandatory and of great interest. Results in this respect are given in Table 4. The inhibitory activity of *E. faecium* NM_2 did not affect by heating and lost by proteolytic enzymes and did not affect by amylase, lipase or organic solvents. This showed that one or more substances of proteinaceous nature were responsible for antibacterial activity. It also indicated on absence of carbohydrate or lipidic moieties in the substance(s) responbsible for antibacterial activity. Consequently the properties of the inhibitory

Table 4: Characteristics of the inhibitory activity of CFS from *Enterococcus faecium* NM_2 using *Enterococcus faecalis* TW_5 as indicator organism

Treatment (°C)	Residual (Au mL^{-1})
Heating for 10 min at	
100	2400
90	2400
80	2400
70	2400
60	2400
Enzyme treatment	
Pepsin	400
Trypsin	0
Alfa-chemotrypsin	0
Protinase K	0
Amylase	2400
Lipase	2400
Organic solvents treatment	
Petroleum ether	2400
Ethyl acetate	2400
Isopropanol	2400
Toluene	2400
Methanol	2400
Hexane	2400
Butanol	2400
Acetone	2400
Stability at different pH-values	
2	2400
3	2400
4	2400
5	2400
6	2400
7	2200
8	0
9	0
10	0

activity coupled with criteria applied for bacteriocin characteristies (Tagg *et al*., 1976). This bacteriocin was thermostable and was stable at acidic levels of pH, but partially degraded at pH 7.0. The bacteriocin activity of *E. faceium* NM_2 was lost at alkaline pH levels.

Further work will be necessary to purify the *E. faecium* NM_2 bacteriocin and to study its characteristics, structure and to elucidate its molecular weight and its application *in vitro* and *in vivo*.

DISCUSSION

Enterococci are part of the normal intestinal microflora (Huycke *et al*., 1998). They can survive in a variety of environments, such as soil, water, food, plants and animals (Graves and Weaver, 2010). In humans, they may become opportunistic pathogens in individuals with serious diseases whose immune systems are compromised and in patients who have been hospitalized for prolonged

periods. Consequently, numerous enterococci were isolated from stool, urine, or blood samples (Castillo-Rojas *et al*., 2013). *Enterococcus faecium* and *Enterococcus faecalis* are the most frequently found species in dairy products (Jamaly *et al*., 2010).

The selection and identification of a bacteriocin producer strain of enterococci is of interest, because it can be used as a probiotic bacterium to inhibit other bacterial pathogens. In this regard, the NM_2 strain isolated from urine of healthy people inhibited other pathogenic bacteria isolated from urine of patients infected by cystitis. It also inhibited other food borne pathogens. This shows the interest in this strain. It could be used as probiotic bacterium and can also be used as food additive. It was necessary to identify the NM_2 strain and other two sensitive strains (TW_5 and TCH_4) strains. Traditional identification protocols including cultural, biochemical and morphological characteristics indicated that the NM_2 strain; TW_5 strain were belonged to *Enterococcus faecium*; *Enterococcus faecalis* and designated *E. faecium* NM_2; *E. faecalis* TW_5 respectively (Facklam and Collins, 1989). Also, the sensitive TCH_4 strain was Gram negative rods, oxidase positive and utilized sorbitol. Additionally, results of carbohydrate fermentation and enzymes abilities were studied using API 20 accoridng to manufacturer's instructions. By surveying literature (Coenye *et al*., 2001) the TCH_4 strain was classified as belonging to *B. cepacia* complex. The *B. cepacia* complex consist of nine genomic species called genomovars (Miller *et al*., 2002). The *B. cepacia* TCH_4 was isolated from urine of cystitis cases in this study and this strain was isolated previously from patients with cystic fibrosis, pyelopnephrities, cystitis where *B. cepacia* have been obtained from catheters, wounds, sputum, urine and blood (Jones *et al*., 2001). It also cause serious infections in neonatal intensive care units. To determine the *Burkholderia* species, the TCH_4 strain was oxidase positive and utilized D-sorbitol, sucrose, inositol and glucose. Following diagnostic key of Bergey's Mannual of Systyematic Bacteriology the TCH_4 strain was identified as *B. cepacia* and designated *B. cepacia* TCH_4.

It was reported that both *E. faecium* and *E. faecalis* showed similar biochemical characteristics (Jamaly *et al*., 2010) and in addition, to the reduction of tetrazolium and potassium telurite which differentiated *E. faecalis* TW_5 from *E. faecium* NM_2 (Table 1), molecular identification was necessary to confirm identification of both TW_5 and NM_2 strains. Because cultural conditions can give ambiguous and speculative biochemical results (Garde *et al*., 1999), molecular idnetfiication was also necessary to confirm identification of *B. cepacia* TCH_4. Consequently, the 16S rRNA gene from TW_5; NM_2; TCH_4 was sequenced and by comparison of their sequences to known ones in Gene Bank, they were identified as belonging to *E. faecalis*; *E. faecium*; *B. cepacia*, respectively (Turner *et al*., 1999).

The inhibitory activity of *E. faecium* NM_2 against different bacterial pathogens noticed in this study could not be attributed to organic acids or hydrogen peroxide produced by the culture as CFS preparations were neutralized (pH 7.0) and treated with catalase. The antibacterial substances produced by *E. faecium* NM_2 was inactivated by proteolytic enzymes, was heat resistant. Consequently, it has been coupled with most definitions of bacteriocins (Klaenhammer *et al*., 1978). It was, therefore, characterized as a bacteriocin. The activity of *E. faecium* NM_2 bacteriocin was not affected by organic solvents, lipase or amylase, probably because of the absence of lipid and carbohydrate moieties in the active molecule. The same was observed for some bacteriocins produced by enterococci (Nettles and Barefoot, 1993) but differs from the bacteriocins brevicin, pediocin N5P, nisin and enterocin (Carolissen-Mackay *et al*., 1997). The bacteriocin employed herein was stable at acidic pH values, but destroyed at neutral and alkaline pH levels and this is promised result since the producer organism: *E. faecium* NM_2 can grow in acidic foods in dairy

fermentations. Ingestion of these foods improve the probiotic capabilities of human gut with inhibition of bactertial pathogens (Enan *et al*., 2012, 2013). This is similar to enterocin S760 (Svetoch *et al*., 2011) and differs from enterocin LR16 (Kumar *et al*., 2010) and enterocin IJ-31 produced by *E. faecium* IJ-31 (Javed *et al*., 2010).

Further work will be necessary to purify the *E. faecium* NM_2 bacteriocin, determine its molecular weight and its amino acid sequence to investigate wheaser it is new one or a variant of other enterocins. This will be important to use *E. faecium* NM_2 in dairy fermentations and as probiotic culture with good capabilities.

CONCLUSION

E. faecium NM_2 isolated from urine of healthy man inhibited both *B. cepacia* TCH_4 and *E. faecalis* TW_5 isolated from urine of patients infected by cystitis. The above strains tested were identified by 16S rRNA sequence analysis. *E. faecium* NM_2 inhibited also other pathogenic and non-pathogenic bacteria from our strain collection. The inhibitory substance was characterized and coupled with most definitions of bacteriocins It was, therefore, characterized as a bacteriocin which showed novel activity against *B. cepacia* isolated from urine.

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