EVALUATION OF ANTIMICROBIAL ACTIVITY AND SAFETY ASPECT OF ENTEROCOCCUS ITALICUS GGN10 STRAIN ISOLATED FROM TUNISIAN BOVINE RAW MILK

NECIMA GAALOUL1, OLFA BEN BRAIEK1, JEAN MARC BERJEAUD2, TIMOTHY ARTHUR3, VERONICA L. CAVERA4, MICHAEL L. CHIKINDAS3, KHALED HANI1 and TAOUFIK GHRAIRI1,5

1Department of Biochemistry, UR12-4503, Faculty of Medicine of Sousse, University of Sousse, Sousse 4002, Tunisia
2Ecologie et Biologie des Interactions, UMR CNRS 7267, Equipe Microbiologie de l’Eau, Poitiers, France
3School of Environmental and Biological Sciences and
4Department of Biochemistry and Microbiology, Rutgers State University, New Brunswick, NJ
5Corresponding author.

Received for Publication February 8, 2014
Accepted for Publication May 22, 2014
doi: 10.1111/jfs.12126

ABSTRACT

Screening of lactic acid bacteria (LAB) from Tunisian raw bovine milk has resulted in the isolation of an Enterococcus italicus strain designated as GGN10, which has demonstrated a bacteriocin-like activity against Listeria spp. Antibacterial activity in the culture supernatant was lost after treatment with proteolytic enzymes, whereas it was stable over a wide range of pH (2–10). The treatment of extracellular extract of this strain at 100°C did not cause loss of antimicrobial activity. The bacteriocin yield reached a maximum of 67,677.1 AU/mL at the beginning of the exponential growth phase and remained stable during 24 h of incubation. Amplification of bacteriocin genes revealed entA and entB genes present in GGN10. In addition, this result was confirmed by the bacteriocin’s reversed-phase high-performance liquid chromatography purification and mass spectrometry analysis. This is the first report on enterocins A and B production by this newly isolated strain. Safety elucidation and antibiotics susceptibility of E. italicus GGN10 was conducted using polymerase chain reaction analysis. No commonly associated pathogenicity islands were identified. An enzymatic study revealed GGN10 as a high producer of acid phosphatase and aminopeptidase, which are important features in flavor and texture development during fermentation. Thus, E. italicus GGN10 produces antimicrobial substances and other biologically active substances of importance for food preservation.

PRACTICAL APPLICATIONS

The potential advantages of discovering new lactic acid bacteria strains offer new possibilities in terms of food application, which may have potent socioeconomic applications. Studies on the local microflora in raw milk contribute to the understanding of both the environment and how selected wild strains can be used with fermented foods. In this study, the strain Enterococcus italicus GGN10 isolated from raw milk was identified as a means of providing a powerful tool for inhibiting pathogenic organisms such as Listeria monocytogenes in dairy foods.

INTRODUCTION

Enterococci are gram-positive, air-tolerant, catalase-negative cocci belonging to the lactic acid bacteria (LAB) group. They differ as a whole from other gram-positive, catalase-negative cocci in several phenotypic traits. They grow in moderately restrictive conditions, including temperature ranges between 10 and 45°C, hyper saline solutions (pH 9.6) and 40% bile. These bacteria are also capable of retaining viability after 30 min of heating at 60°C (Hardie
Enterococci are ubiquitous bacteria but primarily appear in the gastrointestinal tract of humans and animals. These bacteria have also been identified in many foods of animal and vegetable origin (Muller et al. 2001; Ben Omar et al. 2004).

Enterococci are the most controversial species of LAB often identified in food products. They are a component of the microorganisms naturally present in various foods such as artisanal dairy products and commercially available probiotics (Giraffa 1995; Morandi et al. 2006). However, some Enterococcus spp., especially the vancomycin-resistant enterococci (VRE), are recognized as one of the most important nosocomial pathogens causing bacteraemia, endocarditis and urinary tract infections (Morrison et al. 1997). VRE have been isolated from a variety of animal-origin foods. Several studies suggest that their presence is associated with the use of the vancomycin-related glycopeptide avoparcin as a growth promoter in animal production.

Raw milk is colonized by enterococci, which originate from animal feces or from contaminated habitats (soil, surface waters, recipient waters, sewage water). Their recovery and persistence in a variety of cheese, also produced from pasteurized milk, is justified by their ability to resist adverse conditions, such as temperature and salinity. Enterococcus faecalis and Enterococcus faecium are the most frequently occurring enterococcal species in dairy products. However, different Enterococcus spp. strains can also be found in dairy products in which they contribute to the ripening and aroma development. A novel species of Enterococcus italicus was isolated in two artisanal Italian cheese: Toma piemontese and Robiola piemontese (Fortina et al. 2004).

Antimicrobial peptide (bacteriocins) production is widespread among LAB present in milk and dairy products (Vaughan et al. 1994; Coventry et al. 1997). Within the last decade, several papers have been published on bacteriocin-producing enterococci associated with food ecosystems (Batdorj et al. 2006; El-Ghaish et al. 2011). The use of bacteriocinogenic LAB strains as starter or co-culture is a promising alternative in food. Many bacteriocins associated with enterococci have been described. These include enterocin B (Casanova et al. 1997), enterocins L50A and L50B (Cintas et al. 1998), enterocin A (Aymérich et al. 1996), enterocin P (Cintas et al. 1998) and many others. Enterocins are of great interest due to their antimicrobial activity against food spoilage and pathogenic bacteria such as Listeria monocytogenes, Staphylococcus aureus, Clostridium spp. and Bacillus spp. Of equal importance, enterococci could be used as biopreservatives or protective cultures in dairy applications (Giraffa 2003). Characterization of LAB from dairy products manufactured using traditional techniques without commercial starter cultures has been considered essential in the search for novel industrially important microorganisms (Cogan et al. 1997).

The present investigation reports on the isolation and characterization of bacteriocinogenic E. italicus strain as identified from raw Tunisian bovine milk.

## MATERIALS AND METHODS

### Chemicals and Media

All chemicals were purchased from Sigma (St. Louis, MO). Culture media (deMan, Rogosa and Sharpe [MRS], M17, brain–heart infusion [BHI] and All-Purpose Tween 80 [APT]) were obtained from Difco Laboratories (Detroit, MI).

### Bacterial Isolation and Identification

LAB was isolated from the samples of raw bovine milk collected from some farmers of Sousse (Sahel region of Tunisia). Standard (1:5) serial dilutions of milk were made in sterile saline buffer and plated on the MRS and M17. Plates were incubated for 48 h at 30 and 37°C. Several colonies were selected at random, at which time cell morphology, Gram staining and catalase activity were performed. Only gram-positive and catalase-negative isolates were re-cultured on MRS or M17 agar. Purified cultures were kept at −80°C in broth medium supplemented with 30% glycerol.

Isolates were identified using standard morphologic and biochemical procedures. Other tests were conducted according to Schleifer et al. (1984), which included growth at different temperatures (4, 10, 40 and 45°C), tolerance to NaCl (5, 6.5 and 10%) and ability to grow at various pH (3.0, 6.0 and 9.0).

### 16S rRNA Sequencing

The identification of the E. italicus strain was carried out by 16s RNA gene sequencing, as described previously (Weisburg et al. 1991). Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using primers fD1 and rD1 (Weisburg et al. 1991). PCRs were performed using a Multigen Mini thermal cycler (Labnet, Edison, NJ) in a total volume of 25 μL.

Template DNA used for PCR amplification was extracted from overnight E. italicus cultures using Promega DNA purification Kit (Madison, WI). Nucleotide sequences of the amplicon 16S rDNA were determined using an ABI 370
automated sequencer with the Taq Dye-Deoxy TM terminator cycle sequencing kit (PerkinElmer, Boston, MA). The resulting sequences were compared with those available at Genebank using Blastn alignment software (http://www.ncbi.nlm.nih.gov/blast). The Mega 5 computer software program (megasoftware.net) was used for sequence alignment and phylogenetic tree analysis.

Detection of the Inhibitory Activity

Cell-free culture supernatants obtained by centrifugation of an overnight culture at 10,000 x g (4C, 10 min) were adjusted to pH 6 with 1 M NaOH (to eliminate the inhibitory effect of organic acid) and sterilized by filtration (0.22 μm, Millipore, Bedford, MA). The antimicrobial activity of the filter-sterilized supernatant was determined by well diffusion assay (Tagg et al. 1976). Adjacent wells (5 mm) were made in soft agar (0.8%) seeded with the indicator strains listed in Table 1 and 75 μL of the supernatants was poured in the wells. The plates were incubated at the temperatures appropriate for the reference microorganisms. After 24 h, the diameter of the inhibition zone was measured.

Kinetics of Microbial Growth and Bacteriocin Production

Fifty milliliters of MRS broth was inoculated with 1% (v/v) of an overnight culture of \textit{E. italicus} and incubated without shaking at 30C. Samples were taken at 1-h intervals for 24 h. Increase in cellular biomass was measured as absorbance at 600 nm (Jenway 6305, Bibby Scientific Limited, Staffordshire, UK). Bacteriocin production was assayed quantitatively every 2 h by well diffusion method. Twofold serial dilutions in MRS broth of the cell-free supernatant containing bacteriocin were spotted onto the fresh agar plate seeded with \textit{Listeria ivanovii} BUG496. The bacteriocin titer was defined as the reciprocal of the highest dilution showing

**TABLE 1. OLIGONUCLEOTIDE PRIMERS USED IN THE STUDY**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
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<th>References</th>
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<td>Known enterocins</td>
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<td>entA</td>
<td>AF</td>
<td>GGT ACC ACT CAT AGT GGA AA</td>
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<td>58</td>
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</tr>
<tr>
<td></td>
<td>AR</td>
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<td></td>
<td></td>
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<tr>
<td>entB</td>
<td>BF</td>
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<td>56</td>
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<td>BR</td>
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<td>PF</td>
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<tr>
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<tr>
<td>LBS50A</td>
<td>F</td>
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<td>135</td>
<td>50</td>
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</tr>
<tr>
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<td>R</td>
<td>TTT GGT AAT TGC CCA TTC TCC</td>
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<td>LBS50B</td>
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<td></td>
<td>R</td>
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<td>cylB</td>
<td>cylB-F</td>
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<td>McBride et al. (2007)</td>
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<tr>
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<td>psaA F</td>
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<td>psaA R</td>
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<td>HLG R</td>
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<td>62</td>
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</tr>
<tr>
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<td>Van F</td>
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<td>Van B</td>
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<td>CGCAATTCGAAATGATTGAAA</td>
<td>457</td>
<td>57</td>
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</table>

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inhibition of the indicator strain. The amount of bacteriocin is expressed as arbitrary unit per milliliter (AU/mL) of culture (Pascual et al. 2008).

The Bacteriocin’s Sensitivity to Enzymes, pH and Temperature

The producer strain was cultured in MRS broth at 30°C for 16 h. Cells were removed by centrifugation at 10,000×g for 10 min. The filter-sterilized supernatant, which contained the bacteriocin, was used for further characterization. The effect of pH on bacteriocin activity was determined by adjusting the pH of the cell-free supernatant to 2–10 with 1 M HCl or 1 M NaOH. MRS broth adjusted to the same values was used as a control. All samples were incubated at 30°C for 2 h, prior to testing in the well diffusion assay.

Thermostability of bacteriocin was studied at different temperatures. The samples of cell-free supernatant were heated at 60 and 100°C for 10, 30 and 90 min. The bacteriocin’s activity was also assayed after the exposure of the sample to 121°C for 15 and 30 min.

The sensitivity of *E. italicus* bacteriocin to proteolytic enzymes was tested as described by Ghrairi et al. (2008). In brief, stock solutions (1 mg/mL) of proteinase K and trypsin (Sigma) were prepared in 20 mM sodium phosphate buffer (pH 7.0). Proteases were added at a final concentration of 0.1 mg/mL before incubation for 1 h at 37°C. To eliminate possible inhibitory effect due to hydrogen peroxide, the crude bacteriocin was treated with catalase at 0.1 mg/mL for 30 min. After all treatments, the residual activity was assayed by the agar well diffusion method and compared with the activity of the corresponding controls. *L. ivanovii* BUG 496 was used as an indicator strain.

Mode of Action of the Bacteriocin

Ten milliliters of filter-sterilized cell-free supernatant of GGN10 was added to 50 mL of *L. ivanovii* BUG496 in early exponential phase and then incubated at 37°C. Hourly optical density readings were taken at 600 nm. Viable cell counts were recorded on BHI agar. Control cells were treated with the inactive bacteriocin preparation (30 min at 121°C).

Acidification and Enzymatic Activities

The strain GGN10 was initially grown in MRS broth and then in sterile reconstituted skim milk supplemented with yeast extract (3%) and glucose (2%) for two successive subcultures. Sterile reconstituted skim milk (100 mL) was inoculated with 1% of a 24-h activated culture and pH changes were determined using a pH meter during 12-h incubation at 37°C.

The enzymatic profile of *E. italicus* GGN10 strain was studied using the API ZYM kit (BioMérieux, Montalieu-Vercieu, France) according to the manufacturer’s instructions. Enzymatic activity was graded from 0 to 5 with the API ZYM color reaction chart. The approximate number of free nanomole hydrolyzed substrate was approximated from the color strength: 0, no activity; 1, release of 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, ≥ 40 nmol.

Enterocin Typing by PCR Gene Amplification

Total DNA was extracted from overnight culture using Promega DNA purification Kit.

Primer sequences for five structural genes of known enterocins (A, B, P, Q and L050) were used for gene amplification (Table 1). DNA amplifications were performed in a Multigen Mini thermal cycler (Labnet) in a model reaction which contained 12.5 μL of PCR Master Mix (Promega), 2.5 μL of each primer, 5 μL of DNA and 5 μL of nuclease water free in a final volume of 25 μL. Five microliters of the reaction mix was analyzed by electrophoresis on a 1% agarose gel. The PCR products were analyzed on 1.0 % agarose gel with 1X TBE buffer at constant voltage of 100 V. *E. faecium* MM21 was used as positive control (Ghrairi et al. 2008).

PCR-based Detection of Antibiotic Resistance and Virulence Genes

The possible presence of genes responsible for antibiotic resistance to gentamicin (*HLG*), β-lactamase (*blaZ*) and vancomycin (*vanA* and *vanB*), or encoding virulence factors, *cylB* (cytolisin), *ddl* (D-Ala ligase), *esp* (enterococcal surface protein), *gls24-like* (stress and starvation protein), *nucl* (nuclease) and *psaA* (metal binding protein) were investigated for *E. italicus* by specific PCR assays (Table 1). PCR were conducted in a Multigen Mini thermal cycler (Labnet). The following PCR conditions were used: 94°C for 2 min, followed by 25 cycles of 94°C for 30 s; 57°C (for *CylB*, *esp*, *gls24-like*, *psaA*, *VanA*, *VanB*), 51° C (for *ddl*) and 62° C (for *Nuel*, *HLG*, *blaZ*); 72°C for 40 s (except *ddl* for 60 s) and a final extension of 72°C for 5 min. Each PCR contains 5 μL PCR Master Mix (Promega), 2.5 μL of each primer, 5 μL of nuclease-free water and 5 μL of DNA. *E. faecalis* MM4594 was used as a positive control (El-Ghaish et al. 2011).

Bacteriocin Purification

*E. italicus* was evaluated for bacteriocin production following the procedure of (Batdorj et al. 2006). The strain was grown in 1 L of MRS medium at 30°C for 18 h in aerobic conditions. The cells were removed by centrifugation at
12,000×g for 10 min and the supernatant was adjusted to pH 6.8 with 1 N NaOH, and tested against L. ivanovii BUG496.

The proteins were precipitated overnight with 60% ammonium sulfate at 4C. The precipitate was collected by centrifugation at 12,000×g (30 min at 4C). The supernatant was discarded and the precipitate was resuspended in 10–15 mL of sterile ddH₂O per 500 mL of the original suspension.

The resuspended precipitate was filter-sterilized using 0.45-μm filters prior to passing through a reversed phase SPE UPTI-CLEAN (Interchim, Montluçon, France) cartridge C18 equilibrated with 20% H₂O–TFA 0.1% (trifluoroacetic acid), 80% acetonitrile. Elution was performed in steps using different concentrations of acetonitrile (30, 40, 60 and 80%).

Fractions (5 mL) were collected and acetonitrile was removed using a Speed-Vac concentrator (Thermo Scientific, Waltham, MA). The antimicrobial activity of all fractions was tested. The active fraction was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Waters Alliance apparatus with Millennium software (Milford, MA). One hundred microliters of the concentrated bacteriocin was injected into an analytical RP-Nucleosil C18 column (Macherey-Nagel, Bethlehem, PA). The elution was performed at a flow rate of 0.2 mL/min with a linear gradient from 40 to 100% in 30 min. The eluted peaks were detected by spectrophotometer, measuring the absorbance between 210 and 300 nm with a photodiode array detector PDA 996 (Waters) and were collected manually. All fractions were tested against L. ivanovii BUG496; those with activities were kept for further testing with mass spectrometry.

**Mass Spectrometry**

The molecular mass of the purified bacteriocin was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry (MS) using a QSTAR hybrid pulsar instrument (Applied Biosystems, Foster City, CA) equipped with an orthogonal MALDI source. A model VSL-337 ND S-nitrogen laser (337 nm, 3 ns pulse length; Thermo Laser Science, Mountain View, CA) was used. The ion acceleration voltage and laser intensity were set to 20 kV and 35%, respectively. Five microliters of crystal suspension of purified bacteriocin sample was carefully mixed with an equal volume of cyano-hydroxycinnamic acid. One microliter of the prepared solution was spotted onto a Perspective 10×10 MALDI plate and left to dry at room temperature for 10 min before analysis.

**RESULTS**

**Isolation and Identification of the GGN10 Isolate**

A total of 20 LAB samples isolated from raw milk were examined for antimicrobial activity against different food pathogens. From these, the isolate GGN10 was selected as producing a large inhibition zone against L. ivanovii (Table 2). The cells were coccoid in shape, gram-positive and catalase-negative, with the ability to grow at 10 and 45°C as well as in the presence of 6% NaCl (Table 2). The isolate’s ability to grow at pH 9.6 and 45°C allowed its characterization as an *Enterococcus* spp. The GN10 strain was identified as *E. italicus* based on 16S rDNA sequence

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Strain</th>
<th>Source*</th>
<th>Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micrococcus luteus</td>
<td>–</td>
<td>U12-ES03</td>
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</tr>
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<td>Lactococcus lactis sp. cremoris</td>
<td>11603</td>
<td>ATCC</td>
<td>16</td>
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<tr>
<td>Lactococcus lactis sp. lactis</td>
<td>11454</td>
<td>ATCC</td>
<td>14</td>
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<tr>
<td>Enterococcus faecalis</td>
<td>JH-22</td>
<td>U12-ES03</td>
<td>16</td>
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<tr>
<td>Listeria ivanovii</td>
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<tr>
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* ATCC, American Type Culture Collection; DSM, Deutche Sammlung von Mikroorganismen und Zell Kulturen; U12-ES03, Unité de Mycotoxicologie (Sousse, Tunisia).
† Diameter of inhibition zone in millimeters.
analysis (Fig. 1). 16S rRNA sequences of E. italicus GGN10 was deposited to the NCBI Gene Bank with accession number KF734648.

**Spectrum of Inhibitory Activity**

The inhibitory activity of the bacteriocin-producing strain E. italicus GN10 against different bacterial strains was tested by the well diffusion method (Table 2). The inhibitory spectrum was narrow, and the most sensitive strains were L. ivanovii, L. monocytogenes and Micrococcus luteus. A number of other bacteria including enterococci, lactobacilli and lactococci were also sensitive to the cell-free supernatant of GGN10 strain. No activity was detected against gram-negative pathogens.

**Stability of the Bacteriocin Produced and Acidification Ability of E. italicus GGN10**

Treatment of the cell-free supernatant of GGN10 strain at either 60, 90 or 100°C at different exposure time did not result in any loss of antimicrobial activity. The activity decreased only by 50% after 15 min at 121°C but was completely lost after 30 min of incubation at 121°C. These results suggest that the antimicrobial substance produced by E. italicus GGN10 is heat resistant.

To verify the proteinaceous nature of the antimicrobial substance, the effect of two proteolytic enzymes (trypsin and protease K) on its activity was tested. Incubation of the sample for 1 h at 37°C with each of these enzymes completely eliminated antimicrobial activity. These data clearly indicate the proteinaceous nature of the inhibitory substance produced by the GGN10 strain.

The crude bacteriocin retained full proteinaceous activity at pH 2.0, 4.0, 6.0, 8.0 and 10.0, whereas total inactivation was observed at pH 12.

Sterile reconstituted skim milk (100 mL) was inoculated with 1% of a 24-h activated culture of GGN10 strain and pH changes were determined using a pH meter. The pH value reached 5.4 after 12 h of incubation at 37°C (data not shown).

**Enzymatic Profile**

The enzymatic profile of E. italicus GGN10 strain was assayed using the API ZYM kit (BioMérieux), according to the manufacturer’s instructions. The results of 19 enzymes tested are shown in Table 3. The enzymatic profile of E. italicus GGN10 shows that $\alpha$-galactosidase,

<table>
<thead>
<tr>
<th>Test</th>
<th>Reaction or characteristics</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>Cocci</td>
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<tr>
<td>Gram</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
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<tr>
<td>Growth</td>
<td>–</td>
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<td>at 4°C</td>
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<td>at 10°C</td>
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<tr>
<td>at 40°C</td>
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<tr>
<td>at 45°C</td>
<td>+</td>
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<tr>
<td>at pH 3.5–9.2</td>
<td>+</td>
</tr>
<tr>
<td>in 5% NaCl</td>
<td>+</td>
</tr>
<tr>
<td>in 6% NaCl</td>
<td>–</td>
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</tbody>
</table>

*API Zym*:

- Alkaline phosphatase: 30
- Esterase: 5
- Esterase lipase (C8): 5
- Lipase (C14): 5
- Leucine arylamidase: ≥40
- Valine arylamidase: ≥40
- Cystine arylamidase: ≥40
- Trypsin: 5
- $\alpha$-Chymotrypsin 10: 5
- Acid phosphatase: 20
- Naphthol-AS Blphosphohydrolase: 20
- $\alpha$-Galactosidase: 0
- $\beta$-Galactosidase: 5
- $\beta$-Glucuronidase: 0
- $\alpha$-Glucosidase: 20
- $\beta$-Glucosidase: 0
- N-acetyl-$\beta$-glucosaminidase: 5
- $\alpha$-Mannosidase: 0
- $\alpha$-Fucosidase: 0

* Enzyme activity is shown in nanomolar (nM) of chromophore released after 6 h of incubation at 37°C.
+ growth; –, no growth.
β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase were not detected. The activities of esterase, esterase/lipase, trypsin, α-chymotrypsin 10, β-glucosidase and N-acetyl-β-glucosaminidase were weak and were estimated to be in the range of 5 nmol.

However, alkaline phosphatase, leucine arylamidase, valine arylamidase and cystine arylamidase had interesting activities (>40 nM).

**Mode of Action**

The addition of the cell-free supernatant from *E. italicus* to an exponentially growing culture of *L. ivanovii* BUG 496 caused a decrease in the number of viable cells (Fig. 2). There was an approximate 2 log reduction in viable cell number 1 h after the addition of enterocin prepared from *E. italicus* strain. Four hours after the addition of bacteriocin, the difference in cell numbers between the treated sample and the control reached 3.5 log. The viability loss was concomitant with the decrease in optical density. These results indicate a bactericidal mode of action of the bacteriocin produced by *E. italicus* GGN10.

**Growth Pattern and Bacteriocin Production**

The growth kinetics of bacteriocin production in MRS broth were determined by incubating GGN10 isolate at 30°C. As shown in Fig. 3, the bacteriocin production by the GGN10 cells started during the early exponential growth phase (331.54 AU/mL) at 3 h with an absorbance equal to 0.54. Maximum production (67677.1 AU/mL) was reached at the end of exponential phase (7 h). After that, the activity remained stable during stationary phase, before declining to a level of 6,500 AU/mL after 24 h of incubation.

**Identification of Enterocin, Structural Gene by PCR**

Structural genes of well-known enterocins were detected in GGN10 using PCR. Agarose gel electrophoresis revealed two amplicons of 110 and 190 base pairs (bp) in DNA of *E. italicus* GGN10 and the in DNA of the positive control (Fig. 4). These fragments corresponded to PCR signals for enterocins A and B, respectively. This result indicates the presence of *entA* and *entB*.

**Purification of the Bacteriocin**

The bacteriocins produced by *E. italicus* GGN10 were purified using a three-step method. The first step consisted of ammonium sulfate precipitation at 80%, which increased the activity. In the second step, the active fraction was passed through a reversed phase SPE UPTI-CLEAN. The collected fractions were checked by the well diffusion test.
The fraction eluted with 40% acetonitrile exhibited an anti-
Listeria activity. This active fraction was then applied on
a RP-HPLC column. All peaks showing absorption at 220
and 280 nm were individually collected and tested by
the agar diffusion method against *L. ivanovii* BUG 496 (Fig. 5).

Finally, fraction purified by chromatography on a C18
column was re-injected onto the same column under the
same conditions and then analyzed by MALDI–TOF–mass
spectrometry. Two molecular masses were obtained –
5,463.86 Da (Fig. 6) and 4,831.96 Da (Fig. 7) – which corre-
spond to enterocin B and enterocin A, respectively.

**Virulence Determinants and Antibiotic Resistance**

PCR amplification was conducted with several sets of
primers and confirmed the absence of vancomycin resis-
tance genes *vanA* and *vanB*. Moreover, no antibiotic resis-
tance (HLG, *blaZ*) was detected in the GGN10 cells. Absence
of the known virulence determinants was confirmed by
amplification of critical virulence genes such as cytolysin
(*cylB*), hemolysin, enterococcal surface protein (*esp*), genral
stress protein (*gls24*), D-Ala ligase (*ddflaecium*), *nucl* (nuclease)
and *psa* (metal binding protein) with PCR (data not
shown).

**DISCUSSION**

The present study was carried out with the objective of iso-
lation and characterization of indigenous bacterial strains
from bovine raw milk for potential bacteriocin production.
One isolate was selected as it inhibited *L. ivanovii*. This
strain was identified as *E. italicus* by 16S rDNA amplifica-
tion and sequencing. This is the first report on isolation of
E. italicus from Tunisian bovine milk. Fortina et al. (2004) isolated seven atypical Enterococcus strains from artisanal Italian cheese. These were characterized as a novel enterococcal species, named E. italicus (DSM15952). The microbial characteristics of our E. italicus GGN10 strain were compared with those described by Fortina et al. (2004). We found that both strains grow at 45°C, at pH 9.6 and in 6% NaCl. Although, the biochemical pattern of GGN10 isolate has not been studied extensively and may be different from E. italicus DSM 15952.

Enterococcus spp. is a significant part of the normal microbial population of LAB in indigenous dairy products of Tunisia (Ghrairi et al. 2008). Other studies also reported isolation of Enterococcus spp. from non-dairy source. Migaw et al. (2013) isolated three E. faecium from fish viscera. Ben Belgacem et al. (2010) screened and isolated 24 enterococcal strains from artisanal fermented meat and were identified as E. faecium.

Enterococcus spp. play an important role in maturation of some cheese and in several probiotic formulations (Franz et al. 1999). In addition, they produce bacteriocins, specifically called enterocins, which inhibit the growth of pathogenic and food-deteriorating bacteria, including Listeria and Clostridium (Giraffa 1995). In our study, the antibiotic resistance of E. italicus GGN10 strain was assessed by PCR amplification. There was no detection of any of the antibiotic resistance genes. Furthermore, safety aspects tested showed negative PCR results for several virulence genes. Results for detection of virulence determinants were in accordance with the findings of Maietti et al. (2007). Similar results were also found with other Enterococci strains from dairy origins (Sánchez-Valenzuela et al. 2013).
In our study, *E. italicus* GGN10 exhibited the highest antimicrobial activity against *L. ivanovii*. The antibacterial activity was completely diminished after the treatment of the cell-free supernatant with protease K and trypsin, confirming the proteinaceous nature of the antimicrobial agent. The antimicrobial substance was stable over a wide range of pH (2.0–10.0) and was still active after exposure to 121°C for 15 min. This stability is consistent with the stability of other bacteriocins (Cocolin *et al.* 2007). The thermostability of bacteriocins could be a very useful feature for their application as food preservatives since many food-processing procedures involve heating. The pH resistance may also be useful in a variety of food applications.

The bacteriocin production by the GGN10 strain started during the early exponential growth phase. Maximum production was reached at the end of the exponential phase and remained stable (6,500 AU/mL) for a 24-h incubation period. As described in previous studies, bacteriocin biosynthesis may depend on the bacterial growth phase (Hadji-Sfaxi *et al.* 2011). In the present study, *E. italicus* GGN10 cells exhibited high acid phosphatase and aminopeptidase activities, which are important features in flavor and texture development during fermentation. We did not notice esterase activity, which is not in accordance with the literature. Indeed, enterococci show significantly higher esterolytic activity than strains of other genera of LAB (Sarantinopoulos *et al.* 2001). However, the absence of C-14 lipase activity in *E. italicus* was in agreement with the literature as the esterolytic system of enterococci is rather complex and more efficient than their lipolytic system (Giraffa 2003). The acid phosphatase and glucosidase activities of the GGN10 strain may be important for the hydrolysis of phosphopeptides and for utilization of milk sugars during ripening of cheese.

The newly isolated microorganism was slow in lowering pH. This result is in agreement with the literature, indicating that enterococci can be considered as poor acidifying bacteria (Andrighetto *et al.* 2001; Sarantinopoulos *et al.* 2001; Chammas *et al.* 2006). Low acidification activity of enterococci was confirmed also by Giraffa (2003) who showed that the mozzarella cheese isolated enterococci did not decrease pH below 5.5.

The bacteriocinogenic *E. italicus* strain was screened for known enterocins by specific PCR. The 110 and 190 bp DNA fragments were amplified from the genomic DNA of *E. italicus*, which correspond to the enterocins A and B, respectively. Mass spectrometry analysis confirmed two molecular size proteins (4,828 and 5,463 Da) similar to enterocin A and enterocin B. This is the first report of *E. italicus* producer of enterocins A and B. These bacteriocins have been described exclusively in *E. faecium* (Cocolin *et al.* 2007; Ghrairi *et al.* 2008) and *E. faecalis* (Aymerich *et al.* 1996; Casaus *et al.* 1997). In addition, according to Strompfova *et al.* (2008), only 13.8% of *E. faecalis* strains and 86.2% *E. faecium* strains were enterocin A or enterocin B gene positive.

Several studies demonstrated the inhibitory effect of enterocin-producing *E. faecium* or *E. faecalis* strains against *L. monocytogenes* and *S. aureus* in model dairy systems such as milk, soft cheese and soy milk. The anti-listerial activity of enterocins produced by protective cultures in cheese was observed until the end of ripening and no or minor influences were generally reported on both the commercial starter activity and the organoleptic characteristics of the products (Giraffa 1995).

In conclusion, the screening of bacteriocin-producing microorganisms from Tunisian bovine milk led to isolation of an *E. italicus* strain. Based on its technological properties, enzymatic and antibacterial activities, this strain could provide potential applications in the processing of some dairy products.

**ACKNOWLEDGMENT**

This work was partially supported by a grant from the Ministry of High Education, Tunisia.
CONFLICT OF INTEREST

The authors report no conflict of interest in this work.

REFERENCES


