# *Bacteriocin production and safety evaluation of non-starter* Enterococcus faecium *IM1 and* Enterococcus hirae *IM1 strains isolated from homemade Egyptian dairy products*

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ORIGINAL PAPER

# Bacteriocin production and safety evaluation of non-starter *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1 strains isolated from homemade Egyptian dairy products

**S. El-Ghaish · A. El-Baz · N. Hwanhlem · M. Zommara · E. Ayad · Y. Choiset · T. Haertlé · J.-M. Chobert**

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**Abstract** This study presents the characterization of some strains of lactic acid bacteria (LAB) from traditional Egyptian dairy products. Isolated LAB (623 isolates) were studied for their antimicrobial activity against taxonomically related microorganisms. Selected LAB were identified by PCR method as *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1. Partially purified enterocins showed antimicrobial activity against *Lactobacillus bulgaricus* 340, *Lb. brevis* F104 and F145, *Lb. sakei*, *Brochothrix thermosphacta* DSMZ20171$^T$ and DSMZ20599, *Carnobacterium maltaromaticum* CIP103135$^T$, *C. piscicola* S4312, *E. faecalis* JH2-2 and JH2-2rr04, *E. faecium* CTC492/t1362 and P13, *Listeria grayi* CLIP12518, *L. innocua* 1, CIP80.11$^T$, F and P, *L. ivanovii* ATCC and CIP78.42T, *L. monocytogenes* CIP78.35, DSM12464, EGDe, RF124, RF125, RF131, RF132, RF133, RF142, RF151 and RF152, and *Vagococcus penaei* CIP 109914$^T$ but have no effect against *Bacillus subtilis* 168T, *Moellerella wisconsensis* MIP2451, *Morganella psychrotolerans* MIP2488, *Pseudomonas fluorescens* 10, *Salmonella enterica*, *Sal. montevideo*, *Sal. typhimurium* and *Serratia liquefaciens* CIP103238$^T$. The inhibitory activity was not due to hydrogen peroxide for *E. hirae* IM1, but strain *E. faecium* IM1 may excrete diverse antimicrobial compounds such as hydrogen peroxide and bacteriocins. Antimicrobial activity of *E. faecium* IM1 was initially detected during exponential phase of growth, and the maximal level (1,300 AU/mL) was observed at 12 h and remained stable till the end of incubation time (48 h). Maximum of activity of *E. hirae* IM1 was observed during the logarithmic phase (6 h) and then decreased after 12 h. Bacteriocins produced by *E. faecium* IM1 and *E. hirae* IM1 were stable between pH 5 and pH 8 and stable also until 100 °C/20 min. Tested strains were free from virulence determinant genes as well as hemolytic and gelatinase activities. *E. faecium* IM1 was sensitive to penicillin, kanamycin, vancomycin, gentamicin and tetracycline but resistant to ampicillin. Otherwise, *E. hirae* IM1 was sensitive to penicillin, kanamycin, vancomycin and tetracycline but resistant to ampicillin and gentamicin. The isolated cultures with antimicrobial activities may be used as safe and useful starter cultures or co-cultures.

**Keywords** Bacteriocin · Lactic acid bacteria · *Enterococcus* · Egyptian dairy products

S. El-Ghaish · M. Zommara
Department of Dairy Sciences, Faculty of Agriculture, Kafrelsheikh University, B.P. 33516, Kafr el-Sheikh, Egypt

A. El-Baz
Department of Dairy Science, Agriculture Research Center, Animal Production Research Institute, Sakha, Kafr el-Sheikh 33717, Egypt

N. Hwanhlem
Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand

E. Ayad
Department of Food Science, Faculty of Agriculture - Saba Basha, Alexandria University, P.O. Box 21531, Bolkely-Alexandria, Egypt

Y. Choiset · T. Haertlé · J.-M. Chobert (✉)
UR 1268 Biopolymères Interactions Assemblages, Équipe Fonctions et Interactions des Protéines, INRA, 44316 Nantes Cedex 03, France
e-mail: chobert@nantes.inra.fr;
Jean-Marc.Chobert@nantes.inra.fr

## Introduction

In Egypt, various traditional fermented milk and milk products such as Ras cheese, Domiatti cheese, Kareish cheese,

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Zabady and Laban Rayeb are produced. All these products are manufactured from pasteurized milk. Ras cheese is a hard cheese made from cow milk with an enzymatic coagulation. Domiatti cheese is a soft cheese obtained by enzymatic coagulation of cow's, buffalo's and/or both milks. Kareish cheese is a soft cheese obtained by acidic coagulation of skim milk. Zabady and Laben Rayeb are traditional types of yoghurt manufactured in Egypt from buffalo's, cow's and/or both milks [1, 2].

Now, there is an increase in consumers demand for decreasing chemical preservatives in food additives and use of natural preservatives such as lactic acid bacteria (LAB). The use of adjunct starters from LAB has been proposed to improve the sensory properties and shelf-life of dairy products. LAB can inhibit the growth of different microorganisms (bacteria, yeasts and fungi) because they can produce various antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins [3].

The bacteriocins produced by LAB were defined as a large group of ribosomally synthesized, amphiphilic, small, cationic, antimicrobial peptides, produced naturally by the microorganisms, which vary in spectrum and mode of activities, molecular structures and molecular masses, thermo-stabilities, pH range of activities and in genetic determinants. Their activity may be bactericidal (causing cell death) or bacteriostatic (causing a slowdown in growth) [4, 5]. Recently, Zouhir et al. [6] proposed a new classification based on the structure, which includes 12 classes of bacteriocins.

Enterococci are associated with several types of fermented foods and play important roles in flavor and aroma development. Consequently, they could be used as starters or bioprotective cultures [7–11]. The enterococci were suggested for use as starter in the production of Cebreiro cheese, feta cheese and mozzarella [12, 13]. Enterococci are important for environmental, food and clinical microbiology applications. Their technological importance stems from their inclusion in the production of various European fermented foods such as sausages and cheeses, where they are purposefully added to the product as a starter culture [14]. A food company sought clearance from the British "Advisory Committee on Novel Foods and Processes" (ACNFP, [15]) for the use of *E. faecium* strain K77D as a starter culture in fermented dairy products. International Dairy Federation (IDF, [16]) accepted *E. faecium* as a starter. So far in the EU, there have been no regulations that control the use of microbial strains in foods. In the anticipation that such regulation will be undertaken in the future, the establishment of a qualified presumption of safety (QPS) system for microbial strains was suggested, which was based on a safety decision tree. Based on the association of enterococci with human infections, these bacteria cannot be considered without reservation as "safe." For each strain, therefore, its safety needs to be assessed based on the "body of knowledge" of the strain including its known virulence determinants as well as antibiotic resistances. This assessment clearly is the responsibility of the producer of the culture.

Enterococci are known to produce enterocins, which are bactericidal peptides against food spoilage and pathogenic bacteria such as *Listeria* spp., especially against *L. monocytogenes* [17–19]. These enterocins are divided into different groups according to the classification used. Enterocin P (*E. faecium* P13), enterocin A (*E. faecium* DPC1146) and enterocin 31 (*E. faecalis* YI717) are examples of subclass IIa [4, 17, 20, 21]. Enterocin L50 (EntL50A and EntL50B) (*E. faecium* L50), enterocin B (*E. faecium* T136/C492 and BFE900), enterocin Q (*E. faecium* L50) and bacteriocin AS-48 (*E. faecalis*) are examples of subclass IIb [22–25].

Otherwise, a few studies have demonstrated that enterococci could be applied in the foods as a safe starter [26], but they must be free from virulence factors such as aggregation substance protein, gelatinase, cytolysin, enterococcal surface proteins hyaluronidase, accessory colonization factors and endocarditis antigens [27, 28].

The aim of this study was to determine the bacteriocin-producing ability of LAB isolated from traditional Egyptian dairy products. The selected strains were evaluated for their bacteriocinogenic potential as well as for their technological properties (effect of thermal treatments, pH stability and enzymatic treatments) and their effects against foodborne pathogenic bacteria. Moreover, partial purification of the isolated antimicrobial substances was attempted, and their safety traits have been determined.

## Materials and methods

### Materials

All products were of analytical grade and obtained from different companies such as Merck, Germany (skim milk powder); Sigma-Aldrich, USA (glycerol, sodium citrate, gelatin, Tris, ammonium sulfate, trifluoroacetic acid, potassium chloride, ethylenediaminetetraacetic acid, proteinase K, trypsin, α-chymotrypsin, catalase, α-amylase, ampicillin, penicillin G, kanamycin, vancomycin, gentamicin, tetracycline, ethidium bromide); Biokar diagnostics, France (MRS and M17); Fisher Bioblock Scientific, France (agarose and agar); Panréac, France (di-potassium phosphate, potassium phosphate, urea, sodium chloride); Applied Biosystems, Ireland (acetonitrile).

### Origin of isolates

Samples of raw milk (buffalo, cow, goat and sheep) and traditional dairy products were obtained from local markets of

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the following Egyptian cities: Alexandria, Kafr El-Sheikh and El-Mehala. Traditional dairy products included Domiatti cheese, Ras cheese, Kareish cheese and Laban Rayeb. Each sample was collected in sterile cup and was kept at 4 °C until analysis.

### Isolation, purification and pre-identification of LAB cultures

Ten grams from each dairy product was weighted, added aseptically to 90 mL of sodium citrate (2 %, w/v) and homogenized in a sterile bag. The resulting homogenate was decimally and serially diluted using Ringer solution [0.9 % NaCl (w/v)] up to $10^{-8}$. One milliliter from each dilution was plated onto M17 and de Man-Rogosa-Sharpe (MRS) agar. For Laban Rayeb, 10 mL of each sample was pipetted aseptically into 90 mL of Ringer solution and mixed thoroughly. Serial dilutions ($10^{-1}$–$10^{-8}$) were made, and 1 mL of the appropriate dilutions was pour plated onto M17 and MRS agar. All plates were incubated at 37 °C for 48 h, and colonies from M17 and MRS agar plates were selected according to their shape and color from plates with colony numbers between 30 and 300. They were examined microscopically for Gram staining. Catalase activity was determined by placing drops of 3 % $H_2O_2$ (w/v) on cultures. The cultures were classified as Gram-positive, catalase-negative rods and Gram-positive, catalase-negative cocci. For purification, the cultures were streaked on suitable media, and the purified strains were reconstituted in sterile skim milk (12.5 %, w/v) supplemented with 30 % (w/v) glycerol and stored at −20 °C.

### Molecular identification of some selected LAB isolates (Amplification and sequencing of 16S rRNA gene)

DNA was extracted from the isolates according to Delley et al. [29] and was used as a template for 16S rRNA gene amplification. In the reaction, universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′ TAAG GAGGTGATCCAGGC3′) (Qiagen GmbH, Hilden, Germany) were used [30]. DNA amplifications were previously described [10].

### Antimicrobial activity assay

The different isolates (623 isolates) were reactivated by combining 50 µL of the pre-culture with 950 µL of M17 (for coccus isolates) or MRS broth (for rod isolates) and overnight incubation at 37 °C. The antimicrobial activity of cell-free supernatant (CFS) was determined by well diffusion method [31]. CFS was obtained after centrifuging the overnight cultures (16–18 h) at 8,000 rpm for 10 min at 4 °C and adjusting the pH to 6.5. An aliquot of 100 µL overnight culture of *Lactobacillus bulgaricus* 340 was added to 20 mL of soft MRS agar (0.8 %, w/v). Wells were made in the lawn of hardened soft agars in Petri dishes, and aliquots (50 µL) of the supernatant were poured in them. The plates were left for 1 h at room temperature in sterile conditions before incubating at 37 °C for 24 h. A clear zone was accepted as positive.

### Characterization of the behavior of antimicrobial compounds

#### *Effect of enzymes on antimicrobial activity*

The effect of various enzymes on antibacterial activity against *Lb. bulgaricus* 340 was determined according to Noonpakee et al. [32] with slight modification. Filter-sterilized CFS of the isolate (200 mL) was incubated with 20 µL of the following enzymes: proteinase K, trypsin, α-chymotrypsin, catalase and α-amylase at a final concentration of 1 mg/mL in 20 mM phosphate buffer, pH 7. After 2 h of incubation at 37 °C, enzyme was inhibited by heating at 100 °C for 5 min. Positive control was constituted from 200 µL bacterial supernatant and 20 µL sterilized water. Negative control was made with 200 µL M17 broth and 20 µL sterilized water. The residual antibacterial activity was determined by spot-on-lawn method [33].

#### *Effect of pH on antimicrobial activity*

The pH of CFS was adjusted to values between 2 and 10 with NaOH or HCl. After 2 h incubation at 37 °C, the pH was readjusted to 6.5 in all samples and the residual bacteriocin activity was determined as described earlier against *Lb. bulgaricus* 340.

#### *Effect of heat treatment on antimicrobial activity*

CFS (5 mL) was placed in water bath at 100 °C for 10, 20 and 30 min and in an autoclave at 121 °C, for 20 min, and the residual bacteriocin activity was determined as described earlier against *Lb. bulgaricus* 340. Unheated samples were used as controls.

### Determination of LAB growth for maximal bacteriocin production

One milliliter of an overnight culture of the tested strains was added to 30 mL M17 (*E. faecium* IM1) or MRS medium (*E. hirae* IM1) and incubated at 37 °C under non-regulated pH conditions. Cell growth was monitored every 1 h until 12 h by turbidity ($OD_{600nm}$) and pH determination. After 12 h and until 48 h, cell growth was monitored every 12 h by turbidity ($OD_{600nm}$) and pH determination.

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**Table 1** PCR primers and products used for the detection of genes coding virulence factors

| Gene | Primer | Sequence 5′→3′ | Product size (bp) | TM | References |
|---|---|---|---|---|---|
| asa*1* | ASA11 | GCACGCTATTACGAACTATGA | 375 | 56 °C | [28] |
| | ASA12 | TAAGAAAGAACATCACCACGA | | | |
| *gelE* | gel1 | TATGACAATGCTTTTTGGGAT | 419 | 45 °C | [28] |
| | gel2 | AGATGCACCCCAAATAATATA | | | |
| $esp_{fm}$ | ESP14F | AGATTTCATCTTTGATTCTTGG | 510 | 56 °C | [28] |
| | ESP12R | AATTGATTCTTTAGCATCTGG | | | |
| *cyl A* | CYTI | ACTCGGGGATTGATAGGC | 688 | 58 °C | [28] |
| | CYTIIb | GCTGCTAAAGCTGCGCTT | | | |
| *Ace* | ACE-F | GAATTGAGCAAAAGTTCAATCG | 1008 | 56 °C | [52] |
| | ACE-R | GTCTGTCTTTTCACTTGTTTC | | | |

Vankerckhoven et al. [27], Ben Omar et al. [52]

Antimicrobial activity was calculated according to spot-on-lawn method [34] and expressed as arbitrary units per mL (AU/mL). Soft nutrient agar was solidified in a sterile Petri dish after addition of 100 µL of an overnight culture of *Lb. bulgaricus* 340. CFS was serially diluted twofold with M17 or MRS medium. An aliquot (10 µL) of each dilution was spotted onto the plate before overnight incubation at 37 °C. The titer was defined as the reciprocal of the highest dilution ($2^n$) causing the inhibition of the indicator lawn. The AU of antimicrobial activity per milliliter was defined as $2^n \times 1{,}000$ µL/10 µL.

## Detection of virulence factors by PCR amplification

DNA was extracted from overnight *Enterococcus* cultures by Dneasy Blood & Tissue Kit (Qiagen, USA) using *E. faecalis* MM4594 as positive control. Primer sequences (Qiagen) for five known virulence genes, *asal* (aggregation substance), *gelE* (gelatinase), $esp_{fm}$ (enterococcal surface protein), *cylA* (cytolysin) and *ace* (collagen-binding protein), were used (Table 1). DNA extracts of the two strains were used as templates for virulence gene amplification. DNA amplifications were performed in DNA thermal cycler model (Techno, Barloworld scientific). Mix reaction was the same as described above.

In case of the amplifications of genes *asa1*, *ace* and $esp_{fm}$, the thermocycler was programmed as follows: denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min and DNA extension at 72 °C for 1 min. For the amplifications of gene *cylA*, the thermocycler was programmed as follows: denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 58 °C for 45 s and DNA extension at 72 °C for 45 s. For the amplifications of gene *gelE,* the thermocycler was programmed as follows: denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 45 °C for 1 min and DNA extension at 72 °C for 1 min. In all cases, a final additional 10 min extension was applied at 72 °C. Amplicons were analyzed on 1 % (w/v) agarose gel with ethidium bromide (0.5 mg/mL) in 0.5 × TAE (40 mM Tris acetate-1 mM EDTA) buffer for 30 min at 100 V and made visible by UV trans-illumination.

## Disk diffusion antibiotic sensitivity testing

The antibiotic resistances of the isolated strains (*E. faecium* IM1and *E. hirae* IM1) were assessed against ampicillin (MIC ≤ 4 µg/mL), penicillin G (MIC ≤ 4 µg/mL), kanamycin (MIC ≤ 512 µg/mL), vancomycin (MIC ≤ 4 µg/mL), gentamicin (MIC ≤ 32 µg/mL) and tetracycline (MIC ≤ 64 µg/mL) by disk diffusion antibiotic sensitivity testing. The breakpoints were defined according to the National Committee for Clinical Laboratory Standards (NCCLS) method (Karmarkar et al. [35] and European Food Safety Authority [36]. An aliquot (100 µL) of the bacterial culture grown to the early stationary growth phase in suitable medium was added to 20 mL of soft M17 or MRS agar (0.8 %, w/v) and left to solidify. The antibiotic disks (Prat Dumas, Couze-St-Front, France, 9 mm) were placed on the surface of the inoculated media, and aliquots (30 µL) of the antibiotic solutions with different concentrations were poured on the disks. The plates were left for 1 h at room temperature under sterile conditions before incubating for 24 h at 37 °C. A clear zone surrounding the disk was taken as positive.

## Purification of bacteriocin

*E. faecium* IM1 and *E. hirae* IM1 were grown in 1 liter of M17 and MRS, respectively, at 37 °C for 24 h. The culture was centrifuged at 10,000×*g* for 10 min at 4 °C to separate bacterial cells and supernatant. The supernatant was adjusted to pH 6.5 by adding 12 N NaOH and then filtered

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through 1-μm filters (Ø 47 mm, Whatman, England). The neutralized and filtered supernatant was assayed for bacteriocin activity against *Lactobacillus sakei* subsp. *sakei* JCM1157 by agar well diffusion assay.

Ammonium sulfate (Fluka, The Netherlands) was gently added to the supernatant (maintained at 4 °C) to obtain 70 % saturation and stirred overnight. After centrifugation (20 min at 12,000×*g*, 4 °C), the pellet was resuspended in (one-ten) volume 6 M urea pH 6.5 and was then assayed for bacteriocin activity against *Lactobacillus sakei* subsp. *sakei* JCM1157 and different pathogenic strains by agar well diffusion assay as described later in Table 5.

The pellet solution (*E. faecium* IM1) was run on a Sep-Pak $tC_{18}$ 12 cc Vac (5 g) Cartridge (Waters Millipore, MA, USA) equilibrated with acetonitrile (ACN). Elution was performed in steps using different concentrations of acetonitrile (0, 25, 60 % ACN and 80 % ACN/20 % isopropanol) with 0.05 % trifluoroacetic acid (TFA). Fractions were collected, and ACN was removed by a Speed Vac concentrator (SC110A, Savant). Before testing the bacteriocin activity against *Lb. sakei* subsp. *sakei* JCM1157, the pH of the solution was adjusted to 6.5 using 50 mM potassium phosphate buffer (50 mM $K_2HPO_4$, 50 mM $KH_2PO_4$, pH 6.5).

The active fraction was further purified by cation-exchange chromatography (Sartobind® Membrane Adsorber Unit matrix S-type exchanger, S15, Sartorius Stedim Biotech GmbH, Germany). Elution was performed by using 0, 0.2 and 1 M NaCl in 10 mM potassium phosphate buffer (pH 7). Fractions were collected, and bacteriocin activity was tested against *Lactobacillus sakei* subsp. *sakei* JCM1157 by agar well diffusion assay.

The active fraction was loaded on a Sep-Pak $C_8$ 12 cc Vac (5 g) Cartridge (Waters Millipore) equilibrated with ACN for desalting. Elution was performed in steps using different concentrations of acetonitrile (0 and 100 % ACN) with 0.05 % TFA. Fractions were collected, and ACN was removed by a Speed Vac concentrator. Before testing the bacteriocin activity against *Lb. sakei* subsp. *sakei* JCM1157, the pH of the solution was adjusted to 6.5 using 50 mM potassium phosphate buffer.

The active fraction was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Waters Alliance apparatus with Millennium software (Millford, MA, USA). One hundred microliters of concentrated bacteriocin was injected into an analytical RP Nucleosil $C_8$ column (Symmetry® $C_8$, 3.5 μm, Ireland) equilibrated with solvent A (1 % ACN, 99 % $H_2O$ and 0.05 % TFA). Elution was performed at a flow rate of 0.5 mL/min with a linear gradient from 0 to 100 % solvent B (20 % isopropanol, 80 % ACN and 0.03 %TFA). The eluted peaks were detected by spectrophotometry, measuring the absorbance between 210 and 300 nm with a photodiode array detector (PDA996, Waters) and were collected manually. The active fractions were then concentrated in a Speed Vac concentrator. All fractions were recovered and resuspended in 0.1 M potassium phosphate buffer (pH 7), and bacteriocin activity was tested against *Lb. sakei* subsp. *sakei* JCM1157 by agar well diffusion assay as described before. The active fraction was reinjected onto the same column in the same conditions in order to improve the quality of the purification and checked again for its activity.

## Results and discussion

### Isolation and pre-identification of LAB

LAB originally isolated from foods are probably the best candidates for improving the microbiological safety of foods, because they are well adapted to the conditions in these kinds of foods and should therefore be more competitive than LAB from other sources. Six hundred and twenty-three isolates were randomly isolated from Egyptian dairy products collected from different regions. The isolates were classified into rods (180 LAB isolates) and cocci (443 LAB isolates), demonstrating the dominance of cocci as compared to rods (Table 2), which is in agreement with the results of El-Soda et al. [1] and El-Baradei et al. [37].

### Antimicrobial activity

The antimicrobial activity of CFS (623 isolates) was determined against *Lb. bulgaricus* 340. Thirty-five isolates showed antimicrobial activity against *Lb. bulgaricus* 340 after incubation for 24 h at 37 °C. The activity is represented by the diameters of the zones of inhibition in mm: high activity (inhibition zone > 6 mm), medium activity (inhibition zone = 3–6 mm) and low activity (inhibition zone < 3 mm). Isolates can be classified into three groups, high (six isolates), medium (nine isolates) and low activity (20 isolates). Isolates represented rods (six isolates) and cocci (29 isolates).

The active isolates (35 isolates) against *Lb. bulgaricus* 340 were tested further for their inhibitory spectra against strains shown in Table 3, revealing 22 isolates with inhibitory activities against *Lb. brevis* F104, 18 isolates with inhibitory activities against *Lb. brevis* F145, 13 isolates with inhibitory activities against *Lb. sakei*, eight isolates with inhibitory activities against *L. ivanovii* ATCC and six isolates with inhibitory activities against *L. innocua* CIP80.11. These indicator strains were grown in different media as shown in Table 3.

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**Table 2** Pre-identification of LAB isolates in milk and dairy products collected from Egyptian local regions

| City | Milk and dairy products | Number of samples | Bacteria | | LAB | |
|---|---|---|---|---|---|---|
| | | | Cocci | Rods | Cocci | Rods |
| Alexandria | Buffalo milk | 5 | 30 | 13 | 21 | 7 |
| | Cow milk | 5 | 32 | 11 | 24 | 6 |
| | Goat milk | 5 | 13 | 5 | 10 | 3 |
| | Sheep milk | 5 | 21 | 9 | 15 | 7 |
| | Ras cheese | 5 | 13 | 4 | 11 | 2 |
| | Cheddar cheese | 3 | 7 | 0 | 5 | 0 |
| | Domiatti cheese | 5 | 23 | 2 | 18 | 1 |
| | Kareish cheese | 3 | 17 | 6 | 10 | 3 |
| | Laban Rayeb | 4 | 17 | 4 | 15 | 2 |
| Kafr El-Sheikh | Buffalo milk | 5 | 28 | 15 | 18 | 10 |
| | Cow milk | 5 | 31 | 12 | 21 | 8 |
| | Goat milk | 5 | 15 | 6 | 9 | 3 |
| | Sheep milk | 5 | 18 | 7 | 11 | 5 |
| | Ras cheese | 5 | 7 | 6 | 4 | 4 |
| | Cheddar cheese | 3 | 8 | 2 | 6 | 1 |
| | Domiatti cheese | 5 | 43 | 18 | 36 | 12 |
| | Kareish cheese | 3 | 29 | 16 | 20 | 14 |
| | Laban Rayeb | 4 | 31 | 5 | 20 | 3 |
| El-Mehala | Buffalo milk | 5 | 40 | 15 | 28 | 12 |
| | Cow milk | 5 | 24 | 11 | 16 | 9 |
| | Goat milk | 5 | 12 | 9 | 6 | 7 |
| | Sheep milk | 5 | 11 | 6 | 7 | 5 |
| | Ras cheese | 5 | 30 | 7 | 24 | 6 |
| | Cheddar Cheese | 3 | 10 | 4 | 7 | 3 |
| | Domiatti cheese | 5 | 48 | 22 | 38 | 19 |
| | Kareish cheese | 3 | 25 | 20 | 16 | 17 |
| | Laban Rayeb | 4 | 32 | 13 | 27 | 11 |
| | Total | 120 | 615 | 248 | 443 | 180 |

**Table 3** Inhibitory effects of LAB supernatants on Gram-positive indicator microorganisms

| Indicator strains | Source | Media | Temperature of growth (°C) | Number of the supernatant of isolates inhibiting each indicator |
|---|---|---|---|---|
| *Lactobacillus bulgaricus* 340 | Rhodia food | MRS | 37 | 35 |
| *Lactobacillus brevis* F104 | INRA | MRS | 37 | 22 |
| *Lactobacillus brevis* F145 | INRA | MRS | 37 | 18 |
| *Lactobacillus sakei* subsp. *sakei* JCM1157 | INRA | MRS | 37 | 13 |
| *Listeria ivanovii* ATCC | INRA | BHI | 37 | 8 |
| *Listeria innocua* CIP80.11 | ONIRIS | BHI | 37 | 6 |

*ATCC* American Type Culture Collection

*INRA* Institut National de la Recherche Agronomique, Nantes, France

*ONIRIS* Ecole Nationale Nantes Atlantique Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes, France

## Identification of isolates by amplification and sequencing of 16S rRNA gene

The six cocci isolates with inhibitory activities against *L. ivanovii* ATCC and *L. innocua* CIP80.11 were also the most active against all other tested strains. The six antimicrobial active cocci isolates were identified by 16S rRNA gene amplification and sequencing yielding two different strains, *E. faecium* IM1 (five isolates) and *E. hirae* IM1 (one isolate). In agreement with these results, other authors

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described numerous enterococcal strains associated with food systems, mainly *E. faecium* able of producing a variety of enterocins with activity against *Listeria* spp. [8, 33, 34, 38–40]. The observed anti *Listeria* activity supports a potential role of enterococci in preventing listeriosis. The resistance of enterococci to pasteurization temperatures and their adaptability to different substrates and growth conditions (low and high temperatures, extreme pH and salinity) imply that they can be found either in food products manufactured from raw materials (milk or meat) or in heat-treated food products. That means that these bacteria could withstand usual conditions of food production [5].

## Characterization of the behavior of the antimicrobial compounds

In our study, the stability of the antimicrobial components produced by the selected strains (*E. faecium* IM1 and *E. hirae* IM1) was investigated.

### *Effect of enzymes on antimicrobial activity*

In general, proteinases destroy or reduce the antimicrobial activity of CFS if it depends on proteinaceous agents. The activity of the antibacterial compounds produced by *E. faecium* IM1 against *Lb. bulgaricus* 340 disappeared after addition of α-chymotrypsin and was reduced after action of trypsin. Addition of proteinase K leads to a decrease in antimicrobial activity of *E. faecium* IM1 while that of *E. hirae* IM1 disappeared (Table 4). These results indicate that the antimicrobial substances produced by these isolates are of proteinaceous nature and belong to bacteriocins [33].

When the pH of the supernatants was readjusted to 6.5 and catalase was added, there was no reduction in inhibition for *E. hirae* IM1, but a little reduction was observed in the case of *E. faecium* IM1 (Table 4). This indicates that the inhibitory activity is not due to hydrogen peroxide in the case of *E. hirae* IM1, but the strain *E. faecium* IM1 may excrete simultaneously different antimicrobial compounds such as hydrogen peroxide and bacteriocins.

Additionally, the inhibiting activity did not change after action of α-amylase and lipase (Table 4). Our results remain in agreement with those of Ponce et al. [38], Ben Belgacem et al. [41] and Ahmadova et al. [42]. Ponce et al. [38] found that bacteriocin-like substances from *E. hirae* and *E. faecium* from organic leafy vegetables were sensitive to the action of proteases. Ben Belgacem et al. [41] found that the inhibitory activity was lost after 2 h of treatment with proteinase K, trypsin and α-chymotrypsin, whereas it was not affected by catalase. Ahmadova et al. [42] found that antimicrobial activity was stable after treatment of supernatant with α-amylase, lipase and catalase, but it was reduced totally or partially when treated with proteinases.

**Table 4** Effect of enzymes, pH and heat stability on the antimicrobial activity of *E. faecium* IM1 and *E. hirae* IM1 strains isolated from Egyptian dairy products against *Lb. bulgaricus* 340 (data for three replicates)

| Isolated strains | *E. faecium* IM1 | *E. hirae* IM1 |
|---|---|---|
| *Enzymes* | | |
| Control positive | +++ | +++ |
| Control negative | – | – |
| α-Chymotrypsin | – | – |
| Trypsin | + | + |
| Proteinase K | + | – |
| Catalase | ++ | +++ |
| Lipase | +++ | +++ |
| α-amylase | +++ | +++ |
| *pH* | | |
| Control positive | +++ | +++ |
| Control negative | – | – |
| 2 | + | ++ |
| 5 | +++ | +++ |
| 8 | +++ | +++ |
| 10 | + | + |
| *Heat treatments* | | |
| Control positive | +++ | +++ |
| Control negative | – | – |
| 100 °C/10 min | +++ | +++ |
| 100 °C/20 min | +++ | +++ |
| 100 °C/30 min | ++ | ++ |
| 121 °C/20 min | – | + |

Activity is represented as diameters of inhibition zones in mm

–, no inhibition; +, inhibition zone < 3 mm; ++, inhibition zone 3-6 mm; +++, inhibition zone > 6 mm

### *Effect of pH on antimicrobial activity*

These assays were performed using supernatants corresponding to the LAB strains grown overnight at 37 °C. The obtained results show that the activities of the supernatants produced by cultures of *E. faecium* IM1 and *E. hirae* IM1 were stable between pH 5 and pH 8 (Table 4), but their activities decreased at pH 10. Our results are in agreement with those obtained by Yoon et al. [43] and Line et al. [44] and are similar to those presented by Ben Belgacem et al. [41] but differed with those found by Ahmadova et al. [42]. Yoon et al. [43] found that the bacteriocin produced by *E. faecium* was also stable until pH 8 and then decreased at pH 10. Line et al. [44] found that the enterocin isolated from *E. durans* and *E. faecium* was stable in pH range between 5 and 8.7. Ben Belgacem et al. [41] found that the stability of antibacterial substances was unchanged in a wide pH range from 3 to 9, with a maximum of activity between pH 6 and 7. Ahmadova et al. [42] found that the antimicrobial

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activity was stable over a wide range of pH from 3 to 10. These differences may be due to the differences between strains.

*Effect of heat treatment on antimicrobial activity*

Antagonistic activity of *E. faecium* IM1 and *E. hirae* IM1 against *Lb. bulgaricus* 340 was stable at 100 °C for 10 or 20 min (Table 4). It decreased after heating for 30 min. After autoclaving, the antimicrobial activity of *E. faecium* IM1 disappeared totally. However, *E. hirae* IM1 still conserved a slight activity. This could allow its use in foods subjected during processing to heat treatment such as pasteurization. Our results remain in agreement with those of Yoon et al. [43], Ben Belgacem et al. [41] and Cintas et al. [20] but disagree with those of Ahmadova et al. [42] who found that the crude antimicrobials were stable during 30 min at temperatures below 90 °C and that *E. faecium* activity disappeared totally after autoclaving. Ben Belgacem et al. [41] found that the antibacterial substances were stable after a treatment at 100 °C for 15 min but that their activity was reduced after autoclaving. Cintas et al. [20] found that *E. faecium* P13 is heat resistant at 100 °C for 15 min. Ahmadova et al. [42] found that the activity for *E. faecium* AQ71 was stable after treatment in autoclave (121 °C/15 min). The highest stability of isolated strains enterocin could be a good criterion for its use as a food preservative under different hard conditions of food processing including pasteurization, heat treatments, freezing and long storage period.

Determination of LAB growth for maximum bacteriocin production

For *E. faecium* IM1, the profile of the antimicrobial activity of the supernatant was tested against *Lb. bulgaricus* 340. The antimicrobial activity was initially detected in exponential phase of growth, and the maximal levels of antimicrobial activity (1,300 AU/mL) were obtained at 12 h and remained stable till the end of incubation time (48 h) (Fig. 1a). In case of *E. hirae* IM1, the maximum of activity was observed in the logarithmic phase (6 h) and then decreased after 12 h (Fig. 1b). The pH dropped to approximately 5.6 during the same period (24 h). In a similar study on *E. faecalis* strain RJ-11, Yamamoto et al. [34] found that the amount of bacteriocin in the fluid culture reached the maximum level in the early stationary phase. These differences may be due to growth medium pH, changes in the medium composition and to the adsorption of bacteriocin on the cell walls of producing microorganisms. This effect may well be the dominating mechanism causing a decline of bacteriocin activity followed by the proteolysis [38]. These results agree well with those found by Ahmadova et al. [42].

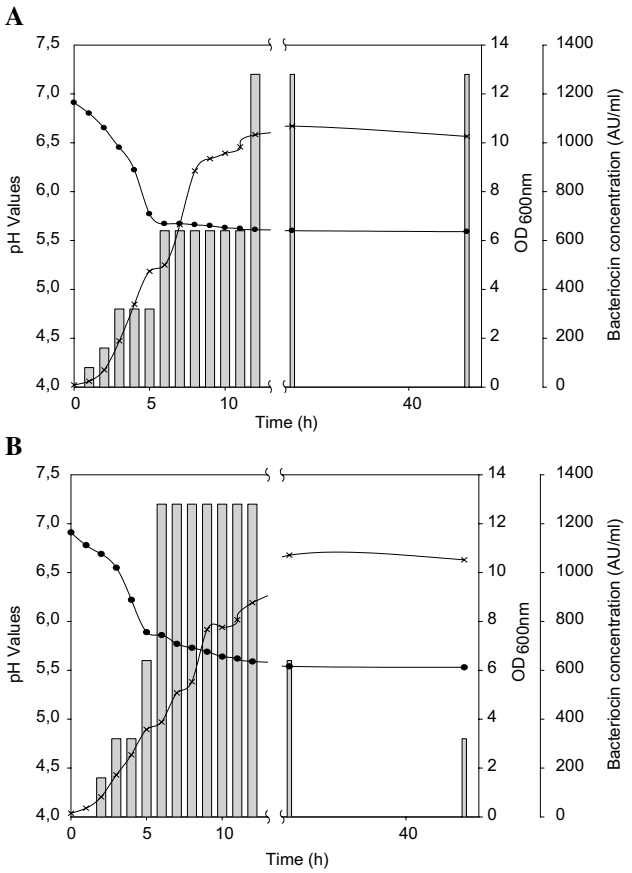


**Fig. 1** Time course of bacteriocin production during the growth of *E. faecium* IM1 (**a**) and *E. hirae* IM1 (**b**) in M17 and MRS broth, respectively, at 37 °C. Optical density (*x*) of culture was measured at 600 nm. Bacteriocin concentration (*bars*) is expressed in arbitrary units per milliliter (AU/mL), and pH is indicated (*circle*). The indicator strain was *Lb. bulgaricus* 340

Detection of virulence factors by PCR amplification of 16S rRNA gene

For safety requirements, it is indispensable to prove the absence of virulence gene factors and transferable antibiotic resistance in the studied strains (*E. faecium* IM1 and *E. hirae* IM1) enabling their acceptable and secure applications as starter cultures or co-cultures in food systems. A virulence factor is an effector molecule that enhances the ability of microorganisms to cause disease beyond that intrinsic to the species background [45]. The presence of five known virulence factors [*asal* (aggregation substance), *cylA* (cytolysin), *ace* (collagen-binding protein), *gelE (gelatinase)* and $esp_{fm}$ (extracellular or enterococcal surface protein)] was tested for two enterococci strains studied by PCR amplification (Fig. 2). Comparison of the electrophoretic patterns of the tested virulence factors in the two studied strains (*E. faecium* IM1 and *E. hirae* IM1) with positive control strain (*E. faecalis* MM4594) indicates that they

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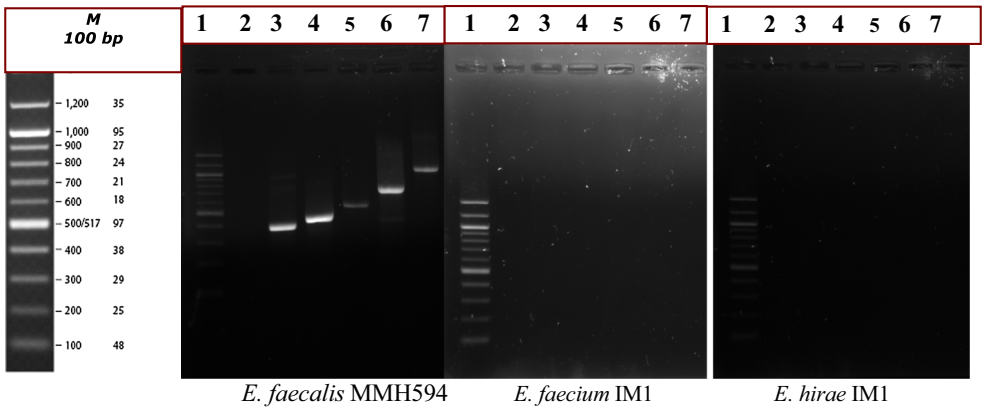


**Fig. 2** PCR product of *E. faecium* IM1, *E. hirae* IM1 and control strain *E. faecalis* MMH594. *Lane 1* molecular marker (100 bp), *lane 2* negative control, *lane 3 asa1*(375 bp), *lane 4 gelE* (419 bp), *lane 5 esp* (510 bp), *lane 6 cylA* (688 bp), *lane 7 ace* (1008 bp)

were free from tested virulence genes. This is in accordance with previous results on similar strains. Generally, *E. faecium* strains of food origin were reported free of the virulence factors with some exceptions [26, 39, 42, 46, 47]. Eaton and Gasson [46] investigated the incidence of known virulence determinants in starter, food and medical strains of *E. faecalis*, *E. faecium* and *E. durans*. *E. faecium* strains were generally free of virulence determinants. They suggested that the use of *Enterococcus* spp. in foods requires careful safety evaluation. Barbosa et al. [27] found that isolated *E. faecium* were generally free of virulence determinants. Ben Belgacem et al. [41] investigated 24 isolates from *E. faecium* for the presence of six known virulence determinants [*asal*, *cylA*, *cylB* and *cylM*, *ace*, $efaA_{fs}$ and $esp_{fm}$ and *gelE*] by PCR and found that ten of these isolates were free from all tested virulence factors. Favaro et al. [47] tested four strains of *E. faecium* and found them free of tested virulence factors [*asal*, *cylA*, *ace*, $efaA_{fs}$ and *gelE*]. Ahmadova et al. [42] found that the cytolysin biosynthetic genes *cylA*, *cylB* and *cylM* were mostly present in clinical isolates of *E. faecium* but not detected in *E. faecium* strain isolated from foods. The absence of cytolysin coding genes (*cylA* and *cylB*) is a positive observation in case of food application. Generally, cytolysin is a bacterial toxin expressed by some isolates of *E. faecalis* that display both hemolytic and bactericidal activities [42]. Moreover, tested strains *E. faecium* IM1 and *E. hirae* IM1 did not show any hemolytic and gelatinase activities (data not shown).

## Antibiotic resistance

The potential role of enterococci as reservoirs of antibiotic resistance genes that can be spread to other strains or species is a matter of concern [48]. The most important factor for the safety evaluation of *Enterococci* spp. is its resistance to glycopeptides such as vancomycin [43]. The resistance or sensitivity depends on minimum inhibitory concentration (MIC) breakpoints as described by the European Food Safety Authority [36]. The results showed that *E. faecium* IM1 was sensitive to penicillin, kanamycin, vancomycin, gentamicin and tetracycline but resistant to ampicillin. Otherwise, *E. hirae* IM1 was sensitive to penicillin, kanamycin, vancomycin and tetracycline but resistant to ampicillin and gentamicin. Resistance of *E. hirae* IM1 to gentamicin and ampicillin could be a disadvantage for its further application as biopreservative in food system. Similar results were observed by Peters et al. [49] and Valenzuela et al. [48]. Peters et al. [49] determined species distribution and antibiotic resistance pattern of enterococci isolated from food of animal origin in Germany. They found that all of studied strains (299 *E. faecalis*, 54 *E. faecium*, 24 *E. durans*, 22 *E. casseliflavus*, 9 *E. avium* and 8 *E. gallinarum*) were sensitive to vancomycin. Valenzuela et al. [48] focused on enterococci isolated from foods (meat, dairy and vegetables foods) in Morocco and determined the incidence of virulence factors and antibiotic resistance. They tested 23 *E. faecalis* and 15 *E. faecium* isolates and found that all isolates were sensitive to penicillin and gentamicin. However, *E. faecium* isolates showed a very low percentage (6.66 %) of resistance to tetracycline. Favaro et al. [47] tested four strains of *E. faecium*, which were sensitive to vancomycin, but for penicillin and tetracycline, the reaction was not specified.

## Partial purification of bacteriocins

The partial purification of the bacteriocins produced by *E. faecium* IM1 and *E. hirae* IM1 was achieved by the four-step method. The first step was a precipitation by ammonium sulfate (70 % saturation), which increased the activity about tenfolds. The antimicrobial activity was tested against different pathogenic bacteria. Data in Table 5 show

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**Table 5** Antimicrobial spectrum of partially purified bacteriocin of *E. faecium* IM1 (A) and *E. hirae* IM1 (B) by using agar well diffusion assay

| Indicator strains | Sources | Growth conditions (°C/h) (BHI media) | Antimicrobial activity[c] | |
|---|---|---|---|---|
| | | | A | B |
| *Bacillus subtilis* 168T | ONIRIS[a] | 37 °C/24 h | − | − |
| *Brochothrix thermosphacta* DSMZ20171$^T$ | ONIRIS | 25 °C/24 h | ++ | ++ |
| *Brochothrix thermosphacta* DSMZ20599 | ONIRIS | 25 °C/24 h | ++ | ++ |
| *Carnobacterium maltaromaticum* CIP103135$^T$ | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Carnobacterium piscicola* S4312 | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Enterococcus faecalis* JH2-2 | ONIRIS | 37 °C/24 h | + | + |
| *Enterococcus faecalis* JH2-2rr04 | ONIRIS | 37 °C/24 h | + | + |
| *Enterococcus faecium* CTC492/t136 2 | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Enterococcus faecium* P13 | ONIRIS | 30 °C/24 h | + + | ++ |
| *Listeria grayi* CLIP12518 | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria innocua* 1 | ONIRIS | 30 °C/24 h | + | ++ |
| *Listeria innocua* CIP80.11$^T$ | ONIRIS | 30 °C/24 h | ++ | + |
| *Listeria innocua* F | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria innocua* P | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria ivanovii* | ONIRIS | 30 °C/24 h | ++ | + |
| *Listeria ivanovii* CIP78.42T | ONIRIS | 30 °C/24 h | + | ++ |
| *Listeria monocytogenes* | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* CIP78.35 | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* DSM12464 | ONIRIS | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* EGDe | ONIRIS | 30 °C/24 h | + | + |
| *Listeria monocytogenes* RF124 | IFREMER[b] | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF125 | IFREMER | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF131 | IFREMER | 30 °C/24 h | ++ | + |
| *Listeria monocytogenes* RF132 | IFREMER | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF133 | IFREMER | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF142 | IFREMER | 30 °C/24 h | + | + |
| *Listeria monocytogenes* RF151 | IFREMER | 30 °C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF152 | IFREMER | 30 °C/24 h | ++ | + |
| *Moellerella wisconsensis* MIP2451 | ONIRIS | 20 °C/24 h | – | – |
| *Morganella psychrotolerans* MIP2488 | ONIRIS | 20 °C/24 h | – | – |
| *Pseudomonas fluorescens* 10 | ONIRIS | 28 °C/24 h | − | − |
| *Salmonella enterica* | ONIRIS | 37 °C/24 h | − | − |
| *Salmonella montevideo* | ONIRIS | 37 °C/24 h | − | − |
| *Salmonella typhimurium* | ONIRIS | 37 °C/24 h | − | − |
| *Serratia liquefaciens* CIP103238$^T$ | ONIRIS | 25 °C/24 h | − | − |
| *Vagococcus penaei* CIP109914$^T$ | ONIRIS | 30 °C/24 h | ++ | + |

[a] *ONIRIS* Ecole Nationale Nantes Atlantique Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes, France

[b] *IFREMER* Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France

[c] Wells (5 mm diameter) were filled with 50 ml of culture supernatant. Diameter of inhibition zone (mm). −, no inhibition zone; +, zone < 3 mm; ++, zone 3–6 mm; +++, zone 7–10 mm; ++++, zone > 10 mm

that *E. faecium* IM1 and *E. hirae* IM1 are active against *B. thermosphacta* DSMZ20171$^T$ and DSMZ20599, *C. maltaromaticum* CIP103135$^T$, *C. piscicola* S4312, *E. faecalis* JH2-2 and JH2-2rr04, *E. faecium* CTC492/t136 2 and P13, *L. grayi* CLIP12518, *L. innocua* 1, CIP80.11$^T$, F, P, *L. ivanovii*, *L. ivanovii* CIP78.42$^T$, *L. monocytogenes*, *L. monocytogenes* CIP78.35, DSM12464, EGDe, RF124, RF125, RF131, RF132, RF133, RF142, RF151 and RF152, and *Vagococcus penaei* CIP109914$^T$. However, they have no effect on *B. subtilis* 168$^T$, *Moellerella wisconsensis*

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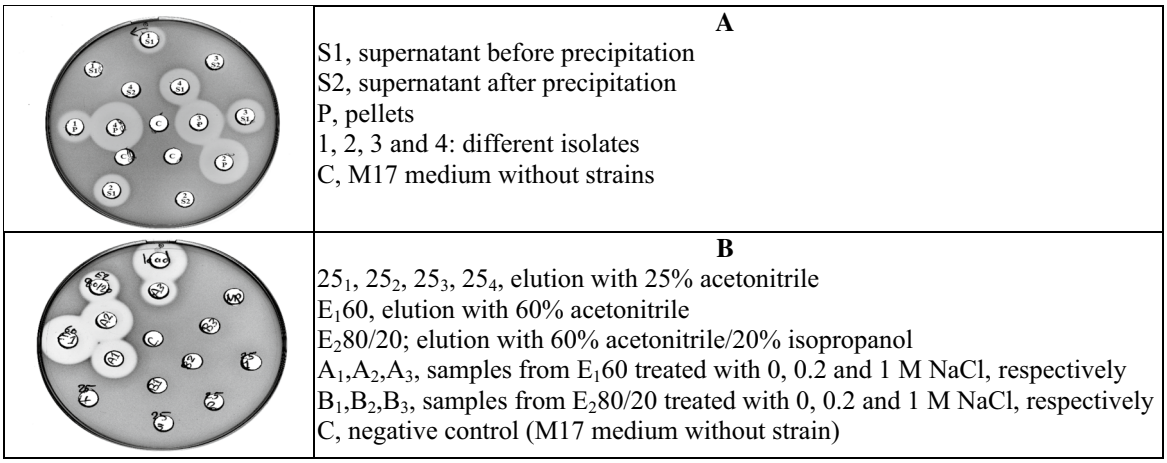


**Fig. 3** Antimicrobial activity against *Lb. sakei* subsp. *sakei* JCM1157 of fractions obtained from supernatant of *E. faecium* IM1 after loading in Sep-Pak $tC_{18}$ 12 cc Vac Cartridge 5 g and cation-exchange column

MIP2451, *Morganella psychrotolerans* MIP2488, *Pseudomonas fluorescens* 10, *Sal. enterica*, *montevideo* and *typhimurium*, and *Serratia liquefaciens* CIP103238$^T$.

The inhibitory spectrum showed a wide range of antibacterial activity against similar bacterial strains, foodborne pathogens and food spoilage bacteria, indicating that it could be a suitable biopreservative to increase the safety and extending the storage life of various food products. In a similar study of Kang and Lee [50], a bacteriocin produced by *E. faecium* GM-1 isolated from an infant was characterized. The bacteriocin showed a broad spectrum of activity against strains of *L. monocytogenes*. Kumar and Srivastava [51] reported that Enterocin LR/6, a purified bacteriocin, exhibited a broad inhibitory spectrum against some foodborne pathogens comprising both Gram-positive and Gram-negative such as *L. monocytogenes*, as well as against several strains of LAB.

The antimicrobial activity of the supernatant extracted from *E. faecium* IM1 before and after precipitation with ammonium sulfate was tested against *Lb. sakei* subsp. *sakei* JCM1157 (Fig. 3). In the second step, the active fraction was separated on a reversed-phase cartridge (Sep-Pak $tC_{18}$ 12 cc Vac Cartridge 5 g). Three fractions were collected and checked for their antibacterial activity by the agar well diffusion assay. The fraction eluted with 60 % ACN showed the highest activity against *Lb. sakei* subsp. *sakei* JCM1157 (Fig. 3). In the third step, the active fractions ($E_1$ 60 % ACN and $E_2$ 80 % ACN/20 % isopropanol) were applied on a cation-exchange column. The fraction eluted with 0.2 M NaCl showed the highest activity against the indicator strain (Fig. 3). In the fourth step, the active fraction was applied on a RP-HPLC column. Two peaks (peak 1 and peak 2, Fig. 4a) were individually collected. The two fractions corresponding to peaks 1 and 2 with retention times of 23 and 25 min showed activity against the

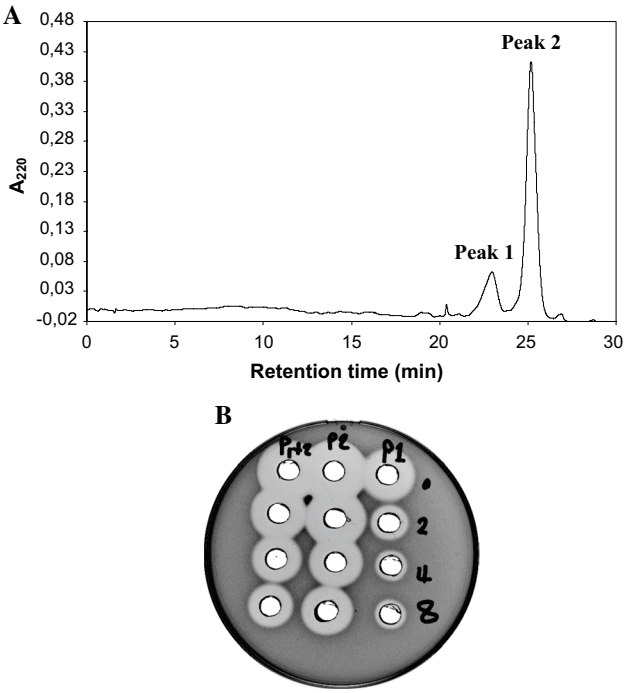


**Fig. 4** **a** Reversed-phase chromatograms of two peaks of bacteriocin of *E. faecium* IM1. The bacteriocin was applied to RP-HPLC (Symmetry $C_8$ 3.5 µm). **b** Activity of two peaks [separated (P1, P2) or together (P1 + 2)] was measured on MRS agar against *Lb. sakei* subsp. *sakei* JCM1157 after 24 h of incubation at 37 °C. First row: crude fractions, 2: diluted twofold; 4: diluted fourfold; 8: diluted eightfold

indicator strain. According to this result, the combinations of peaks 1 and 2 were tested against *Lb. sakei* subsp. *sakei* JCM 1157 (Fig. 4b). It was found that the combination of peaks (P1 + 2) did not show synergy as compared to that of peak 1 or peak 2 tested individually. Finally, these active fractions were concentrated and rechromatographed on the

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same column to check their purity. Each successive purification step increased the specific activity against *Lb. sakei* subsp. *sakei* JCM1157 and reduced the amount of contaminating non-bacteriocin proteins.

## Conclusion

In the present study, two active strains (*E. faecium* IM1 and *E. hirae* IM1) among 623 isolates of LAB studied were isolated from Egyptian dairy products. Bacteriocin-producing *E. faecium* IM1 and *E. hirae* IM1 did not present any genes coding virulence factors neither transferable antibiotic resistance (especially vancomycin resistance genes). *E. hirae* IM1 was resistant to gentamicin and ampicillin what could be a disadvantage for its further application as biopreservative in food system. Consequently, additional experiments are required to confirm that selected strains might be applied in fermented dairy foods as efficient biopreservatives cultures.

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**Conflict of interest** None.

**Compliance with Ethics Requirements** This article does not contain any studies with human or animal subjects.

## References

1. El-Soda M, El-Ziney M, Awad S, Osman G, Omran N, Gamal G, Ezzat N, El-Shafei H (2003) A culture collection of lactic acid bacteria isolated from raw milk and traditional Egyptian dairy products. Egypt J Dairy Sci 31:23–41
2. Ayad EHE, Nashat S, El-Sadek N, Metwaly H, El-Soda M (2004) Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Micro 21:715–725
3. Sánchez-González L, Saavedra JIQ, Chiralt A (2014) Antilisterial and physical properties of biopolymer films containing lactic acid bacteria. Food Control 35:200–206
4. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12:39–86
5. Foulquié Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of *Enterococci* in food and health. Int J Food Microbiol 106:1–24
6. Zouhir A, Hammami R, Fliss I, Ben Hamida J (2010) A new structure-based classification of Gram-positive bacteriocins. Protein J 29:432–439
7. Franz CM, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety. Int J Food Microbiol 47:1–24
8. Giraffa G (2003) Functionality of enterococci in dairy products. Int J Food Microbiol 88:215–222
9. Ayad EHE (2009) Starter culture development for improving safety and quality of Domiati cheese. Food Microbiol 26:533–541
10. El-Ghaish S, Hadji-Sfaxi I, Ahmadova A, Choiset Y, Rabesona H, Sitohy M, Haertlé T, Chobert J-M (2011) Characterization of two safe *Enterococcus* strains producing enterocins isolated from Egyptian dairy products. Benef Microbes 2:15–27
11. Hadji-Sfaxi I, El-Ghaish S, Ahmadova A, Batdorj B, Le Blay-Lalibré G, Barbier G, Haertlé T, Chobert J-M (2011) Antimicrobial activity and safety of use of *Enterococcus faecium* PC4.1. Food Control 22:2020–2027
12. Centeno JA, Menendez S, Rodriguez-Otero JL (1996) Main microbial flora present as natural starters in Cebreiro raw cow's milk cheese (Northwest Spain). Int J Food Microbiol 33:307–313
13. Litopoulou-Tzanetaki E, Tzanetakis N, Vafopoulou-Mastrojiannaki A (1993) Effect of type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese. Food Microbiol 10:31–41
14. Bover-Cid S, Hugas M, Izquierdo-Pulido M, Vidal-Carou MC (2001) Amino acid decarboxylase activity of bacteria isolated from fermented pork sausages. Int J Food Microbiol 66:185–189
15. Advisory Committee on Novel Foods and Processes (ACNFP) (1997) Report on *Enterococcus faecium*, strain K77D. Ergon House c/o Nobel House, 17 Smith square, London SW1 3JR, UK
16. International Dairy Federation (IDF) (1997) Dairy starter cultures lactic acid bacteria (LAB), Standard 149A. International Dairy Federation, Brussels
17. Aymerich T, Holo H, Havarstein LS, Hugas M, Garriga M, Nes IF (1996) Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl Environ Microbiol 62:1676–1682
18. Aymerich T, Garriga M, Ylla J, Vallier J, Monfort JM, Hugas M (2000) Application of enterocins as biopreservatives against *Listeria innocua* in meat products. J Food Prot 63:721–726
19. Franz CM, van Belkum MJ, Holzapfel WH, Abriouel H, Gálvez A (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31:293–310
20. Cintas LM, Casaus P, Havarstein LS, Hernandez PE, Nes IF (1997) Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl Environ Microbiol 63:4321–4330
21. Ennahar S, Asou Y, Zendo T, Sonomoto K, Ishizaki A (2000) Biochemical and genetic evidence for production of enterocins A and B by *Enterococcus faecium* WHE 81. Int J Food Microbiol 70:291–301
22. van Belkum MJ, Hayema BJ, Jeeninga RA, Kok J, Venema G (1991) Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl Environ Microbiol 57:493–498
23. Tahara T, Yoshioki S, Utsumi R, Kanatani K (1997) Isolation and partial characterization of bacteriocin produced by *Lactobacillus gasseri* JCM 2124. FEMS Microbiol Lett 148:97–100
24. Floriano B, Ruiz-Barba JL, Jiménez-Diaz R (1998) Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl Environ Microbiol 64:4883–4890
25. Cintas LM, Casaus P, Herranz C, Håvarstein LS, Holo H, Hernandez PE, Nes IF (2000) Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the sec-dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J Bacteriol 182:6806–6814
26. Giraffa G (2002) Enterococci from foods. FEMS Microbiol Rev 26:163–171

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27. Barbosa J, Gibbs PA, Teixeira P (2010) Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21:651–656
28. Vankerckhoven V, van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H (2004) Development of a multiplex PCR for the detection of $asa_1$, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J Clin Microbiol 42:4473–4479
29. Delley M, Mollet B, Hottinger H (1990) DNA probe for *Lactobacillus delbrueckii*. Appl Environ Microbiol 56:1967–1970
30. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic analysis. J Bacteriol 173:697–703
31. Schillinger U, Lucke F (1989) Antimicrobial activity of *Lactobacillus sakei* isolated from meat. Appl Environ Microbiol 55:1901–1906
32. Noonpakee W, Santivarangkna C, Jumriangrit P, Sonomoto K, Panyim S (2003) Isolation of nisin-producing *Lactococcus lactis* WNC20 strain from nham, a traditional Thai fermented sausage. Int J Food Microbiol 81:137–145
33. Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Métro F, Prévost H, Chobert JM, Haertlé T (2006) Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. J Appl Microbiol 101:837–848
34. Yamamoto Y, Togawa Y, Shimosaka M, Okazaki M (2003) Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. Appl Environ Microbiol 69:5746–5753
35. Karmarkar MG, Gershom ET, Mehta PR (2004) Enterococcal infections with special reference to phenotypic characterization and drug resistance. Indian Med Res 119:22–25
36. European Food Safety Authority (2008) Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. Eur Food Saf Auth 732:1–15
37. El-Baradei G, Delacroix-Buchet A, Ogier JC (2008) Bacterial biodiversity of traditional Zabady fermented milk. Int J Food Microbiol 121:295–301
38. Ponce AG, Moreira MR, del Valle CE, Roura SI (2008) Preliminary characterization of bacteriocin-like substances from lactic acid bacteria from organic leafy vegetables. LWT Food Sci Technol 44:432–441
39. Ben Belgacem Z, Abriouel H, Ben Omar N, Lucas R, Martínez-Canamero M, Gálvez A, Manai M (2010) Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic *Enterococcus faecium* from artisanal Tunisian fermented meat. Food Control 21:462–470
40. de Vuyst L, Vandamme EJ (1994) Antimicrobial potential of lactic acid bacteria. In: de Vuyst L, Vandamme EJ (eds) Bacteriocins of lactic acid bacteria, microbiology, genetics and applications. Blackie Academic & Professional, London, pp 91–142
41. Ben Belgacem Z, Ferchichi M, Prévost H, Dousset X, Manai M (2008) Screening for anti-listerial bacteriocin-producing lactic acid bacteria from "Gueddid" a traditionally Tunisian fermented meat. Meat Sci 78:513–521
42. Ahmadova A, Todorov SD, Choiset Y, Rabesona H, Zadi TM, Kuliyev A, Franco BDGM, Chobert JM, Haertlé T (2013) Evaluation of antimicrobial activity, probiotic properties and safety of wild strain *Enterococcus faecium* AQ71 isolated from Azerbaijani Motal cheese. Food Control 30:631–641
43. Yoon MY, Kim YJ, Hwang HJ (2008) Properties and safety aspect of *Enterococcus faecium* strains isolated from Chungkukjang, a fermented soy product. LWT Food Sci Technol 41:925–933
44. Line JE, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Levchuk VP, Svetoch OE, Seal BS, Siragusa GR, Stern NJ (2008) Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 52:1094–1100
45. Mundy L, Sahm D, Gilmore M (2000) Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev 13:513–522
46. Eaton TJ, Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol 67:1628–1635
47. Favaro L, Basaglia M, Casella S, Hue I, Dousset X, de Melo Franco BDG, Todorov SD (2014) Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese. Food Microbiol 38:228–239
48. Valenzuela AS, Ben Omar N, Abriouel H, López RL, Veljovic K, Cañamero MM, Topisirovic MKL, Antonio G (2009) Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. Food Control 20:381–385
49. Peters J, Mac K, Wichmann-Schauer H, Klein G, Ellerbroek L (2003) Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int J Food Microbiol 88:311–314
50. Kang JH, Lee MS (2005) Characterization of a bacteriocin produced by *Enterococcus faecium* GM-1 isolated from an infant. J Appl Microbiol 98:1169–1176
51. Kumar M, Srivastava S (2010) Antilisterial activity of a broad-spectrum bacteriocin, enterocin LR/6 from *Enterococcus faecium* LR/6. Appl Biochem Biotechnol 162:698–706
52. Ben Omar N, Castro A, Lucas R, Abriouel H, Yousif NM, Franz CM, Holzapfel WH, Pérez-Pulido R, Martínez-Cañamero M, Gálvez A (2004) Functional and safety aspects of enterococci isolated from different Spanish foods. System Appl Microbiol 27:118–130