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# PARTIAL CHARACTERIZATION OF BACTERIOCINS PRODUCED BY TWO STRAINS OF LACTIC ACID BACTERIA, ISOLATED FROM TRADITIONAL EGYPTIAN DAIRY PRODUCTS

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## ABSTRACT

This study presents the characterization of strains of lactic acid bacteria (LAB) from traditional Egyptian dairy products. 623 isolates of lactic acid bacteria (LAB) were studied for their antimicrobial activity against taxonomically related microorganisms. Selected LAB were identified by PCR method as *Enterococcus faecium* IM1and *Enterococcus hirae* IM1. Partial purified enterocin from tested strains showed antimicrobial activity against different indicators such as: *Brochothrix thermosphacta* DSMZ, *Carnobacterium maltaromaticum* CIP103135$^T$, *Carnobacterium piscicola* S4312, *Enterococcus faecalis* JH2, *Listeria grayi* CLIP12518, *Listeria innocua*, *Listeria ivanovii* ATCC**,** *Listeria monocytogenes* and *Vagococcus penaei* CIP109914$^T$ but have no effect against *Bacillus subtilis*, *Moellerella wisconsensis*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Salmonella montevideo*, *Salmonella typhimurium* and *Serratia liquefaciens* CIP103238$^T$. The inhibitory activity was not due to hydrogen peroxide for *Enterococcus hirae* IM1, but strain *Enterococcus faecium* IM1 may excrete diverse antimicrobial compounds such as hydrogen peroxide and bacteriocins. Bacteriocins produced by isolated strains were stable between pH 5 and 8 and stable until 100°C/ 20 min. Tested strains were free from some of virulence determinant genes. They may be useful as starter cultures and co-cultures. Consequently, the selected strains can be applied in fermented dairy foods as efficient biopreservatives cultures.

**Keywords: Enterococci, enterocins, Listeria ssp.**

## INTRODUCTION

In Egypt, a lot of traditional fermented milk and milk products such as: Ras cheese, Domiatti cheese, Kareish cheese, Zabady and Laban Rayeb were existed. All of these products manufactured from pasteurized milks. Ras cheese is a hard cheese, enzymatic coagulation and made of cow milk. Domiatti cheese is a soft cheese, enzymatic coagulation and manufactured of cow's, buffalo's and/or both milks. Kareish cheese is a soft cheese, acidic coagulation and made of skim milk. Zabady and Laben Rayeb are traditional types of yoghurt manufactured in Egypt from buffalo's, cow's and/or both milks (El-Soda *et al*., 2003).

Now, there is an increase in consumer demand for substituted chemical preservatives used in food additives by using natural preservatives such as 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 a lot of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins (Sánchez-González *et al*., 2014).

The bacteriocins produced by LAB were defined as a large group of ribosomally synthesized, amphiphilic, small, cationic, antimicrobial peptides, produced naturally by the microorganisms. They 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) (Klaenhammer, 1993; Foulquié Moreno *et al*., 2006). Recently, Zouhir *et al*. (2010) proposed a new classification based on the structure, which includes 12 classes from bacteriocins.

Enterococci are associated with several types of fermented foods and play important roles in flavor and aroma development. Therefore, it could be used as starters or bio protective cultures (Franz *et al*., 1999; Giraffa, 2003; El-Ghaish *et al*., 2011; Hadji-Sfaxi *et al*., 2011). Enterococci are known to produce enterocins, which are bactericidal peptides against food spoilage and pathogenic bacteria such as *Listeria* ssp., especially against *Listeria monocytogenes*. (Aymerich *et al*.,1996, 2000; Franz *et al*., 2007). This enterocins are divided into two subclasses (IIa and IIb). The enterocin P (*Enterococcus faecium* P13), the enterocin A (*Enterococcus faecium* DPC1146) and enterocin 31 (*Enterococcus faecalis* YI717) are examples of this subclass IIa (Klaenhammer, 1993; Aymerich *et al*., 1996; Cintas *et al*., 1997; Ennahar *et al*., 2000). The enterocin L50 (A and B) (*Enterococcus faecium* L50), the enterocin B (*Enterococcus faecium* T136/C492 and BFE900), the enterocin Q (*Enterococcus faecium* L50), the bacteriocin AS-48 (*Enterococcus faecalis*) are examples of the sub-class IIb (van Belkum *et al*., 1991; Floriano *et al*., 1998; Cintas *et al.* 2000). Few studies however, indicated that entercocci could be applied in the foods as a safe starter (Giraffa, 2002), but it must be free from virulence factors such as aggregation substance protein, gelatinase, cytolysin, enterococcal surface proteins

hyaluronidase, accessory colonization factors and endocarditis antigens (Barbosa *et al*., 2010; Vankerckhoven *et al*., 2004).

The present study was, therefore undertaken 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 food borne pathogenic bacteria. Moreover, partial purification of the isolated antimicrobial substances was attempted, and their safety traits have been determined.

## MATERIALS AND METHODS

**Isolation, purification and identification of LAB cultures**

Samples of raw milk (buffalo, cow, goat, and sheep) and traditional dairy products were obtained from local markets of Alexandria, Kafr El-Sheikh, and El-Mehala. Traditional dairy products included Domiatti cheese, Ras cheese, Kareish cheese and Laban Rayeb. Each samples were collected in sterile cup and was kept at 4ºC until analysis. LAB were isolated and pre-identified by morphological and physiological tests as described with El-Ghaish *et al*. (2011). For purification, the cultures were streaked on suitable media (MRS and M17) 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. DNA was extracted from the isolates according to Delley *et al*. (1990), and was used as a template for 16S rRNA gene amplification. In the reaction, universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-TAAGGAGGTGATCCAGGC-3') were used (Weisburg *et al*., 1991). DNA amplifications were described with El-Ghaish *et al*. (2011).

**Antimicrobial activity**

Indicator strains were grown on different media (MRS and M17) as shown in Tables (3 and 5). For antimicrobial activity assay, the different isolates were reactivated by combining 50 µL of the pre-culture with 950 µL of M17 or MRS broth and incubation at 37 °C for overnight. The antimicrobial activity of cell-free supernatant (CFS) was determined by well diffusion method (Schillinger and Lucke, 1989).A clear zone was accepted as positive.

**Characterization of the antimicrobial compounds**

The effect of various enzymes (proteinase K, trypsin, α-chymotrypsin, catalase and α-amylase) on antibacterial activity against *Lactobacillus bulgaricus* 340 was determined according to Noonpakee *et al.* (2003). Negative control was made with 200 µL M17 broth and 20 µL sterilized water. Positive control was constituted from 200 µL bacterial supernatant and 20 µL sterilized water. The residual antibacterial activity was determined by spot-on-lawn method (Batdorj *et al*., 2006). The stability of antimicrobial agent towards pH was determined as described with El-Ghaish *et al*. (2011) against *Lactobacillus bulgaricus* 340. The stability of antimicrobial agent for heat-treatment was determined as described with El-Ghaish *et al*. (2011) against *Lactobacillus bulgaricus* 340.

For the determination of LAB growth for maximal bacteriocin production, one millilitre of an overnight culture of the tested isolate was added to 30 mL M17 or MRS medium and incubated at 37°C under non-regulated pH conditions. Cell growth was monitored every 1 h by turbidity ($OD_{600nm}$) and pH determination. Antimicrobial activity was calculated according to spot-on-lawn method (Yamamoto *et al.* 2003) and expressed as arbitrary units per mL (AU $mL^{-1}$). The AU of antimicrobial activity per millilitre was defined as $2^n \times 1000$ µL/10 µL.

**Detection of some virulence factors by PCR amplification**

DNA was extracted from overnight *Enterococcus* cultures by using Dneasy Blood & Tissue Kit (Qiagen) using *Enterococcus faecalis* MM4594 as positive control. Primer sequences for five known virulence genes, *asal* (aggregation substance), *gelE* (gelatinase), *$esp_{fm}$* (enterococcal surface protein), *cyl* A (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 described with Vankerckhoven *et al*. (2004); Ben Omar *et al*. (2004).

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

| Gene | Primer | Sequence 5' ⟶ 3' | Product size (bp) | TM | Ref. |
|---|---|---|---|---|---|
| asa*1* | ASA11 | GCACGCTATTACGAACTATGA | 375 | 56°C | 1 |
| | ASA12 | TAAGAAAGAACATCACCACGA | | | |
| *gelE* | gel1 | TATGACAATGCTTTTTGGGAT | 419 | 45°C | 1 |
| | gel2 | AGATGCACCCCAAATAATATA | | | |
| *$esp_{fm}$* | ESP14F | AGATTTCATCTTTGATTCTTGG | 510 | 56°C | 1 |
| | ESP12R | AATTGATTCTTTAGCATCTGG | | | |
| *cyl A* | CYTI | ACTCGGGGATTGATAGGC | 688 | 58°C | 1 |
| | CYTIIb | GCTGCTAAAGCTGCGCTT | | | |
| *ace* | ACE-F | GAATTGAGCAAAAGTTCAATCG | 1008 | 56°C | 2 |
| | ACE-R | GTCTGTCTTTTCACTTGTTTC | | | |

**1: Vankerckhoven et al. (2004)** **2: Ben Omar et al. (2004)**

**Disk diffusion antibiotic sensitivity testing**

The antibiotic resistances of the isolated strains were assessed against ampicillin, penicillin G, kanamycin, vancomycin, gentamicin and tetracycline by disk diffusion antibiotic sensitivity testing. The antibiotic resistances of the isolated strains were described with El-Ghaish *et al*. (2011).

**Purification of bacteriocin**

*Enterococcus faecium* IM1 and *Enterococcus hirae* IM1 were grown in 1 litre of M17 and MRS, respectively, at 37°C for 24 h. Purification of bacteriocin from isolated strains was carried out as described by Hwanhlem *et al*. (2013) with slight modification.

**The modifications are:**

**A:** after centrifugation (20 min at 12,000 x g, 4°C), the pellet was re suspended 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.

**B:** The pellet solution 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).

**C:** 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 and testing the bacteriocin activity against *Lactobacillus sakei* subsp. *sakei* JCM1157, the pH of the solution was adjusted to 6.5 using 50 mM potassium phosphate buffer.

**D:** One hundred microliters of concentrated bacteriocin was injected into an analytical (RP-HPLC) 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).

## RESULTS AND DISCUSSION

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 lactobacilli (Table 2), which is in agreement with the results of El-Soda *et al*. (2003) and of El-Baradei *et al.* (2008). 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. This means that these bacteria could withstand usual conditions of food production (Foulquié Moreno *et al*., 2006).

**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** |

**Antimicrobial activity**

The antimicrobial activity of isolates (623 isolates) was determined against *Lactobacillus bulgaricus* 340 after adjusting the pH to 6.5 with 1 N NaOH. Thirty-five isolates showed antimicrobial activity against *Lactobacillus bulgaricus* 340 after incubation for 24 h at 37°C (Table 3). 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 (6 isolates), medium (9 isolates) and low activity (20 isolates). Isolates represented lactobacilli (6 isolates) and cocci (29 isolates).

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

| Indicator strains | Source | Media | Temp. (°C) | No. |
|---|---|---|---|---|
| *Lactobacillus bulgaricus* 340 | Rhodia Food, France | MRS | 37 | 35 |
| *Lactobacillus brevis* F104 | INRA, Nantes, France | MRS | 37 | 22 |
| *Lactobacillus brevis* F145 | INRA, Nantes, France | MRS | 37 | 18 |
| *Lactobacillus sakei* | INRA, Nantes, France | MRS | 37 | 13 |
| *Listeria ivanovii* ATCC | INRA, Nantes, France | BHI | 37 | 8 |
| *Listeria innocua* CIP80.11 | ONIRIS, Nantes, France | BHI | 37 | 6 |

**ATCC: American Type Culture Collection No.: Number of isolates inhibiting each indicator**
**INRA : Institut National de la Recherche Agronomique Temp.: Temperature of growth**
**ONIRIS: Ecole Nationale Nantes Atlantique Vétérinaire, Agroalimentaire et de l'Alimentation**

The active isolates (35 isolates) against *Lactobacillus bulgaricus* 340 were further tested for their inhibitory spectra against some of indicator strains in Table 3, revealing 22 isolates with inhibitory activities against *Lactobacillus brevis* F104, 18 isolates with inhibitory activities against *Lactobacillus brevis* F145, 13 isolates with inhibitory activities against *Lactobacillus sakei*, 8 isolates with inhibitory activities against *Listeria ivanovii* ATCC, and 6 isolates with inhibitory activities against *Listeria innocua* CIP80.11. These indicator strains were grown in different media as shown in Table 3.

The six cocci isolates with inhibitory activities against *Listeria ivanovii* ATCC and *Listeria innocua* CIP80.11 were also the most active against all other tested strains. These results are in agreement with previous studies indicating that enterococci produce bacteriocins, termed enterocins, which are small peptides with activities against closely related Gram-positive bacteria including spoilage or pathogenic bacteria, such as *Listeria* spp. (Batdorj *et al.*, 2006; Ben Belgacem *et al.*, 2010; de Vuyst and Vandamme, 1994; Giraffa, 2003; Yamamoto *et al*., 2003). The 6 antimicrobial active cocci isolates were identified by 16S rDNA amplification and sequencing yielding two different strains, *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1. In agreement with these results, other authors described numerous Enterococcal strains associated with food systems, mainly *Enterococcus faecium* able of producing a variety of bacteriocins, called enterocins with activity against *Listeria* spp. (Ponce *et al.*, 2008).

**Characterization of the antimicrobial compounds**

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

For enzymes, in general, proteinases destroy or reduce the antimicrobial activity of (cell free supernatant (CFS) if they depend on proteinaceous agents. The antimicrobial activities of *Enterococcus faecium* IM1, and *Enterococcus hirae* IM1 were sensitive to the action of proteases (Table 4).When the pH of the supernatants was readjusted to 6.5 and

catalase was added, there was no reduction of inhibition for *Enterococcus hirae* IM1, but a little reduction was observed in case of *Enterococcus faecium* IM1 (Table 4).

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

| Isolated strains | *Enterococcus faecium* IM1 | *Enterococcus 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 | - | + |

**-, no inhibition; +, inhibition zone < 3mm; ++, inhibition zone 3-6mm; +++, inhibition zone > 6mm. Activity is represented as diameters of inhibition zones in mm**

This indicates that the inhibitory activity is not due to hydrogen peroxide in case of *Enterococcus hirae* IM1 but the strain *Enterococcus faecium* IM1 may excrete simultaneously different antimicrobial compounds such as hydrogen peroxide and bacteriocins. The activity of the antibacterial compounds produced by *Enterococcus faecium* IM1 against *Lactobacillus bulgaricus* 340 was destroyed by α-chymotrypsin, or decreased by proteinase K and trypsin while for *Enterococcus hirae* IM1, the antimicrobial activity against *Lactobacillus bulgaricus* 340 disappeared after action of proteinase K and α-chymotrypsin, but decreased after action of trypsin. These results indicate that the antimicrobial substances produced by these isolates are of proteinaceous nature and belong to bacteriocins (Batdorj *et al*., 2006). Additionally, the inhibitory activity did not change after action of α-amylase

and lipase (Table 4). Our results are in agreement with those of Ponce *et al*. (2008), Ben Belgacem *et al.* (2008) and Ahmadova *et al*. (2013). Ponce *et al*. (2008) found that the antimicrobial activities of *Enterococcus hirae* IM1 and *Enterococcus faecium* IM1 were found to be sensitive to the action of proteases. Ben Belgacem *et al*. (2008) found that the inhibitory activity was lost after 2 h of treatment with proteinase K, trypsin and $\alpha$-chymotrypsin whereas it was not affected by catalase. Ahmadova *et al*. (2013) 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.

Effect of pH on antimicrobial activity, these assays were performed using supernatants corresponding to the LAB strains grown at 37°C for 24 h, since this led to maximum antimicrobial activity. The obtained results show that the activities of the supernatants produced by cultures of *Enterococcus faecium* IM1 and *Enterococcus 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.* (2008) and Line *et al.* (2008), and are similar to those presented by Ben Belgacem *et al*. (2008), but differences with those found by Ahmadova *et al*. (2013). Yoon *et al*. (2008) found that the bacteriocin produced by *Enterococcus faecium* was also stable until pH 8 and then decreased at pH 10. Line *et al*. (2008) found that the enterocin isolated from *Enterococcus durans* and *Enterococcus faecium* was stable in pH range between 5 and 8.7. Ben Belgacem *et al*. (2008) 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*. (2013) found that the antimicrobial activity was stable over a wide range of pH from 3 to 10. These differences may be to the differences between strains.

Effect of heat treatment on antimicrobial activity, Antagonistic activity of *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1 against *Lactobacillus bulgaricus* 340 was stable at 100 °C for 20 min (Table 4). It decreased after heating for 30 min. After autoclaving, the antimicrobial activity of *Enterococcus faecium* IM1 disappeared totally. However, *Enterococcus 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*. (2008), Ben Belgacem *et al*. (2008), Cintas *et al.* (1997), but disagree with those of Ahmadova *et al*. (2013), who found that the crude antimicrobials were stable during 30 min at temperatures below 90°C, and that *Enterococcus faecium* activity disappeared totally after autoclaving. Ben Belgacem *et al*. (2008) 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*. (1997) found that *Enterococcus faecium* P13 is heat resistant at 100°C for 15 min. Ahmadova *et al*. (2013) found that the activity for *Enterococcus faecium* AQ71 was stable after treatment in autoclave (121°C / 15 min). These differences may be due to differences in the selected isolates.

For the determination of LAB growth for maximum bacteriocin production, for *Enterococcus faecium* IM1*,* the profile of the antimicrobial

activity of the supernatant was tested against *Lactobacillus bulgaricus* 340. The antimicrobial activity was initially detected in exponential phase of growth and the maximal levels of antimicrobial activity (1300 AU $mL^{-1}$) was obtained at 12 h and remained stable till the end of incubation time (48 h) (Fig. 1A). In case of *Enterococcus hirae* IM1, the maximum of activity was observed in the logarithmic phase (6 h) and it decreased after 12 h (Fig. 1B). The pH dropped to approximately 5.6 during the same period (24 h).

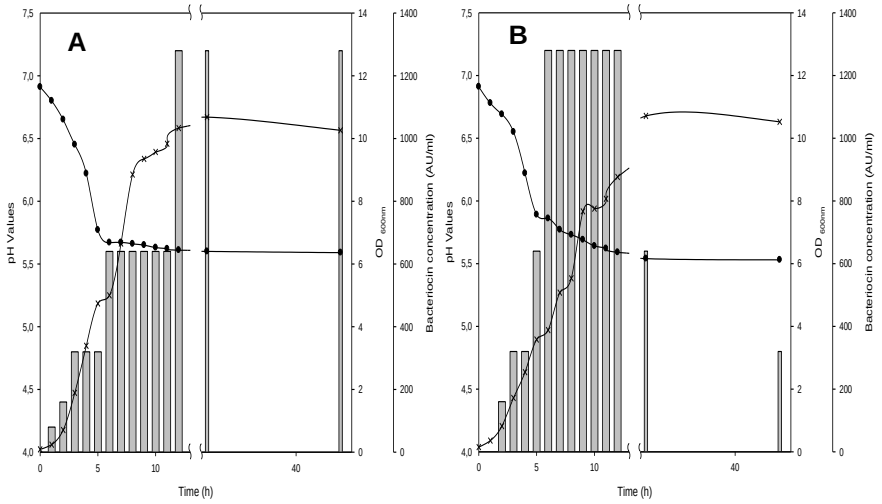



**Figure (1): Time course of bacteriocin production during the growth of *Enterococcus faecium* IM1 (A) and *Enterococcus hirae* IM1 (B) in M17 and MRS broth, respectively, at 37°C. Optical density of culture was measured at 600 nm. Bacteriocin concentration is expressed in arbitrary units per milliliter (AU $mL^{-1}$). The indicator strain was *Lactobacillus bulgaricus* 340.**

In a similar study on *Enterococcus faecalis* strain RJ-11, Yamamoto *et al*. (2003) found that the amount of bacteriocin in the fluid culture reached the maximum level in the early stationary phase. These differences might 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 (Ponce *et al*., 2008). These results agree well with those found by Ahmadova *et al*. (2013).

**Detection of some virulence factors by PCR amplification of 16S rDNA**

For safety requirements, it is indispensable to prove the absence of virulence gene factors and transferable antibiotic resistance in the studied strains (*Enterococcus faecium* IM1 and *Enterococcus 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 (Mundy *et al.*, 2000). The presence of five known virulence factors

[*asaI* (aggregation substance), *cyl* A (cytolysin), *ace* (collagen-binding protein), *gelE (Gelatinase*), and $esp_{fm}$ (extracellular or enterococcal surface protein)] were tested for two Enterococci strains studied by PCR amplification (Fig. 2).

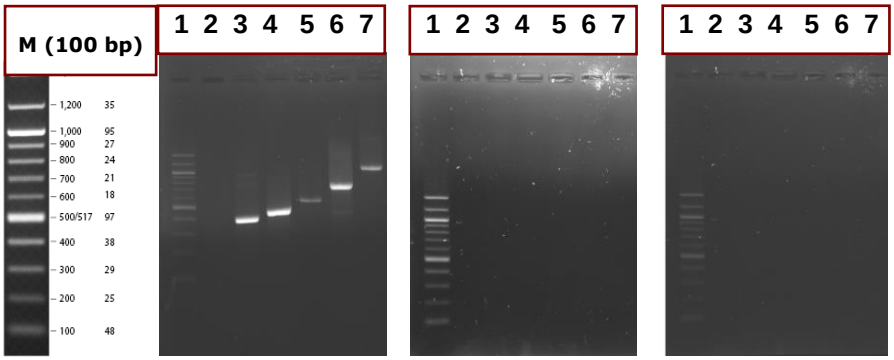



**Figure (2): PCR product of *Enterococcus faecium* IM1 (B), *Enterococcus hirae* IM1 (C) and control strain *Enterococcus faecalis* MMH594 (A). Lane 1, molecular marker (100 bp); lane 2, negative control; lane 3, *asa1*(375 bp); lane 4, *gelE* (419 bp); lane 5, *esp* (510 bp); lane 6, *cylA* (688 bp);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

Comparison of the electrophoretic patterns of the tested virulence factors in the two studied strains (*Enterococcus faecium* IM1 and *Enterococcus hirae* IM1) with positive control strain (*Enterococcus faecalis* MM4594) indicates that they were free from tested virulence genes. This is in accordance with the previous results on similar strains. Generally, *Enterococcus faecium* strains of food origin were reported free of the virulence factors with some exceptions (Eaton and Gasson, 2001; Yoon *et al.*, 2008; Barbosa *et al.*, 2010; Ben Belgacem *et al*., 2010; Favaro *et al*., 2014). Eaton and Gasson (2001) investigated the incidence of known virulence determinants in starter, food, and medical strains of *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus durans*. *Enterococcus faecium* strains were generally free of virulence determinants. They suggested that the use of *Enterococcus* spp. in foods requires careful safety evaluation. Yoon *et al.* (2008) found that tested *Enterococcus faecium* strains showed resistance against simulated gastrointestinal conditions such as acidic environment and the presence of bile salts. These strains also showed bile salt hydrolase activity but neither hemolytic activity nor virulence determinant such as *gelE* and *efaAfm*. All strains were susceptible to glycopeptides and lacked potential as vancomycin-resistant enterococci (VRE). Two strains, S2C10 and S2C11 inhibited the viability of *Listeria monocytogenes* in vitro. The ability was probably due to the production of bacteriocin. For this reason, the strains could be used as selected starters or protective cultures in soybean fermented food production. Barbosa *et al.* (2010), found that isolated *Enterococcus faecium* were generally free of

virulence determinants. Ben Belgacem *et al*. (2010) investigated 24 isolates from *Enterococcus faecium* for the presence of six known virulence determinants [*asal*, *cyl* A, *cyl* B and *cyl* M, *ace*, $efaA_{fs}$, $esp_{fm}$ and *gelE*] by PCR and found that 10 of these isolates were free from all tested virulence factors. Favaro *et al*. (2014) tested four strains of *Enterococcus faecium* and found them free of tested virulence factors [*asal*, *cyl* A, *ace*, $efaA_{fs}$, and *gelE*].

**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 (Valenzuela *et al*., 2009). The most important factor for the safety evaluation of *Enterococci* spp. is its resistance to glycopeptides such as vancomycin (Yoon *et al*., 2008). The resistance or sensitivity depends on minimum inhibitory concentration (MIC) breakpoints as described by the European Food Safety Authority (EFSA, 2008). The results showed that *Enterococcus faecium* IM1 was sensitive to penicillin, kanamycin, vancomycin, gentamicin, and tetracycline but resistant to ampicillin. Otherwise, *Enterococcus hirae* IM1 was sensitive to penicillin, kanamycin, vancomycin, and tetracycline but resistant to ampicillin and gentamicin. Similar results were observed by Peters *et al.* (2003); Valenzuela *et al*. (2009). Peters *et al.* (2003) 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 *Enterococcus faecalis*, 54 *Enterococcus faecium*, 24 *Enterococcus durans*, 22 *Enterococcus casseliflavus*, 9 *Enterococcus avium* and 8 *Enterococcus gallinarum*) were sensitive to vancomycin. Valenzuela *et al*. (2009) 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 *Enterococcus faecalis* and 15 *Enterococcus faecium* isolates and found that all isolates were sensitive to penicillin and gentamicin. However, *Enterococcus faecium* isolates showed a very low percentage (6.66%) of resistance to tetracycline. Favaro *et al*. (2014) tested four strains of *Enterococcus 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 *Enterococcus faecium* IM1 and *Enterococcus 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 ten-folds. The antimicrobial activity was tested against different pathogenic bacteria (Table 5).

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

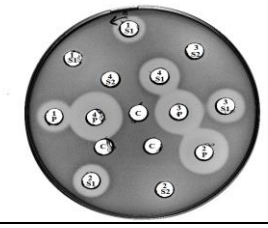
| Indicator strains | Sources | C | D | |
|---|---|---|---|---|
| | | | A | B |
| *Bacillus subtilis* 168[T] | ONIRIS | 37°C/24 h | - | - |
| *Brochothrix thermosphacta* DSMZ20171[T] | ONIRIS | 25°C/24 h | ++ | ++ |
| *Brochothrix thermosphacta* DSMZ20599 | ONIRIS | 25°C/24 h | ++ | ++ |
| *Carnobacterium 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/t1362 | 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 | 30°C/24 h | ++ | ++ |
| *Listeria monocytogenes* RF131 | 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* spp. | ONIRIS | 37°C/24 h | - | - |
| *Serratia liquefaciens* CIP103238[T] | ONIRIS | 25°C/24 h | - | - |
| *Vagococcus penaei* CIP109914[T] | ONIRIS | 30°C/24 h | ++ | + |

**ONIRIS: Ecole Nationale Nantes Atlantique Vétérinaire, Nantes, France.**
**IFREMER: Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France**
**C: Growth conditions (°C/h) (BHI media)**
**D: Antimicrobial activity [wells (5 mm diameter) were filled with 50 ml of culture supernatant].**
**-, no inhibition zone; +, zone < 3mm; ++, zone 3-6mm; +++, zone 7-10mm; ++++, zone > 10mm**.

Data in Table 5 show that *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1 are active against *Brochothrix thermosphacta* DSMZ20171[T], *Brochothrix thermosphacta* DSMZ20599, *Carnobacterium maltaromaticum* CIP103135[T], *Carnobacterium piscicola* S4312, *Enterococcus faecalis* JH2-2, *Enterococcus faecalis* JH2-2rr04, *Enterococcus faecium* CTC492/t136 2,

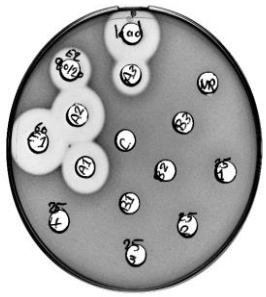
*Enterococcus faecium* P13, *Listeria grayi* CLIP12518, *Listeria innocua* 1, *Listeria innocua* CIP80.11$^T$, *Listeria innocua* F, *Listeria innocua* P, *Listeria ivanovii*, *Listeria ivanovii* CIP78.42$^T$, *Listeria monocytogenes* (different strains) and *Vagococcus penaei* CIP109914$^T$ but no effect on *Bacillus subtilis* 168$^T$, *Moellerella wisconsensis* MIP2451, *Morganella psychrotolerans* MIP2488, *Pseudomonas fluorescens* 10, *Salmonella* spp. and *Serratia liquefaciens* CIP103238$^T$.

The antimicrobial activity of the supernatant extracted from *Enterococcus faecium* IM1 before and after precipitation with ammonium sulfate was tested against *Lactobacillus sakei* subsp. *sakei* JCM1157 (Fig. 3 A). In the second step, the active fraction was separated on a reversed phase cartridge (Sep-Pak $tC_{18}$ 12 cc Vac Cartridge 5g). Three fractions were collected and checked for their antibacterial activity by the agar well diffusion assay. The fraction eluted with 60% of acetonitrile showed the highest activity against *Lactobacillus sakei* subsp. *sakei* JCM1157 (Fig. 3B). In the third step, the active fractions ($E_1$ 60% 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. 3B).



**A**

S1, supernatant before precipitation
S2, supernatant after precipitation
P, pellets
1, 2, 3 and 4: different isolates
C, M17 medium without strains



**B**

$25_1$, $25_2$, $25_3$, $25_4$, elution with 25% acetonitrile
$E_1$60, elution with 60% acetonitrile
$E_2$80/20; elution with 60% acetonitrile/20% isopropanol
$A_1$,$A_2$,$A_3$, samples from $E_1$60 treated with 0,0.2and1MNaCl, respectively
$B_1$,$B_2$,$B_3$, samples from $E_2$80/20 treated with 0,0.2and1MNaCl, respectively
C, negative control (M17 medium without strain)

**Figure (3, A and B): Antimicrobial activity against *Lactobacillus sakei* subsp. *sakei* JCM1157 of fractions obtained from supernatant of *Enterococcus faecium* after loading in Sep-Pak $tC_{18}$ 12 cc Vac Cartridge 5g and cation-exchange column.**

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 indicator strain. According to this result, the combinations of peaks 1 and 2 were tested against *Lactobacillus 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.

Finally, these active fractions were concentrated and re chromatographed on the same column to check their purity. Each successive purification step increased the specific activity against *Lactobacillus sakei* subsp. *sakei* JCM1157 and reduced the amount of contaminating non-bacteriocin proteins.

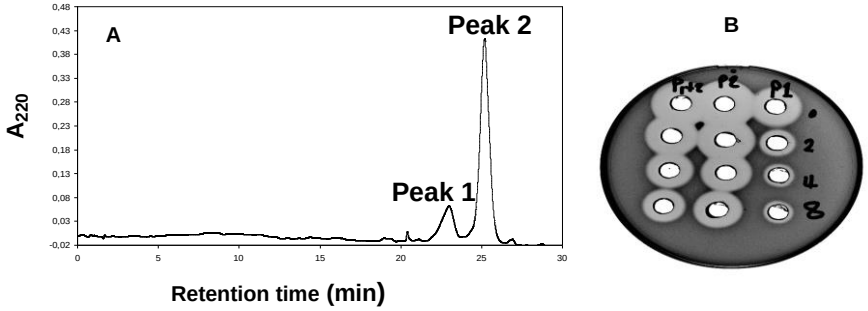



**Figure (4A): Reversed-phase chromatograms of two peaks of bacteriocin of *Enterococcus faecium* IM1. The bacteriocin was applied to RP-HPLC (Symmetry $C_8$ 3.5 µm). (4B): Activity of separated two peaks was measured on MRS agar against *Lactobacillus sakei* subsp. *sakei* JCM1157 after 24 h of incubation at 37°C.**

In the present study, two active strains (*Enterococcus faecium* IM1 and *Enterococcus hirae* IM1) were isolated from Egyptian dairy products. Bacteriocin-producing *Enterococcus faecium* IM1 and *Enterococcus hirae* IM1 did not present any genes coding virulence factors (tested only) neither transferable antibiotic resistance (especially vancomycin resistance genes). They may be useful as starter cultures and co-cultures. Consequently, the selected strains can be applied in fermented dairy foods as efficient biopreservatives cultures.

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# توصيف للبكتيروسين الناتج من سلالتين من بكتيريا حمض اللاكتيك التى تم عزلها من منتجات الألبان المصرية التقليدية

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تهتم هذه الدراسة بتوصيف سلالتين من بكتريا حامض اللاكتيك (LAB) التى تم عزلها من منتجات الألبان المصرية التقليدية. تم دراسة التأثير المثبط لـ ٦٢٣ عزلة من بكتريا حامض اللاكتيك على بعض الميكروبات الممرضة . تم تعريف العزلات التى اعطت نتيجة ايجابية على معظم الميكروبات الممرضة بإستخدام الوراثة الجزيئية وكانت *Enterococcus faecium* IM1 و *Enterococcus hirae* IM1 . تم تنقية الانتيروسين من السلالات التى تم تعريفها و كانت لها نتيجة ايجابية على تثبيط بكتريا *Brochothrix thermosphacta* DSMZ, *Carnobacterium maltaromaticum* CIP103135$^{T}$, *Carnobacterium piscicola* S4312, *Enterococcus faecalis* JH2, *Listeria Listeria ivanovii* ATCC, *Listeria grayi* CLIP12518, *Listeria innocua*, *monocytogenes* and *Vagococcus penaei* CIP109914$^{T}$ و كان لها نتيجة سلبية على تثبيط بكتريا *Bacillus subtilis*, *Moellerella wisconsensis*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Salmonella montevideo*, *Salmonella typhimurium* and *Serratia liquefaciens* CIP103238$^{T}$ . التأثير المثبط لـ *Enterococcus hirae* IM1 يمكن ان يرجع الى الانتيروسين فقط بينما التأثير المثبط لـ *Enterococcus faecium* IM1 يمكن ان يرجع الى الانتيروسين وفوق اكسيد الايدروجين. الانتروسين المستخلص من السلالات كان ثابت على أس هيدروجينى مابين ٥ الى ٨ و يتحمل درجة حرارة حتى ١٠٠ درجة مئوية لمدة ٢٠دقيقة. السلالات المختارة كانت خالية من ال virulence factors . لذلك يمكن استخدام هذه السلالات كبادئات او مع البادئات الاصلية فى صناعة منتجات الألبان المتخمرة كمواد حافظة طبيعية.