Determination of bacteriocin-encoding genes of lactic acid bacteria isolated from traditional dairy products of Luxor province, Egypt

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Abstract

Background: Researchers have focused on isolating and identifying the bacteriocin producing lactic acid bacteria from various food systems especially dairy products. Molecular techniques have been recently used for rapid identification of bacteriocins rather than time-consuming biochemical characters. Global climate disturbances can affect the diversity of beneficial microorganisms in dairy and their products, especially lactic acid bacteria, so it is worth to evaluate their bacteriocinogenicity in different climates. Thus, the aim of this study was to screen for predominant bacteriocin producing lactic acid bacteria (LAB) in traditional dairy products of Luxor governorate at Upper Egypt and determine their bacteriocin-encoding genes.

Results: Eighty-six strains of the LAB were isolated from raw milk and traditional dairy product of Luxor province, Egypt, in which 76.1% and 23.9% were identified as lactic acid bacilli and cocci, respectively. On the basis of their antibacterial potentials, 30 out of 68 LAB isolates were found to be antimicrobial producers. These isolates exhibited a potential antibacterial activity against Salmonella paratyphi B, Escherichia coli, Staphylococcus aureus, and Proteus mirabilis, except for Listeria monocytogenes. LAB isolates were analyzed using species-specific PCR results emphasized that 22 of isolates were identified as Lactobacillus plantarum, while 8 were Leuconostoc mesenteroides. According to the sequencing of isolates, two strains named Lactobacillus plantarum Egypt 2018 (accession no. MH817034) and Leuconostoc mesenteroides Egypt 2018 (accession no. MH817035) were identified. Detection of bacteriocin-encoding genes was performed by polymerase chain reaction (PCR). The results emphasized that almost all tested Lb. plantarum strains (n = 10) possess both plcA and plcEF genes, whereas the gene encoding mesentericin Y105 was detected in one Lc. mesenteroides of the examined isolates.

Conclusions: This study was effective for the rapid detection of bacteriocin producing strains within dairy products. Extracted bacteriocin could be a valuable source of natural food biopreservative.

Keywords: Bacteriocin gene, Egypt dairy products, Lactic acid bacteria, Mesentericin Y105, Plantaricin EF, Plantaricin A, Species specific primer

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Background

Fresh and unpasteurized milk in Egypt is frequently used to prepare traditional products such as White, Karish, Mish, and Ras cheese, yogurt, Rayeb, Labneh, and butter. The local homemade dairy products in Upper Egypt depend on the spontaneous activity of the milk indigenous microbial population itself. Lactic acid bacteria (LAB) are occurring naturally as indigenous microflora in raw milk which can be taken as starter strains with unique characteristics. In addition, metabolic products produced by LAB related to the organoleptic, textural profile, and shelf life of the foods [1]. LAB are generally recognized as safe (GRAS) status, which increases their application in industrial-scale especially health-promoting products [2]. According to the dairy technology point of view, the subsequent genera are considered the foremost LAB: Aerococcus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, and Streptococcus [3].

European Union has developed a project under ECLAIR programme, AGRE-0064 for the isolation of new starter cultures from traditional cheese and fermented milk across Europe [4]. Nevertheless, in Egypt, traditional LAB flora still waits for scientific attention because of uncontrolled industrialization.

The majority of starter cultures which are food grade LAB, applied in the dairy industry, have enormous antimicrobial potential. Bacteriocins which are ribosomally synthesized peptides or proteins are produced by most of the LAB species. These bacteriocins are able to kill phylogenetically related strains. Owing to their antagonistic activities, bacteriocins can be used as non-thermal means to prevent spoilage [5].

Based on molecular weight, genetics, and chemical properties, bacteriocins are classified to four major groups: class I which is small peptide inhibitors termed as lantibiotics; class II comprises small heat-stable proteins that has five subclasses; class III contains large, heat-labile protein, and this class has two subclasses; and finally class IV that are complex bacteriocins containing lipid or carbohydrate sections [6].

The traditional screening strategies consume time and effort. The researchers are continuously trying to develop rapid screening methods like polymerase chain reaction (PCR) method. Some researchers have designed specific primers that amplify particular genera or species of LAB [7]; others have developed specific primers for bacteriocin coding genes or regulation related genes for rapid detection of bacteriocins [8, 9]. These advanced methods have led to a better understanding of the microbial communities existing in a variety of ecosystems, like the gut microbiota [10].

Little is known about the LAB microbial population in Luxor province at Upper Egypt. Luxor has a hot desert climate. During daytime, temperatures exceed 40 °C (104 °F); this will give an opportunity to isolate LAB strains, which are able to tolerate high temperatures. Thus, the aim of this study was exploring LAB strains found in raw milk and traditional dairy products of Luxor province, evaluating their antibacterial activity, using molecular techniques (PCR) for rapid screening of these strains at the species level by using a species-specific LAB primer and for determining their bacteriocins-encoding genes.

Methods

Collection of samples

This study was conducted from March to May 2018. A total of 23 different traditional fermented milk dairy samples consist of 6 samples of raw milk (cow, buffalo, and goat), 8 samples of Laban rayeb (cow, buffalo, and goat), 1 sample of cheddar cheese, 3 samples of Karish cheese, 3 samples of Mish, and 2 samples of butter, which were collected from the surrounding area of the city of Luxor, Egypt. Samples were collected in sterilized bottles and delivered to laboratory using icebox for microbiological examination. Samples were kept in a refrigerator (around 4 °C) until the analysis began.

Isolation and enumeration of lactic acid bacteria

For isolation of LAB, approximately 10 g of each sample was homogenized with sterile physiological peptone water (10%) and serially diluted in the same diluent. A volume of 0.1 ml of appropriate dilutions was spread plated on MRS (Oxoid) agar media [11] and incubated under aerobic and anaerobic conditions (an anaerobic gas pack system-Oxoid) at 37 °C for 2–5 days. Typical LAB characteristic colonies were enumerated (cfu/ml), then randomly picked up and purified by streaking two or three times on fresh MRS agar plates. Isolates were preserved at 4 °C in MRS agar plates (Oxoid) [12], followed by macroscopic and microscopic examinations.

Phenotypic characterization

The colonies displaying the general characteristics of LAB were chosen from each plate for physiological and biochemical tests. LAB were characterized by Gram staining, catalase reaction, oxidase test, milk coagulation activities, motility, esculin hydrolysis, and growth for 48 h at 37 and 45 °C for mesophilic and thermophilic LAB, respectively [13, 14].

Detection of antibacterial activity

Antibacterial activity of all LAB isolates was first screened with agar spot diffusion [15]. Then, potential antibacterial LAB strains were investigated by well diffusion methods [16].

Indicator strains used Listeria monocytogenes, Staphylococcus aureus, Proteus mirabilis, Salmonella paratyphi B, and Escherichia coli, which were kindly
obtained from central laboratories in Cairo, were inoculated in 100 ml of peptone water, and the concentration was matched against 0.5 Mcfarland Standard and then diluted by 1:100 to obtain a concentration of 1.0 × 10⁶ CFU/ml.

For the preparation of crude bacteriocin, 10 ml of MRS broth was inoculated with 0.1 ml of freshly prepared culture of isolated strains individually, then incubated for 18 h at 37 °C. The grown culture was centrifuged at 4000×g for 4 min at room temperature in the centrifuge tubes. The supernatant of overnight culture of isolates was heated at 80 °C for 10 min and the pH adjusted to 6.5 using 1 M NaOH, to obtain crude bacteriocin [17].

**Agar spot diffusion method**

Muller Hinton Agar (MHA) was poured in plates and surface seeded with indicator strains. Ten microliters of crude bacteriocin or of positive gentamicin disc (10 μg) and negative (sterilized MRS broth) controls were spotted on the surface of inoculated agar, and plates were incubated overnight at 37 °C for 24 h. After incubation, inhibition was indicated by a clear zone around spots.

**Well diffusion method**

Wells were made on MHA plates containing indicator strains, and each well was filled with 50 μl of crude bacteriocin. Eventually, after an incubation period (37 °C for 24 h), inhibition was indicated by a clear zone around each well.

**Extraction of bacteriocin and determination of its antibacterial activity titer**

Bacteriocin was precipitated from the crude extract by the addition of ammonium sulfate; a 24-h old culture of the isolate at the species level. Primers used were for Leuconostoc (Le) mesenteroides, Lactobacillus (L.) plantarum, and Lactococcus lactis [19]. The sequences of PCR primers are shown in Table 1. According to Emerald Amp GT PCR Master Mix (Takara), 25 μl of reaction mixture containing 6 μl of bacterial DNA template, 1 μl of each primer, 12.5 μl of Emerald Amp GT PCR mastermix (2× premix), and 4.5 μl of PCR grade water were prepared. The thermal cycling program was followed according to PCR mastermix kit that briefly consisted of an initial denaturation step at 94 °C for 5 min and 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension for 7 min at 72 °C. The amplified products were visualized by electrophoresis in 1.5% agarose gels stained with 0.5 μg/ml ethidium bromide. The gel was photographed by a gel documentation system [20], and the data was analyzed through computer software (reference lab for veterinary quality control on poultry production, Animal health research institute, Egypt).

**Sequences analysis**

For further identification of the lactobacilli examined by species-specific primers, we amplified and sequenced two strains of Lb. plantarum and Lc. mesenteroides isolates using the recA gene sequencing. A purified RT-PCR product was sequenced in the forward and/or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA). A BLAST® analysis (Basic Local Alignment Search Tool) (NCBI, http://www.blast.ncbi.nlm.nih.gov) was initially performed to establish sequence identity to GenBank accessions [21].

**Phylogenetic analysis**

A comparative analysis of sequences was performed using the Clustal W multiple sequence alignment.
program, version 1.83 of MegAlign module of the Laser-gene DNAStar software Pairwise, and phylogenetic analyses were done using neighbor-joining tree with Maximum Composite Likelihood substitution model in MEGA6. Numbers at nodes represent measures of robustness depend on 1000 bootstrap replications in MEGA6 [22].

Detection of genes encoding bacteriocin production
PCR assay was used to determine bacteriocin genes, including mesY, plnA, plnEF, and plnC. PCR primers (Metabion, Germany) were used according to [23–25] and are presented in Table 1. PCR conditions were similar to all the genes: an initial denaturation step of 94 °C for 5 min, 34 cycles of 94 °C for 30 s, annealing 55 °C for 30 s, and extension 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. The amplified products were visualized by electrophoresis in 1.5% agarose gels as previously described [9].

Results
Isolation and identification of LAB from dairy samples
The microbial population of LAB cultivated on MRS revealed that Karish and cheddar cheese presenting the

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Table 1 Specific oligonucleotide primers sequences used throughout this study

| Primer (Target gene) | Primer sequence | Amplified product | Reference |
|----------------------|-----------------|-------------------|-----------|
| Bacterial strain gene | *Lb. plantarum, recA* gene | F: 5’CAGAATTGAGCTGGTGCTGG3’<br>R: 5’TGTACCTTTGCAACCCAGAT3’ | 210 bp | [19] |
| Lactococcus lactis subsp. Lactis, recA gene | F: 5’TATCCGAACACTTGGACGA3’<br>R: 5’CAACCTTTGAATGGTGAGGAG3’ | 262 bp | |
| *Lc. mesenteroides*, recA gene | F: 5’SATACGGGCACACGGGATTA3’<br>R: 5’GGGTGTAAGTTTCTGGGTTTC3’ | 269 bp | |
| Bacteriocin gene | *MesY* | F: 5’CATACATGAAATGAGGAG3’<br>R: 5’TTCACCAATCCATTTCGC3’ | 186 bp | [23] |
| *PlnEF* | F: 5’GGCATAGTTAAATCCTCCCCC3’<br>R: 5’RCAGGTGTCGGCAGCAGAG3’ | 428 bp | [25] |
| *PlnA* | F: 5’GTACAGTACAATGCGGAG3’<br>R: 5’CITAGCCGATCTAATAGG3’ | 450 bp | |
| *PlnC* | F: 5’GGTGGGCACAGAGATTTA3’<br>R: 5’AGAAACGCGTTCGAGTTTA3’ | 353 bp | [24] |

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Table 2 Samples, sampling locations, and lactic acid bacteria (LAB) count (log CFU/ml or g) of milk and dairy products

| Sample types | Sampling location | No. of samples | Sample codes | Lactic acid bacteria count (log CFU/ml) |
|--------------|------------------|----------------|--------------|---------------------------------------|
| Raw milk     | Elbaqala         | 4              | GM19, CM5, CM3, BM2 | 3.05 ± 1.02 | 1.60–4.00 |
|              | Rayina           | 1              | CM8          | 2.40 ± 0.09 | 2.35–2.49 |
|              | Luxor            | 1              | CM12         | 3.20 ± 0.02 | 3.18–3.22 |
| Rayeb        | Elbaqala         | 4              | GR20, BR6, LR7, LR4 | 5.18 ± 4.52 | 0.60–9.30 |
|              | Rayina           | 1              | LR17         | 1.80 ± 0.02 | 1.78–1.82 |
|              | Luxor            | 1              | LR15         | 2.70 ± 0.09 | 2.61–2.79 |
|              | Elmeris          | 1              | LR21         | 1.30 ± 0.09 | 1.21–1.39 |
|              | Esna             | 1              | LR1          | 2.70 ± 0.04 | 2.66–2.74 |
| Karish cheese| Elbaqala         | 1              | KC16         | 5.60 ± 0.09 | 2.51–2.69 |
|              | Rayina           | 1              | KC9          | 13.80 ± 0.02 | 13.78–13.82 |
|              | Elmeris          | 1              | KC23         | 4.20 ± 0.02 | 4.18–4.22 |
| Cheddar cheese| Luxor           | 1              | CC18         | 5.80 ± 0.02 | 5.78–5.82 |
| Mish         | Elbaqala         | 2              | MI13, MI14   | 1.67 ± 1.20 | 0.82–2.52 |
|              | Elroziqat        | 1              | MI10         | 2.09 ± 0.00 | 2.09 |
| Butter       | Elbaqala         | 1              | BU11         | 1.82 ± 0.02 | 1.80–1.84 |
|              | Elmeris          | 1              | BU22         | 4.00 ± 0.00 | 4.00 |

BM buffalo milk, CM cow milk, GM goat milk, LR cow rayeb, KC Karish cheese, CC cheddar cheese, MI Mish, BU butter
highest bacterial count, followed by Laban rayeb, while the least count recorded with Mish (Table 2). A total of 119 colonies were recovered from 23 fermented milk samples. Sixty-eight isolates only were considered as LAB, which was characterized by positive Gram, negative catalase, not motile, and ability to live in microaerophilic conditions. The colony morphology and microscopic characteristic were considered (Fig. 1). The most frequently isolated colonies which were observed in different samples were those with milky-white, circular, convex, and moist colonies, followed with a creamy, smooth, and slimy colonies. It was detected from the microscopic characteristics of LAB that rod shape LAB were as many as 51 isolates (75%), and coccus-shaped were 17 isolates (25%). The bacterial cells were mostly long rods, and sometimes they were coccoid, while coccoid cells were in single and diplo shapes. The majority of isolates were able to grow at 45 °C, while the other isolates had an optimum temperature around 37 °C. Fifty-nine percent of bacilli were found to be thermophilic, and 14% were mesophilic, while 10% of the cocci were thermophilic, and 17% were a mesophilic type. Thirteen percent of the bacilli and 8% of the cocci had the ability to hydrolyze bile esculin salts.

Antibacterial activity
According to the agar spot diffusion test, all the 68 LAB isolates displayed an inhibitory effect against tested foodborne indicator strains by initial screening of supernatant. The antibacterial activities of the crude bacteriocin of 68 LAB strains were further investigated by well diffusion method. The results show that only 30 LAB strains have antibacterial activity (Table 3). The highest antibacterial activity was recorded against S. paratyphi B (29 LAB strains) and Staph. aureus (26 LAB strains) at various degrees. The highest inhibition zone is recorded against Staph. aureus, mainly by isolates from Laban rayeb and Karish cheese. However, none of the isolates displayed antibacterial activity against L. monocytogenes.

Molecular identification
PCR analysis was carried out for 30 selected isolated strains by using species-specific primers for Lc. mesenteroides, Lb. plantarum, and Lactococcus lactis (Table 4 and Fig. 2). Results revealed that isolates contain 8 coccoid isolates which were identified as Lc. mesenteroides, and 22 bacilli isolates were identified as Lb. plantarum, while neither of them belongs to Lactococcus lactis.

For further study, we selected randomly strain no. (1) Lb. plantarum and strain no. (2) Lc. mesenteroides for sequencing analysis. The sequence identity of Lb. plantarum Egypt 2018 was deposited in NCBI under GenBank accession number of MH817034, and the accession number for the Lc. mesenteroides Egypt 2018 is MH817035.

Lb. plantarum Egypt 2018 has a 100% similarity with the nucleotide sequence of Lb. plantarum subsp. plantarum ATCC 14917T (GenBank: AJ621668.1) among other closely related strains (Fig. 3), while the nucleotides sequence of Lc. mesenteroides subsp. jonggajikimchii strain DRC1506 with accession no. CP014611.1 in Korea comprises a similarity of 99.3% to our strain Lc. mesenteroides Egypt 2018 (Fig. 4).

Detection of bacteriocin activity
Based on the results of antibacterial activity, bacteriocin activity was measured for 11 selected potent antibacterial strains. The results are presented in the Table 5. Strain no. 13 recorded the highest antibacterial activity (160 AU/ml) against indicator strain (Staph. aureus). While the lowest antibacterial activity was recorded by strains no. 1 and 18, other strains showed a similar moderate antibacterial activity of 80 (AU/ml).

Detection of the bacteriocin genes
Selected strains were tested for the presence of genes encoding bacteriocin: plnA, plnC and plnEF, and mesY gene. Results emphasized that plnEF is detected in all tested Lb. plantarum strains, except for strain no. 19. PlnA genes were also tested for some plnEF gene-positive strains, and they gave positive results of plnA in all tested strains, while mesY gene was found in one of the two strains of Lc. mesenteroides (Table 5, Fig. 5).
Table 3 Antibacterial activity of isolated lactic acid bacterial strains by diffusion test and well diffusion test

| Sample no. | Species | S. paratyphi B | E. coli | Staph. aureus | Pr. mirabilis | L. monocytogenes |
|------------|---------|---------------|---------|--------------|--------------|----------------|
|            |         | S  W          | S  W    | S  W         | S  W         | S  W           |
| 1          | P       | +  +          | +  +    | +  +         | +  +         | +  +           |
| 2          | L       | +  +          | +  +    | +  +         | +  +         | +  +           |
| 3          | P       | +  +          | +  +    | +  +         | +  +         | +  +           |
| 4          | P       | +  +          | −  −    | −  −         | +  +         | −  −           |
| 5          | L       | +  +          | −  −    | −  −         | +  +         | −  −           |
| 6          | L       | ++ +          | −  −    | −  −         | +  +         | −  −           |
| 7          | P       | ++ ++         | −  −    | ++ ++        | +++ ++       | +  +++         |
| 8          | P       | ++ ++         | −  −    | ++ ++        | −  −         | −  −           |
| 9          | P       | ++ ++         | −  −    | +  +         | ++ ++        | −  −           |
| 10         | P       | ++ ++         | −  −    | −  −         | −  −         | −  −           |
| 11         | L       | −  −          | −  −    | −  −         | −  −         | −  −           |
| 12         | P       | ++ +          | −  −    | +  +         | −  −         | +  ++          |
| 13         | P       | +  +          | −  −    | +  +         | −  −         | +  ++          |
| 14         | P       | −  −          | +  +    | +  +         | +  +         | −  −           |
| 15         | P       | ++ ++         | +  +    | ++ ++        | +++ ++       | +  +           |
| 16         | L       | ++ ++         | −  −    | ++ ++        | +++ ++       | +  +           |
| 17         | L       | ++ ++         | −  −    | +  +         | +  +         | −  −           |
| 18         | P       | ++ ++         | +  +    | +  +         | +++         | −  −           |
| 19         | P       | ++ ++         | −  −    | +  +         | −  −         | −  −           |
| 20         | L       | ++ +          | −  −    | ++ ++        | −  −         | −  −           |
| 21         | P       | ++ +          | −  −    | +  +         | +++         | −  −           |
| 22         | P       | ++ +          | +  +    | +++         | −  −         | +  +           |
| 23         | P       | +  +          | +  +    | +  +         | −  −         | +++           |
| 24         | P       | +  +          | +  +    | +++         | −  −         | −  −           |
| 25         | P       | +  +          | −  −    | +++         | −  −         | −  −           |
| 26         | P       | ++ +          | −  −    | +  +         | ++         | −  −           |
| 27         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 28         | P       | +  +          | +  +    | +++         | +  +         | −  −           |
| 29         | L       | ++ ++         | −  −    | +  +         | +  +         | −  −           |
| 30         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 31         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 32         | L       | ++ ++         | −  −    | +  +         | +  +         | −  −           |
| 33         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 34         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 35         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 36         | L       | ++ ++         | −  −    | +  +         | +  +         | −  −           |
| 37         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 38         | L       | ++ ++         | −  −    | +  +         | +  +         | −  −           |
| 39         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |
| 40         | P       | ++ +          | +  +    | +++         | +  +         | −  −           |

S spot diffusion test, W well diffusion test, P Lb. plantarum, L Lc. mesenteroides
Diameter of the inhibition zone: (+) weak (3–5) mm, (++) intermediate (6–10) mm, (+++) strong > 10 mm, (−) no growth
Discussion

The rich biodiversity of natural dairy products can be considered as excellent sources of LAB strains [26]. Karimi et al. [27] described factors influencing the dairy product ecosystem of probiotics count in milk products which could be microbial interactions, acidity, oxygen, growth promoters, hydrogen peroxide, ripening factors, food additives, salt, and storage temperature. The microbial content of raw milk is important for the production of hygienic dairy foods [28]. The results found in this research revealed that Karish cheese samples have the highest bacterial count, while Mish samples have the lowest LAB count, and that may be related to the high salt content of Mish. High numbers of LAB were mentioned previously in Egypt \((1.1 \times 10^7 \pm 1.6 \times 10^6 \text{ CFU/g})\) and were recorded for Karish cheese, Whey, and Ras cheese in Gharbiya and Minufiyah [29]. Also, a high and significant diversity of LAB was identified in the samples of Coalho cheese produced in the State of Paraíba from \(10^8\) to \(10^{11}\) CFU/g, which also presented an elevated number of colonies that may be as a consequence of the traditional manufacturing process [30]. Besides, the fact that raw milk is rich in lactic and non-lactic bacteria, which may originate from the mammary gland, the environment, water, or other tools involved in the milk production process. This non-pasteurized milk is the main source of the Karish cheese microbiota, by means it was processed with. All these factors could be a reason behind the elevated numbers of colonies detected in Karish cheese.

The identification of predominant bacteriocin producing LAB was started by looking at the shape of the colonies of each sample followed by Gram staining, catalase testing, and then determining its antibacterial activity. Results of this examination indicated that 68 out of 161 isolates were considered as LAB. In harmony with our results, Moulay et al. [31] had isolated microorganisms from goat milk and found that only 13 isolates from a total of 138 isolates belonged to LAB group. Isolated LAB strains were classified to either cocci, bacilli, or coco bacilli. In general, the dominant LAB were those with rod-shaped. A similar observation was noted by Yelnetty et al. [32] who found that rod shape represents 61.5% of LAB strains isolated from spontaneous fermented local goat milk.

Thermophilic bacilli were the dominant microflora of dairy products and raw milk samples of Luxor and that was expected from such high temperature climate. Similar observations of species isolated from fermented foods by Jagadeeswari et al. [33]. Otherwise, Franciosi et al. [34] found that the mesophilic cocci were dominant over thermophilic cocci, and the mesophilic rods were dominant over thermophilic rods in fermented food.

Consumers are always concerning about the microbiological hazardous of raw milk involved in the manufacture of fermented dairy products like cheeses because of the absence of thermal inactivation of pathogenic microbiota. Nevertheless, Brooks et al. [35] showed that all raw milk cheeses were negative for pathogens such as *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7. Unexpected, pasteurized milk cheeses were found to be a source of infection of Listeria-associated foodborne illnesses outbreaks in Germany from 2006 till 2007 [36]. The maintenance of the hygiene and safety of dairy products could be related to the bacteriocins produced by LAB strains.

The isolated 68 LAB displayed antibacterial activity against 4 of indicator strains. It is known that LAB are able to produce several antimicrobial compounds which include hydrogen peroxide and organic acids. In order to ensure that the inhibition was caused by the bacteriocin only, preparation of cell free-supernatant at pH 6.5 was carried out to eliminate the effect of acids produced by lactic acid bacteria against indicator strains. Also, to exclude the effect of hydrogen peroxide, producer strains

### Table 4 Molecular identification of lactic acid bacterial isolates by using species-specific primers

| Sample No. | Sample ID | *Lb. plantarum* | *L. lactis* | *Lc. mesenteroides* |
|------------|-----------|-----------------|-------------|---------------------|
| 1          | LR1**     | +               | N           | N                   |
| 2          | LR11**    | N               | N           | +                   |
| 3          | LR12**    | N               | N           | N                   |
| 4          | BM2**     | N               | N           | N                   |
| 5          | CM3       | N               | -           | +                   |
| 6          | CM3**     | N               | -           | +                   |
| 7          | LR4       | +               | N           | N                   |
| 8          | CM8       | +               | N           | N                   |
| 9          | MI10      | +               | N           | N                   |
| 10         | MI10**    | N               | -           | +                   |
| 11         | MI10      | N               | -           | +                   |
| 12         | BU11**    | +               | N           | N                   |
| 13         | BU11      | +               | N           | N                   |
| 14         | BU11      | +               | N           | N                   |
| 15         | CM12      | N               | N           | N                   |
| 16         | MI13      | N               | -           | +                   |
| 17         | MI13**    | N               | -           | +                   |
| 18         | MI14      | N               | N           | N                   |
| 19         | LR15      | N               | N           | N                   |
| 20         | KC16**    | -               | N           | N                   |
| 21         | KC16      | N               | N           | N                   |
| 22         | GM19      | N               | N           | N                   |
| 23         | LR21      | N               | N           | N                   |
| 24         | BU22      | N               | N           | N                   |
| 25         | CM8       | N               | N           | N                   |
| 26         | KC16**    | N               | N           | N                   |
| 27         | LR7       | N               | N           | N                   |
| 28         | LR17      | N               | N           | N                   |
| 29         | LR7       | N               | -           | +                   |
| 30         | CC18      | +               | N           | N                   |

(1): gene present, (·): absence of gene, (N): not performed PCR. (**): *Lb. plantarum* Egypt 2018, (**): *Lc. mesenteroides* Egypt 2018.
were incubated in anaerobic conditions, which may also reduce the effect of hydrogen peroxide against the indicators [37]. After the elimination of possible other antibacterial factors, only 30 LAB strains show antagonistic activity against 4 foodborne pathogens and recorded a different inhibitory spectrum of activity against Gram-positive as well as Gram-negative bacteria. In this context, Mohamed et al. [38] revealed that cell free supernatant of lactic acid strains had varying degrees of inhibition towards both Gram-positive and Gram-negative bacteria. In harmony with our results, Azizi et al. [39] have found that Staph. aureus ATCC 25923 was more sensitive to bacteriocin of isolated LAB strains from Iranian raw milk Motal cheese.

Crude bacteriocin of isolated LAB strains in this study shows no activity against L. monocytogenes. This result
resembles the outcomes obtained by CFN supernatants of strains isolated from Korycinski cheese [40]. Also, Sip et al. [41] found that only 7 out of 800 LAB strains have the ability to inhibit the growth of *L. monocytogenes*. Demir and Baspılbıl [42] anticipated that some bacteriocins have very narrow targets, so it is a key point to choose sensitive indicator strains. Bacteriocin production also influenced by many other factors like pH, nutrient source, and incubation temperature [43].

It has been proposed that the recA gene could be used as a phylogenetic marker for distantly related species, and it has previously given adequate results for many bacterial genera [44]. The choice of specific recA gene-based primers was based on phenotypic characterization and the available database gathered about the frequently identified isolates from fermented milk products. The results emphasize that the predominant bacteriocin producers comprise *Lb. plantarum* (32%) and *Lc. mesenteroides* (11.7%). These findings are familiar with those reported by other workers, where the percentage of incidence was reported within the range 9–23% for *Lb. plantarum* [45, 46], which as well agreed with an earlier report about the identification of *Lb. plantarum* as the predominant species isolated from other milk cheeses [47], also with Arizcun et al. [48] who found that the predominant species in two types of cheeses were *Lb. plantarum*, *Lb. casei*, and *Lc. mesenteroides*.

In the present study, *Lb. plantarum* strains showed antibacterial activity against 4 studied indicators; these results present a close similarity with Azizi et al. [39] as they establish that all *Lb. plantarum* isolated from Iranian raw milk Motal cheese possess antibacterial activities against 3 indicators. Furthermore, Sankar et al. [49] observed that *Lb. plantarum* isolates from raw cow milk samples had powerful antimicrobial activity against some of the indicator microorganisms.

From the populous *Lactobacillus*, *Lb. plantarum* is the most valuable species with beneficial properties, which is abundant in fermented foodstuffs from various habitats such as milk and cheese. *Lb. plantarum* can grow at temperatures between 15 and 45 °C and under acidic conditions with a pH as low as 3.2 [50]. Former studies demonstrated that the number of *Lb. plantarum* cells increase considerably during cheese ripening among nonstarter LAB identified along with other species [51, 52]. On the other hand, *Leuconostoc* species are used as flavor forming culture due to their ability to enhance buttery aroma, produce a number of volatile flavor compounds, and iconic slimy texture in many dairy products.
Leuconostocs have proved to increase the safety of food by producing a variety of antimicrobial compounds including hydrogen peroxide and antimicrobial peptides [53, 54]. The low numbers of leuconostocs identified from milk may possibly be explained by the complex nutritional requirements of these bacteria [55]. In recent years, isolation of *Lc. mesenteroides*, *Lc. dextranicum*, and *Lc. pseudomesenteroides* is more common from starter cultures or from cheese derivatives [56–58]. It is worth to mention that some strains of *Lb. plantarum* and *Lc. mesenteroides* are exopolysaccharides producers; exopolysaccharides are large, structurally diverse polysaccharides that permeate the extracellular environment in the form of capsules or biofilms, and these molecules can help bacteria to survive extreme environmental conditions [59], which suggested that these exopolysaccharides might be the reason for the predominance of these two species in this study. Also, it could be supposed that the microbial diversity of these genera is strongly related to the source from which it was isolated as well as the environment climate.

LAB strains were analyzed by PCR for rapid screening of bacteriocins-encoding genes; this technique can be an alternative method to indicate the possibility of bacteriocin production, especially when multiple species present in natural samples such as milk and fermented food [60]. According to the present study, we have

**Table 5** PCR amplification of bacteriocin genes from *Lb. plantarum* and *Lc. mesenteroides* isolates

| Strain no. | Bacteriocin gene | Bacteriocins activities (AU/ml) |
|-----------|------------------|---------------------------------|
|           | PInA             | PInEF | PInC | MesY |
| 2         | –                | N     | N    | 80   |
| 16        | +                | N     | N    | 80   |
| 1         | N                | N     | N    | 60   |
| 7         | N                | +     | +    | 80   |
| 13        | N                | +     | N    | 160  |
| 19        | N                | –     | N    | 80   |
| 15        | N                | +     | –    | 80   |
| 18        | N                | +     | –    | 40   |
| 22        | N                | +     | –    | 80   |
| 24        | N                | +     | –    | 80   |
| 27        | N                | +     | –    | 80   |

(+) gene present, (−) absence of gene, (N) not performed PCR for determine that carried genes bacteriocin

Fig. 4 Phylogenetic tree showing the phylogenetic placement of *Lc. mesenteroides* Egypt 2018 (MH817035) as inferred by the neighbor-joining method based on recA gene sequence analyses. Bootstrap values of 1000 replicates are given.
avoided choosing of subclass IIa genes of bacteriocin in *Lb. plantarum* strains, which are known to be active against *L. monocytogenes*. From BAGEL and BLAST bacteriocin screens conducted by Collins et al. [61], we were able to possibly predict bacteriocin operons from the *Lactobacillus* Genus Complex. Thus, the most common bacteriocin’s genes which were previously detected for *Lb. plantarum* strains were mostly related to class IIb bacteriocin. Besides, some studies have reported that certain strains of *Lb. plantarum* are capable to produce more than one peptide with synergistic antibacterial effect, probably class IIb bacteriocins [62]. Based on the observation of the PCR product, it revealed the high incidence of these two non-lantibiotic bacteriocin’s genes (*pln* A and *pln* EF) in *Lb. plantarum* isolates among them strain *Lb. plantarum* Egypt 2018, while these bacteriocin’s genes were absent in one strain (no. 19). Plantaricin EF is a two-peptide bacteriocin (*pln*E and *pln*F) that depends on the complementary and synergistically action of two different peptides to function. These two-peptide bacteriocins act by binding to a specific membrane protein (a bacteriocin receptor) that leads to membrane leakage and cell death [63]. *L. plantarum* strains have reported to produce more than one bacteriocin. For example, *Lb. plantarum* C-11 isolated from fermented cucumbers [64] produces two-peptide bacteriocins, plantaricin EF, and plantaricin JK. On the other hand, plantaricin A, previously incorrectly identified as the bacteriocin responsible for inhibitory activity by *Lb. plantarum* C11, may be considered a group II one-peptide bacteriocin, although it is a less potent antagonist than *pln*EF and *pln*JK, but it gives an additive antibacterial activity [65]. Sharing our results, Azizi et al. [39] confirmed the presence of *pln*EF and *pln*A genes in all identified *Lb. plantarum* strains. Unlikely, plantaricin C, a lantibiotic bacteriocin produced by a *Lb. plantarum* strain of dairy origin [66] was absent in tested strains.

Whereas *mesY* gene was found in one of two strains of *Lc. mesenteroides*. The *mesY* gene encodes mesentericin Y105 (Mes-Y105) is a class IIa bacteriocin produced by *Lc. mesenteroides* Y105 [67]. Hechard et al. [68] indicated that mesentericin Y105 decreases membrane potential and oxygen consumption of Listeria cells by using flow cytometry and oximetry analysis. Thus, existing of
this gene with no anti-listeria activity may be related to the development of natural resistance in *listeria* species to this bacteriocin [69]. When studying strains from the animal and human microbiota encoding bacteriocins, Collins et al. [60] concluded that there maybe is a link between the strains’ environment and bacteriocin production. Also, the present study suggests the same theory. The environmental factors and sources could not only control the biodiversity of LAB strains but also the distribution of bacteriocin-encoding genes. Yet, the presence of bacteriocin genes does not necessarily reflect their antibacterial potential as they also depend on other environmental factors and genetic mutations. So combining both molecular techniques and in vitro screening approach has enabled us to explore the persistence of bacteriocins across the genus.

**Conclusion**

Up to the best of our knowledge, the present study is the first report on the isolation and screening of bacteriocinogenic LAB from Upper Egypt dairy products and detecting their bacteriocin-encoding genes. *Lb. plantarum* and *Lc. mesenteroides* were the predominant bacteriocin producers. The identified isolates recorded a good antibacterial activity mainly against Gram-positive indicators and some Gram-negative ones. The presence of 22 *L. plantarum* strains and most of those strains shared the presence of two non-lantibiotic bacteriocin’s genes (plnA and plnEF) suggested that there might be a strong link between the strains’ environment and their distribution as well as their ability to produce bacteriocins. The present study was useful and timely to develop a rapid and reliable method that could be used for the identification of common LAB species and also to determine their bacteriocinogenicity in various fermented milk samples. More studies are needed to be conducted about the safe application of these bacteriocins as an important source for using as biopreservatives in foods or as a natural substitute for antibiotics.

**Abbreviations**

LAB: Lactic acid bacteria; GC: Glycine-cysteine; GRAS: Generally recognized as safe; PCR: Polymerase chain reaction; ECLAR programme: European Collaborative Linkage of Agriculture and Industry through Research 1988–1993; recA: Recombinase A; MRS: Man-Rogosa and Sharpe; CFU: Colony forming unit; pH: *Pondus hydrogenii* (quantity of hydrogen); AU: Arbitrary unit; DNA: Deoxyribonucleic acid; Lb: Lactobacillus; bac+: bacteriocin positive; bp: Base pair; rRNA: Ribosomal ribonucleic acid; NCBi: National Center for Biotechnology Information; SD: Standard deviation

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**Authors’ contributions**

SAA and MAM conceived and designed the experiments. RR performed the experiment. RR and SA wrote the manuscript. SAA, MAM, and HMA analyzed the data. SA reviewed and revised the manuscript, and HIMA supervised and validated the data. All authors have read and approved the manuscript.

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**Availability of data and materials**

Authors declare that all generated and analyzed data are included in the article. The genome sequences of *Lactobacillus plantarum* Egypt 2018 and *Leuconostoc mesenteroides* Egypt 2018 have been deposited in GENBANK under accession numbers of MH817034 [https://www.ncbi.nlm.nih.gov/nuccore/MH817034] and MH817035 [https://www.ncbi.nlm.nih.gov/nuccore/MH817035], respectively.

**Ethics approval and consent to participate**

Not applicable

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**Competing interests**

The authors declare that they have no competing interests

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