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# Molecular Detection of Plantacirin C Produced from *Lactobacillus Plantarum* Isolated from Dairy Products Sold in Assuit City, Egypt

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**ABSTRACT**

Last decade, bacteriocins have most concern due to their benefits in human health and immunity. One of the most important bacteriocin-producing bacteria is *Lactobacillus plantarum* (*L. plantarum*). Thus, this study aimed to screen for plantacirin C (*plnc*) predominant bacteriocin produced from *L. plantarum* in traditional dairy products (Milk, Cheese and yoghurt) of Assuit city in Upper Egypt. Molecular method has been used for the detection of bacteriocin-encoding genes (*plnc*) produced after isolation *L. plantarum* and identified by biochemical methods.

The findings showed that, at a proportion of 54.3%, 51 strains of *L. plantarum* were isolated from raw milk and conventional dairy products. Polymerase chain reaction (PCR) was used to find the genes encoding bacteriocin. Based on the molecular technique, 6 out of 51 isolates were carried *plnc* gene. The results concluded that raw milk is the highest sample containing *plnc* gene. More studies for molecular identification of plantacirin c in Egyptian dairy products should be advised. Bacteriocin has a lot of benefits for animal and human health so should be used as a natural bio-preservative, medicinal and a natural substitute for antibiotics.

## INTRODUCTION

Due to their numerous roles in food fermentation and preservation of food in addition to their existence in the intestinal tract of human, lactic acid bacteria (LAB) are significant microbes [1]. Mostly LAB is non-pathogenic and generally recognized as safe microorganisms (GRAS). According to biochemistry, Lactic acid bacteria comprises both homo-fermentative processes that primarily establish lactic acid and hetero-fermentative processes that produce a broad spectrum of fermentation products in addition to lactic acid, including acetic acid, ethanol, carbon dioxide, and formic acid [2]. The genera Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus contain the species of LAB. Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus, and Weissella are recent instances of other genera proposed as LAB [3] Numerous LAB have been identified as probiotic microorganisms that, when given in sufficient quantities, boost the host's health [4]**.** Lactic acid bacteria are useful in the food manufacturing. They lower the pH of food to a level low enough to prevent the growth of the majority of other microbes, including usual human diseases, improving the shelf life of fermented foods. [5].

*Lactobacillus plantarum* (*L. plantarum*) is a versatile and flexible bacteria found in various environments and foods, as well as in the normal microbiota of humans and animals [5]. It is well-known for producing antibacterial compounds, particularly bacteriocins, which are natural peptides with high commercial value and safety status [6]. *L. plantarum*-produced bacteriocins have broad-spectrum antibacterial effects and have a low capacity to generate resistance, making them beneficial in therapeutic applications alongside antibiotics. [7]. Plantaricin is one of the most essential bacteriocins developed by *L. plantarum*, and the bacteria can generate at least six distinct kinds of it. [8]. *L. plantarum* bacteriocin-producing strains are frequently used in the production of food. Additionally, probiotic *L. plantarum* strains have been shown to improve a variety of medical ailments such as diarrhea, psoriasis, allergies, and nervous system diseases [9, 10].

*L. plantarum* from dairy origin produces bacteriocinin named plantaricin C (*pln*C),which is lantibiotic. It possesses a broad spectrum of antibacterial activity against both bacteria responsible for food spoiling and bacteria that cause food poisoning.. *Pln*C activity is sensitive to pronase, trypsin, and α-chymotrypsin, but unaffected by other proteolytic enzymes, amylase, or lipase. The bacteriocin remained stable under storage at 4 ºC and -20 ºC, and after being heated to 100 ºC for 1 hour or 121 ºC for 10 minutes. The optimal pH for PlnC activity is acidic or neutral, and its activity diminishes at alkaline pH [11]. Studies indicate that *Pln*C works by being bactericidal or bacteriolytic [12].

In the realm of animal production and food, however, it is best to avoid using bacteriocins simultaneously as medicinal agents and food preservatives. As the mechanisms of target cell recognition, producer cell self-protection (immunity), and bacteriocin resistance of sensitive bacterial cells become better understood in the field of bacteriocin research, such developments would also make it possible to design efficient bacteriocins against undetected sensitive bacteria and to overcome bacterial resistance

[13]. The identification of Lactobacillus plantarum and detection of the plnC gene in different dairy milk products were the objectives of this research. Also, the inhibitory effect of the isolated strains was determinted against some foodborne pathogens.

## MATERIALS AND METHODS

### Collection of samples:

This research was done from October 2021 to April 2022. A total of various traditional dairy samples consist of 28 raw milk samples (cow, buffalo, camel and goat), 33 samples of each cheese and yoghurt, which were collected from Assuit city, Egypt. Sterilized bottles were used to collect samples and delivered to the laboratory using an icebox and were preserved in a refrigerator (around 4°C) until the microbiological examination analysis began.

### Isolation and identification of *lactobacillus plantarum*

The diluted test samples were performed according to Patil, M.M., et al., [14] then the next procedure done according to Farber et al. and El-Shafei et al., [15, 16].The morphology of Lactobacillus colonies should appear white, smooth and convex with regular edges.The colonies displaying the general characteristics of *Lactobacillus plantarum* were chosen from each plate for physiological and biochemical tests. *Lactobacillus plantarum* were characterized by Gram staining, catalase reaction, oxidase test and glucose fermentation [17, 18]

### Molecular identification

### Genomic DNA extraction

Counted cells of interest (either suspension or trypsinated adherent cells) and pellet cells ($10^6$ to $10^7$) were centrifuged (600g, 5 min in tabletop centrifuge) using a labeled 1.5 ml safe-lock tube. The pellets were resuspended into 100µl of PBS. Tubes were placed at 95ºC for 15 min. And were then centrifuged at >10.000 g for five min. To pellet the cellular debris. The lysate (supernatant) is transferred into a correctly labelled (with sample name) 1.5 ml Eppendorf . Lysate is keeped at temperature (-20 to 4ºC ).

### Genes encoding bacteriocin production detection:

The PCR assay performed was used according to [19, 20] for determination of the bacteriocin gene which contain plnC. PCR primers (Metabion, Germany) were F (5'-GGTGGCGACAGGAGATTTAC-3') and R- (5'-AGAAACGCGTTCCGATTTTA-3'). The final volume of PCR mixture is 25 µl composed of 12.5 µl of 2X PCR master mix (Green Master, Promega, USA), 150 ng of the DNA template, 0.5 µM of each primer and complete the mixture up to 25 µl Nuclease free water were mixed in a PCR tube. The amplification was performed in a programmable heating block (Gradient Thermal Cycler, Veriti Applied Biosystem, USA). The conditions of PCR were beginging by initial DNA denaturation and enzyme activation steps were proceeded at 95 ºC for 5 min., followed by 40 cycles of denaturation at 95 ºC for 30 s., annealing at 52 ºC for 30 sec. and extension at 72 ºC for 30 sec., and a final extension at 72ºC for 10 min. The amplified product size was 353 bp.

The PCR products were separated by electrophoresis on 1% agarose.

Loading Marker and running was on the power supply (Biometra, Germany) for 60 min, 110 volts, 120 amp. The image was visualized by the transilluminator (biometraT12) and the documentation system (Viber Loumat Kaiser).

**Plantacirin C preparation:**

After *L. plantarum* propagation in MRS broth, the culture was subjected to centrifuge at 10000 rpm for 20 min under cooling (4 ºC) using cooling centrifuge (Jouan, UK) to remove cells. The cells free supernatant (CFS) was passed through membrane filters with pore diameter of 0.22 µm and then stored at 4 ºC till use as pure bacteriocin [21].

Detection of the antimicrobial activity of plnC against S. aureus and E.coli O157:H7 using well diffusion method.

Wells were made on MHA plates containing indicator strains (*Staphylococcus aureus* and *Eschericia coli* O157:H7), 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 [22].

## RESULTS

*L. plantarum* isolation and identification from dairy products.

The lactobacilli's and *L.plantarum* microbial population cultivated on MRS revealed that the highest bacterial level was represented in raw milk followed by yoghurt than cheese (Table1). This research showed that the Lactobacilli were the most common bacteria isolated (72 samples out of 94), The predominant lactic acid bacteria found in milk and milk products was *L. plantarum*.. Out of 94 samples, only fifty one was considered as *L. plantarum*( Table 2), which has a distinctive feature of positive Gram stain, negative for catalase, negative oxidase and positive glucose fermentation. The Gram stain of the isolates was assessed by light microscopy after the staining. *L. plantarum* is known to be Gram+ve (Photo 1 ).

Photo 1. Microscopic characterization of isolated strain (*L. plantarum*)

**Table 1: Prevalence of *Lactobacilli spp. in milk and some dairy products.***

| Type of sample | sample no. | No. of positive samples | Percentage(%) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Milk | 28 | 28 | 100 |
| Yoghurt | 33 | 23 | 69.7 |
| Cheese | 33 | 21 | 63.7 |
| Total | 94 | 72 | 76.6 |

**Table 2: Prevalence of *L. plantarum in milk and some dairy products.***

| Type of sample | Sample no. | No. of positive samples | Percentage (%) |
|---|---|---|---|
| Milk | 28 | 21 | 75 |
| Yoghurt | 33 | 16 | 48.5 |
| Cheese | 33 | 14 | 42.4 |
| Total | 94 | 51 | 54.3 |

**Table 3: Frequency distribution of *plnC gene in milk and some dairy products.***

| Sample Type | Positive samples for *L. plantarum* | Positive samples for *plnC* | Percentage (%) |
|---|---|---|---|
| Milk | 21 | 5 | 23.8 |
| Yoghurt | 16 | 1 | 6.3 |
| Cheese | 14 | 0 | 0 |
| Total | 51 | 6 | 11.8% |

## Molecular identification:

### Bacteriocin genes' detection:

It was determined whether 51 chosen strains contained the *plnC* bacteriocin gene.The findings revealed the fact that only 6 of the *L. plantarum* strains investigated at 353 bp have *plnC*. Out of 6 tested *L. plantarum*, 5 samples belong to milk samples, and one is from yoghrt samples(Table 3).

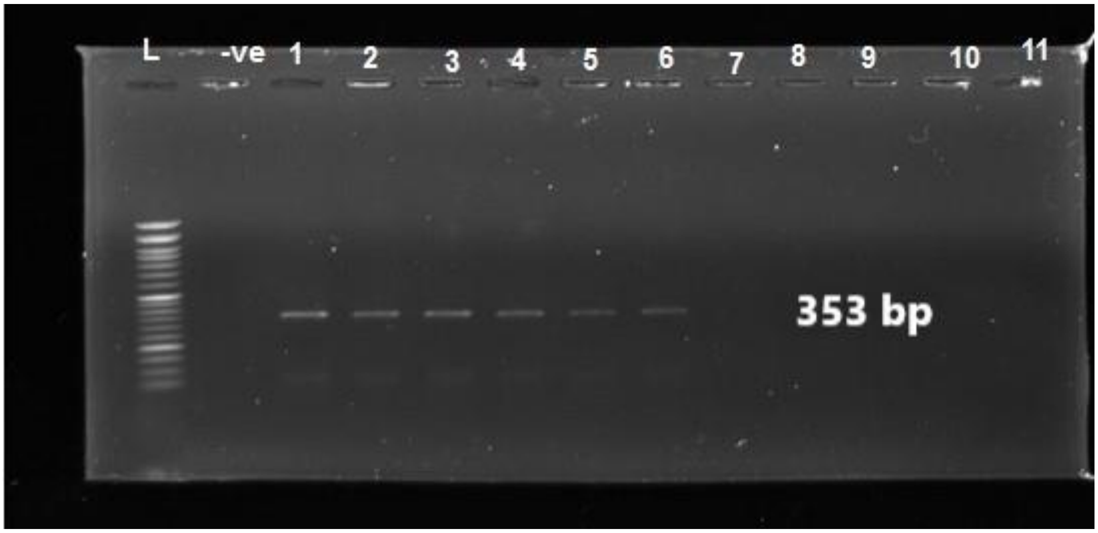


**Photo 2.** Electrophoresis gel of PCR product of *pln*C fragment lane L: ladder (50 bp), Lane -ve: negative control; Lane (1-6): positive for *pln*C with the specific band at 353 bp, Lane (7-11): negative samples.

The inhibitory effect of bacteriocin of *L. plantarum* was determined by using two reference strains as *E.coli* O157:H7 and *S. aureus*.The diameter of inhibitory zone against *E.coli* O157:H7 was 10±1.9mm, on the other side, the result of *S. aureus* was 8.7± 2.5mm.

## DISCUSSION

*L. plantarum* is a crucial species within the food and dairy fermentation industry among all other species of Lactobacillus. It is extensively utilized in the production of cheeses, yoghurt, fermented milk, and other fermented food products. Despite having comparable dietary and growth requirements and minor differences in phenotypic traits among all *Lactobacillus spp.*, identifying all of them through classical microbiological methods can be challenging. Conventional methods tend to produce unclear outcomes and even incorrect identifications [10].

The results of this study indicated that 72 out of 94 isolates were *Lactobacillus spp.*,this result was similar to El-Shafei et al.[16] , Abd El Gawad et al. [23] and Refay et al. [24] shown that dairy products contain a greater percentage of *Lactobacillus spp*. El-Shafei et al. [16] also noted that among isolated LAB, *Lactobacillus spp*. predominated because milk and milk products are a wide range of bacteria. Additionally, the common use of fermented milk produced from cow milk may encourage the proliferation of these species.The high prevelance of *lactobacillus spp*. In milk is due to neutral pH of milk also *lactobacillus spp*. Can resist low pH. [25].

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, oxidase reaction and glucose fermentation test. In the current study, the percentage of *L. plantarum* was 56.6% (51 samples out of 94 samples). In order to survive the digestive process, lactic acid bacteria in fermented milk must be present in high quantities. These bacteria have the greatest impact on gastrointestinal functions and serve as probiotics. Moreover, despite being few in number, lactobacilli are the most common bacteria in a normal fasting stomach. [12]. Also, this disagreed with Heller et al. [26] reported that the low amounts of Lactobacilli may be due to probiotic bacteria participating actively in fermentation, on the other hand, Probiotic organisms' growth will be slowed down or completely inhibited as a result of the antagonistic interactions between starter cultures and probiotics.

During the current study, as indicated in (Table 2), after the isolation of *L. plantarum* from a total of 94 samples (28 raw milk, 33 yogurt and 33 cheese) samples, a total 51 (54.26%) samples were phenotypically positive for *L. plantarum* as 21, 16, 14 samples from milk, yogurt and cheese respectively, which were characterized as creamy-white, circular smooth, low convex on selective MRS agar, Gram positive rods strucured as chains/double/single and catalase negative reaction. By using the PCR technique for molecular isolation, only 6 samples (11.76%) were positive for *L. plantarum* as followed (5 milk and 1 yogurt sample). This result is lower than the result postulated by Refay et al.[24], who detected *L. plantarum* in 22 (95%) of the examined traditional dairy products samples . However, the obtained result of this study was higher than the result done by Herreros et al. [27], Fguiri et al.[28], Yam et al. [29], Karakas-Sen [30], Fahem [31] Ahmed et al. [32].

Based on the above results, it is clear that the highest percentage of *L.plantarum* were in raw milk then yoghurt carried *pln*C gene, while the isolates strains from cheese samples couldn't be detect *plnC* gene in them, total only 6 isolates out of 54 contained this gene.

Bacteriocin-producing genes may be located on the chromosomal DNA or plasmid of the bacteria. The plantarici.n C locus, which was composed of six genes arranged in an operon-like configuration, was discovered in an 18 kbp-long contig in the plasmid of *L. plantarum*. The bacteriocin structural gene (*pln*C), a gene expressing a LanM-like protein hypothesised to be critical in the maturation of plantaricin C, and four downstream genes encoding ABC-type transporter components were all found at this locus. These genes likely belonged to the proposed immunity and export mechanism. *Pln*C encodes a precursor to bacteriocin, a 58-amino acid peptide with a 27-amino acid core peptide and a 31-amino acid double-glycine leader peptide. [33]

The plantaricin C protein could enter the susceptible organisms through the cytoplasmic membranes in a voltage-independent manner without the need for protein receptors. There, it presumably polymerizes, forming a barrel structure that causes pores to form where the plasma content is discharged [34]. The result is that the cell dies and, occasionally, lysis is caused by the food-borne and spoilage bacteria. Therefore, the results recorded before in this study confirmed the previous fact.

Accroding to well defusion test to detecte the inhibitory effect of bacteriocin against the indicator microorganisms, the plantacirin C have inhibitory effect against *S. aureus* and *E.coli* O157:H7. Many previous studies described the antibacterial effect of plantacirin C against Gram-positive as *S. aureus, Listeria sp.*, *Streptococcus sp.* and *Enterococcus sp.* [34], while the same authors couldn't obtained any inhibitory effect against Gram-negative bateria as *E. coli*.

*Lactobacillus spp. and L.plantarum* were highest in raw milk samples and that can be explained because raw milk has neutral pH and not subjected to heat treatment.

## CONCLUSION

The increasing demand for products associated with health benefits has increased the research on underexplored foods and their potential as probiotic sources. In the present work, milk and some dairy products, were examined for the presence of lactic acid bacteria. Results revealed that most isolated strains of lactic acid bacteria followed *L. plantarum*. Some of the isolated strains carried the plantaricin C gene, this gene responsible for producing plantaricin C bacteriocin. In addition, plantaricin C had inhibitory effect against food poisoning bacteria as *S. aureus* and *E.coli* O157:H7. So, the production of bacteriocin is a useful source of natural food preservatives to reduce the effect of chemical preservtion.

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