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Impact of pH and temperature on bacteriocin activity and plantaricin C gene expression of *Lactobacillus plantarum* bacteria

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ABSTRACT

This study aimed to investigate the effect of different pH (7 and 4) and temperatures (4 and 40°C) on bacteriocin activity and plantaricin C (*plnC*) gene expression of Lactobacillus plantarium. Six strains of *L. plantarum* were used in these trials. The bacteriocin activity was measured after the different treatments by well diffusion test using indicator bacteria (Staphylococcus aureus and Escherichia coli O157:H7). Moreover, the *plnC* gene expression was determined by real-time PCR using 16S rRNA primers for universal bacteria and plantacirin C primers. The results declared a significant difference between the different pH and temperatures. In addition, the downregulation of *plnC* gene expression in pH 4. The upregulation of the same gene was applied to 40 °C during bacteria incubation. Also, there is no correlation between the bacteriocin activity and *plnC* gene expression after applying different pH and temperatures in *L. plantarum* bacteria.

Introduction

Bacteriocins, antimicrobial peptides produced by various bacteria, are gaining attention for their potential health benefits and therapeutic applications. Lactic acid bacteria, Specifically *Bifidobacterium* and *Lactobacillus* genera, are recognized for produce bacteriocins, which play a crucial role in microbial antagonistic relationships (Darbandi *et al*., 2022). These compounds, along with other antimicrobial substances, contribute to organisms' immune responses and defensive systems, enhancing their ability to compete for resources and space (Roces *et al*., 2012).

Lactic acid bacteria (LAB) bacteriocins, with their stability and narrow to broad-spectrum antimicrobial activity, are being researched extensively for their potential as new therapeutic antibiotics and natural food preservatives (Perez *et al*., 2014). Despite their antimicrobial properties, bacteriocins show no evidence of inducing bacterial resistance, likely due to factors such as rapid pore formation and enzymatic degradation (Dicks *et al*., 2018). LAB bacteriocins have been found to inhibit various intestinal infections, highlighting their potential as antibiotics for gastrointestinal illnesses (Alvarez-Sieiro *et al*., 2016). *L. plantarum*, a common LAB species, produces a bacteriocin known as plantaricin C (*plnC*). This bacteriocin is classified as a lantibiotic, indicates a wide range of inhibition against Gram-positive bacteria and is highly stable under various environmental conditions (Darbandi *et al*., 2022). *plnC*'s mechanism of action involves pore formation in the target cell membrane, leading to cell death. This bacteriocin has shown promise in food preservation and infection control due to its resistance to environmental factors and storage conditions (Ibrahim *et al*., 2021).

pH and temperature effect on *plnC* and its gene expression are critical for understanding its stability and potential applications. Research has shown that *plnC* remains stable under acidic and neutral pH environments but loses effectiveness at alkaline pH. Moreover, *plnC*'s activity is not affected by lipase, α-amylase, and proteinase K, but is eliminated by protease, trypsin, and α-chymotrypsin treatments (Abdulhussain Kareem and Razavi, 2020). This information is valuable for optimizing the use of *plnC* in various applications, such as food preservation and infection control. This study investigated the impact of pH and temperature on the bacteriocin activity produced by *L. plantarum* and *plnC* gene expression.

Materials and methods

Ethical approval

Guidelines from the Molecular Biology Research and Studies Institute at Assiut University, Egypt were followed in conducting this study (IORG0010947-AB-21-23-A).

Preparation of L. plantarum

Six strains of *L. plantarum* were isolated from milk products and identified by microbiological and molecular techniques in the Molecular Biology Research Unit, at the Molecular Biology Research and Studies Institute, Assiut University, Egypt according to (Gad *et al*., 2023).

Preparation of indicator strains

Staph. aureus (ATTTC: 29213) and *E. coli* O157:H7 (ATCC:43888 tm) were used as indicator strains in this experiment. They were obtained from Licensed Food Lab at Animal Health Research Institute, Giza, Egypt, and inoculated in 100 ml sterile peptone 0.5 Mcfarland standard and diluted to obtain a concentration of 1.5×10^7cfu /ml (Elsherif *et al*., 2024).

Effect of pH and temperature on bacteriocin activity and plnC gene expression (Syrokou et al., 2021)

To investigate the effect of pH on the production of bacteriocin, six distinct sets of flasks containing 100 ml of sterilized MRS broth were inoc-

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ulated with different 6 strains of *L. plantarum*. Then the pH was adjusted to 4 and 7 by using 6 M HCl and NaOH. The treated broths were incubated at 37ºC for 24 hours. Another six flasks inoculated by the same previous 6 *L. plantarum* strains were incubated without aeration at different temperatures of 40 and 4ºC for a duration of 20 hours to determine the influence of temperature on bacteriocin activity. Following incubation, the medium's bacteriocin titer was measured. The activity of bacteriocin was evaluated by the agar well diffusion method. Also, the gene expression of plantacirin C gene was determined by using real-time PCR.

Bacteriocin preparation

After *L. plantarum* preparation in MRS broth, the broth was centrifuged at 10,000 rpm for 20 minutes at 4ºC in a cooling centrifuge (Jouan, UK). The supernatant was passed through membrane filters with pore diameter of 0.22 Mm and then stored at 4ºC.

Bacteriocin activity assay

The bacteriocin activity was determined by the well diffusion method. After forming wells on MHA plates with indicator strains and adding 50 µl of crude bacteriocin to each well, the inhibition was eventually visible as a clear zone surrounding each well following a 24-hour incubation period at 37ºC.

Determination of plantacirin C gene expression after treatment with different pH and temperatures

Extraction of RNA from *L. plantarum* strains

Total RNA was extracted from 10 ml of the previously treated broths of *L. plantarum* using the ABT total RNA mini extraction kit (Applied Biotechnology, Cat.No. ABT002).

Converting RNA to cDNA

The cDNA was obtained by using ABT H-minus cDNA synthesis kit (Applied Biotechnology, Cat.No. ABT009). The total volume of this reaction was 20 µl. The mixture contained 4µl 5×RT BUffer, 0.5 µl Hminus MMLV (200 unit)2µl Oligo (dt) 18 primer (10mm), 2ml of NTPs mixture (10 m M) 1.5µL deionized water and 10 µl of isolated RNA.

Real-time PCR

In a thermal cycler, the real-time PCR amplification was carried out (Stepone™ Thermal Cycler, Applied Biosystems, USA). A 25 µl final volume Real-Time PCR reaction was carried out, containing 12.5 µl of 2×Sybregreen master mix (Hera, England), 150 ng of cDNA template (spectrophotometer, Gene Quant 1300, England), 1µl of every primer, and the volume was used 25 µl by Nuclease- free water. The thermal profile was done as followed: 95ºC for five min of initial denaturation; 40 cycles of 95ºC for 30 sec of denaturation, the annealing was 52ºC for 30 s, and extension at 72ºC for thirty seconds, then the final extension at 72ºC for 5 min. The fluorescent product was detected during the annealing step of each cycle. Amplification, detection, and data analysis were performed on a Stepone plus TM real-time PCR (Applied Biosystems, USA) version 2.0 . The relative fold differences in each treated sample were determined by deducting the CT value of each gene expression of plantacirin C (*plnC*) gene from the CT value of 16S rRNA gene of bacteria.

Statistical analysis

The bacteriocin activity was determined by using GraphPad Prism 9.5.1 software (GraphPad Software Inc., San Diego, CA, USA) by mean, standard deviation and significance.

Results

Table 1 shows the effect of different pH and temperatures on the activity of bacteriocin produced by *L. plantarum*. The data illustrated no significant difference between the bacteriocin activity produced by incubated bacteria at pH 7 and 4 on *Staph. aureus* bacteria, while the bacteriocin activity on *E. coli* O157:H7 showed a significant difference between the two pH treatments. Moreover, the bacteriocin produced after incubation of *L. plantarum* at 4 and 40°C had a significant difference between the two temperatures when applied to the indicator bacterium.

Table 1. Effect of different pH and temperatures on bacteriocin activity of *L. plantarum* strains.

Treatment		*Staph. aureus*	*E. coli* O157:H7
pH	At pH 7	12.33±1.25	11.11±1.05*
	At pH 4	12.17±1.17	10.33±0.75
Temperature	At 4°C	11.47±1.93*	9.47±1.36*
	At 40°C	10.08±1.66	8.08±1.71

Data are expressed as Means±S.D. Inhibition zones of different treatment on *plnC*at Maccferlan (1.5x107cfu/ml) against *Staph. aureus* and *E. coli* O157:H7.

By Real-time PCR using 16s rRNA primers for universal bacteria and *plnC* primers, from the obtained results of the gene transcription due to the effect of applying different pH values (7 and 4) that illustrated the downregulation of *plnC* gene at acidic pH (pH 4) it was 0.0108. While at normal pH (pH 7) the fold change of the same gene was 1.0000. In addition, the low temperature (4ºC) can downregulation of *plnC* gene expression in contrast the high temperature (40ºC) can upregulation of the same gene, the fold change became 2.302 (Table 2).

Table 2. Effect of different pH and temperatures on *plnC* gene expression of *L. plantarum* strains.

Treatment		Fold change ($2^{-\Delta\Delta ct}$)
pH	At pH 7	1
	At pH 4	0.01
Temperature	At 4°C	1.01
	At 40°C	2.30

Discussion

The fact that *L. plantarum* has shown an inhibitory effect on these bacteria suggests that it has antibacterial action. The test organisms contain the compound bacteriocin, according to the results. It has been found that bacteriocins inhibit several different microorganisms. According to Flythe and Russell (2004), the presence of bacteriocin by *L. plantarum* suggests that the bacteria have potential applications as both probiotics and biopreservatives.

According to our research, *L. plantarum* inhibits S. aureus, because probiotic bacteria primarily work to inhibit pathogens by producing compounds like hydrogen peroxide, organic acids, low molecular weight antimicrobial substances (Pancheniak and Soccol, 2005).The obtained result agreed with Li *et al.* (2023) who reported that *L. plantarum* has inhibitory effect on some pathogenic bacteria as S. aureus.

Initial pH values have a pronounced effect on bacteriocin activity. In the present study, two pH values, namely, 7.0 and 4.0 applied. The data obtained revealed a higher effect against *Staph. aureus* and *E. coli* O157 were obtained by incubation *L. plantarum* at pH 7. This result agreed with Syrokou *et al.* (2021) who claimed higher levels of plantaricin activity by the four *L. plantarum* strains after growth in a medium adjusted to an initial pH of 6, compared to that at pH 5.

Temperature constitutes a crucial factor in cell growth and bacteriocin production. Several authors have suggested that maximum plantaricin

activity levels are recorded at temperature values below the optimum one for cell growth (Mataragas *et al*., 2003; Sidooski *et al*., 2019). However, this is not a rule, since there are cases of maximum bacteriocin activity levels recorded at temperature values close to the optimum for cell growth. In this study, temperatures applied were 4 and 40ºC. Results obtained in the present study revealed that the bacteriocin of *L. plantarum*, produced at 4ºC more effective against the two indicators microorganisms than that produced after incubation at 40ºC. These previous results disagreed with (Syrokou *et al*., 2021) who revealed that the four strains of *L. plantarum* LQC 2441, 2422, 2485 and 2516, give low planctaricin activity when these strains grow at 30 and 37ºC, while the high activity was obtained after incubation at 20 ºC.

According to Zhang *et al*. (2018), who conducted their investigation, bacteriocin J23, produced by *L. plantarum* J23, isolated from traditional fermented milk products in China, demonstrated pH stability in the range of 2.0 to 12.0 and heat stability at temperatures below 100°C for 30 minutes. While we found that bacteriocin of *plnC* produced by *L. plantarum* increased by heat at 40°C and decreased by increasing acidity.

Additionally, it was demonstrated that two bacteriocins, JW3BZ and JW6BZ, generated by *L. plantarum* (isolated from Bulgarian boza) were effective against various kinds of Gram-positive bacteria (Von Mollendorff *et al*., 2006). According to Arena *et al*. (2016), Gram-positive bacteria appear to be more susceptible to the effects of *L. plantarum* bacteriocin than Gram- negative and this agreed with our result which showed the sensitivity of *Staph. aureus* and *E. coli* and observed high sensitivity at temperatures 4º and 40ºC against *Staph. aureus* and the strong inhibitory activity for *E. coli* at pH 4 and 7 at Macferlan (1.5×10^7cfu/ml).

The decrease in the pH value from 7 to 4 had downregulation in the *plnC* gene expression. This result was parallel the result carried out by Syrokou *et al.* (2021), they found that four strains of *L. plantarum* were downregulated of *plαD* gene when they decreased the pH from 6 to 5. Moreover, the temperature increases lead to the upregulation of *plnC* expression. This result agreed with Syrokou *et al*. (2021) who described that the effect of high temperature on gene expression especially *plnG*, *plnY*, *plnI* and *plnM*, the previous genes showed upregulation in its expression.

Summarizing the results of the present study, the major findings revealed that the lack of correlation between plantaricin C activity and gene expression due to post-transcriptional mRNA or post-translational of plantaricin C protein (Picard *et al*., 2009) after different treatments of pH and temperatures, in addition, the extracted bacteriocin contains many types of proteins rather than plantaricin C which may be not affected by different pH or temperatures. Also, the obtained results were similar to results carried out by Syrokou *et al*. (2021), they found that there is no correlation between the different plantaricin protein activity and gene transcription of these genes.

The data discussed in this study suggest that various food conditions may affect the prevalence of certain strains of *L. plantarum* that are capable of competing with other bacterial populations in addition to responding to particular adverse circumstances and that agree with Cao *et al*. (2013).

Conclusion

Plantaricin C is one of bacteriocin produced by *L. plantarum*. The activity of this protein can be affected by pH and temperature their significant difference between the different treatments. Moreover, downregulation of *plnC* gene expression due to decreased pH (4), while upregulation of the same gene when the bacteria incubated at 40°C. There is no correlation between bacteriocin activity and gene expression under the effect of different pH and temperatures.

Conflict of interest

The authors declare that they have no conflict of interest.

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