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## ORIGINAL ARTICLE

# Inhibitory Effects of *Lactococcus lactis* and its Supernatant on *Listeria monocytogenes*

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## SUMMARY

***Background:*** **The application of biological compounds generated by lactic acid bacteria, especially *Lactococcus lactis*, is recently considered to be a natural preservative for improving quality and health of food. The purpose of this study is to investigate the inhibitory potential of *L. lactis* supernatant on the expression of *inlA*, *plc*, and *hly* genes related to *L. monocytogenes* virulence capacity.**
***Methods: L. lactis*** **was cultured under anaerobic conditions for 16 - 18 hours. The supernatant and live bacteria were then separated by centrifuge. The anti-listeria effects of *L. lactis* and supernatant were measured using the agar diffusion technique, and the effect on the expression of the virulence-related genes was calculated by real-time PCR. Also, the effects of live bacteria and its supernatant on the microbial count of milk and sausage infected by *L. monocytogenes* was evaluated by the colony count assay.**
***Results:*** **After 24 hours, the highest non-growing hole diameter was obtained in the presence of acidic supernatant (pH = 3.5). The microbial count showed the inhibitory effect on the eighth day after incubation with *L. lactis*. qPCR data revealed a down-regulation of virulence-related genes of *inlA* (8 fold), *hly* (6 fold), and *plc* (1 fold) in *L. monocytogenes* after 24-hour incubation with the supernatant.**
***Conclusions:*** **Our findings showed that the supernatant of *L. lactis* has an effective inhibitory role in the growth of *L. monocytogenes*. In the presence of supernatant, among *plc*, *inlA* and *hly* genes, the expression of *inlA* and *hly* genes decreased after 2 hours, which could indicate the molecular inhibitory mechanism of *L. lactis* in *L. monocytogenes*.**
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*Listeria monocytogenes*, *Lactococcus lactis*, supernatant, real-time PCR

## INTRODUCTION

Food-borne diseases (FBDs) are still a serious concern for the human population and, as one of the most widespread health problems, they have various implications both on health and development [1]. Due to the lack of foodborne disease surveillance systems, the frequency

of FBDs has been under-estimated; however, it is believed that FBDs are 300 - 350 times more frequent than the global reports [2]. Microbial contamination of foods can occur at any point along the production chain - during production, processing, distribution, or preparation - in the factories. On the other hand, contaminating bacteria in raw or processed materials may exist; therefore, there should be monitoring measures from the beginning of the food production cycle [3].

*L. monocytogenes* is a major bacterial foodborne pathogen which is an opportunistic pathogenic gram-positive, optional anaerobe, without spores, catalase and negative oxidase bacterium [4-8]. Unlike many bacteria, *L. monocytogenes* grows well at refrigerated temperatures, is able to survive under different conditions such as in the temperature range (0 - 42°C), in pH range of, and in high salt concentration. *L. monocytogenes* is abundantly found in the surroundings including soil, water, vegetables, and foods, especially ready-to-eat (RTE) foods [9]. Its ability to grow at refrigeration temperature and bacterial resistance against these environmental factors makes it difficult to control in the food industry [10]. The use of unpasteurized milk for cheese manufacturing and the environment are two major sources of finished product contamination with *L. monocytogenes* [11]. Listeriosis is a severe foodborne disease with high incidence and with high hospitalization cases (~300 cases/ year) and high fatality rates (20 - 30%) [10]. Transmission of the disease occurs through the consumption of contaminated foods such as ready to cook meat, seafood, and dairy products [12]. However, investigation in processing plants producing pasteurized fresh cheese has revealed that food contact surfaces may facilitate biofilm formation [13]. Therefore, further studies are needed to figure out effective methods to control *L. monocytogenes* in foods and food production plants. Various preservation technologies have been introduced in many reports regarding the inhibition of *L. monocytogenes.* Control of *L. monocytogenes* in a variety of RTE seafood or meat products by application of bio preservation approaches such as protective microorganisms have been indicated as an efficient strategy [14,15]. Bacteriostatic potentials against many bacterial species have been found in lactic acid bacteria (LAB) as excellent candidates for preventing the growth of pathogenic bacteria in food products. LAB exert their effects through various mechanisms and do not cause unfavorable sensory changes in foodstuffs [10]. Antimicrobial compounds such as bacteriocins, bacteriocin-like inhibitory substance, reuterin, organic acid or hydrogen peroxide have been demonstrated to have growth inhibition ability against the target bacteria which is usually described as Jameson effect [16-19].

Many studies on natural substances have recently been conducted on the control of the growth of food pathogens, including *Listeria*, among which the effectiveness of bio-pro-active lactic acid bacteria and its metabolites has been proven [12]. However, the molecular bases through which the production of inhibiting metabolites is not evidenced and must be tested. Bacterial pathogenic factors play an important role in the development of disease caused by *L. monocytogenes*. Overall, *L. monocytogenes* invasion by a large number of pathogenic factors including inlA, hlyA, and plc mediates host cell attack, escape from the host cell, enter the host cell, and leads to survival in the host [4,20].

This study was designed to evaluate the antimicrobial activity of *L. lactis* and its supernatant on *L. monocytogenes* through diffusion assay, expression assay of virulence-related genes, and colony count assay.

## MATERIALS AND METHODS

### Bacterial strains, culture media, and conditions

*Lactococcus* strain (prepared from the microbial collections of the University of Tehran, Iran) and *L. monocytogenes* were prepared. The *L. lactis* strain ATCC11454 was cultured in de Man, Rogosa and Sharpe (MRS) agar for 24 hours and gram staining was performed after one culture passage (sub-culture). In addition, *L. monocytogenes* strain ATCC1298 was prepared at the microbiological department of Iran University of Medical Sciences (Tehran, Iran), and cultured in Brain Heart Infusion (BHI) agar for 24 hours, and different examinations were carried out following one sub-culture. All the experimental stages were conducted in the Department of Medical Bacteriology and Cellular and Molecular Research Center of Iran University of Medical Sciences.

### The growth curve of *L. lactis*

To obtain the growth curve of *L. lactis*, dissolve a colony of bacteria in 15 mL of MRS broth and incubate in a shaker incubator at 37°C at 150 rpm for 24 hours. The culture of *L. lactis* is then diluted 1 to 100 and inoculated in 50 mL of MRS broth and transferred to a shaker incubator at 37°C at 150 rpm for up to 24 hours and at 4, 8, 12, 16, and 24 hours serial dilution and colony count were done.

### Antimicrobial assays

The presence of antimicrobial compounds in *L. lactis* and its supernatant or, in other words, determining the sensitivity of *L. monocytogenes* was evaluated by using well diffusion assay. In summary, fresh *L. monocytogenes* suspension in aerobic conditions in BHI broth culture medium and *L. lactis* suspension in 13 mL MRS medium were prepared and incubated for 48 hours in a shaker incubator. The prepared microorganisms were then transferred equally into sterile test tubes and simultaneously co-cultured for 24 hours at 37°C. Also, to determine the inhibitory effect of *L. lactis* supernatant, at the first step, one mL of a suspension containing $10^6$ CFU $mL^{-1}$ of *L. monocytogenes* was poured in 15 mL BHI agar plates and kept at room temperature for 15 - 20 minutes. In the next step, a culture medium containing *L. lactis* in its exponential growth phase was re-

moved from the incubator and was centrifuged at 1,000 rpm for 10 minutes. The supernatant consisting of all bacterial metabolites and the bacterial pellet were isolated to use in downstream treatments. The supernatant was then filter sterilized (0.45 μm) and ten microliters of filtered supernatant and ten microliters of neutralized supernatant that was neutralized by addition of 5 mol/L NaOH were dropped (as a spot) onto the solidified BHI agar, and the plates were incubated 24 hours at 37°C to detect inhibition zones around the spots. The mean diameter of the inhibition zone was measured 24 hours after inoculation of *L. monocytogenes* with *L. lactis*. MRS medium was used as negative control.

**Colony count assay**

Colony counting method was used to assess the inhibitory effects of *L. lactis* in milk and sausage. For this purpose, effective probiotics against *L. monocytogenes*, sterilized milk and autoclaved sausage samples were inoculated with *L. monocytogenes* at concentrations of $10^3$ CFU/mL simultaneously in the presence of *L. lactis* at concentrations of $10^7$ or its supernatant. The growth rate of *L. monocytogenes* was measured at 1, 8, and 16 days after inoculation at 37°C by colony count compared to the negative control samples. All the experiment values were independently determined three times

**Reverse-transcriptase real-time PCR (RT-qPCR)**

RT-qPCR was performed to measure the expression status of virulence-related genes of *L. monocytogenes* in the presence of *L. lactis* or its not neutralized and neutralized supernatant. Therefore, the total RNA was extracted from treated and untreated *L. monocytogenes* samples using GenElute™ Total RNA Purification Maxi Kit (Sigma-Aldrich, USA) based on the manufacturer's instructions. The quantity and quality of extracted RNAs were evaluated by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). In addition, the extracted RNA was electrophoresed on 1% agarose gel to assess its integrity. In the next step, cDNA synthesis was performed on 500 ng RNA using cDNA synthesis kit according to manufacturer's guideline. Specific primers were designed using Oligo 7 software (Table 1). The expression analysis of target genes including *hly*, *plcA*, and *inlA* was carried out by using the elected primers via RT-qPCR assay. The target genes were amplified in 20 μL reaction mix containing 2 μL cDNA (40 ng/μL), 10 μL RealQ Plus Master Mix Green (Amplicon), 1 μL of each primer (5 pmol/μL), and 6 μL ddH2O in a Qiagen ROTOR-GENE Q thermocycler according to the following cycling program: initial heating at 95°C for 15 minutes, and 40 cycles of denaturation at 95°C for 50 seconds and annealing/extension at 60°C for 30 seconds. Melting curve analysis was drawn to detect any non-specific amplification or any primer-dimers. 16SrRNA was used as the reference control gene for internal calibration of gene expression. The expression alterations were calculated using the Livak method [21].

**Statistical analysis**

Data of growth curves were expressed as the mean of the three independent experiments. Data obtained from the mRNA expression analysis were presented as means ± standard error of three independent assessments by Prism 8 (GraphPad Software, Inc).

## RESULTS

**The growth curve of *L. lactis***

As shown in Figure 1, *L. lactis* grows up to 16 hours in a logarithmic or exponential phase. In the first 4 hours, the growth of the bacteria was low; however, the logarithmic phase begins from about 4 to about 16 hours.

**Antimicrobial assays findings**

In order to maximize the inhibitory effects of *L. lactis* on the growth of *L. monocytogenes*, the supernatant of *L. lactis* in the logarithmic phase of growth which contains the highest amount of metabolite was used. The sensitivity of *L. monocytogenes* to *L. lactis* and its supernatant was assessed by measurement of the inhibition zone diameter (IZD). In the presence of a live bacterium, the IZD was about 8 ± 0.5 mm within 24 hours. On the other hand, the IZD was 6.6 ± 1.15 in the presence of neutralized *L. lactis* supernatant which showed less inhibitory effect on *L. monocytogenes* than live bacteria. The inhibitory activity of cell free supernatant (CFS) of *L. lactis* and the effect of the acidic medium showed that the IZD increased compared to the crude CFS by decreasing the pH to 3.5. The inhibitory effects of living bacteria, supernatant, and pH on *L. monocytogenes* growth are shown in Table 2 and Figure 2.

**Colony count assay**

The 24 h cultures of *L. monocytogenes* were inoculated into pasteurized milk and autoclaved sausage at concentrations of $10^3$ CFU/mL and *L. lactis* at a concentration of $10^7$ CFU/mL and its CFS were added to the inoculated milk and sausage. The inhibitory activity of each treatment was evaluated in 37°C at 1, 8, and 16 days by using colony count assay in Palcam Agar. The inhibitory effects are demonstrated in Figure 3.

**Quantitative real-time PCR findings**

The results of the expression analysis of *hly*, *inlA*, and *plcA* genes showed that the expression level of these genes was down-regulated in the presence of CFS of *L. lactis*. Our data revealed a one-fold increased expression of *hly* in the time = 2, while the expression level of *inlA* and *plcA* was increased to 2-fold compared to *hly* at the same time. Assessment of gene expression pattern at time = 8 hours showed an 8-fold decrease in *inlA* gene and the expression level of *plcA* and *hly* was decreased to 2- and 5-fold. The expression of *inlA* was about 2-fold in 24-treated *L. monocytogenes*, which show lower down-regulation than time = 8 hours. After 24 hours' treatment with CFS, *plcA* gene expression

**Table 1. The primer characteristics used in the current study.**

| Primers | Sequence (5'→3') | Annealing Tm (°C) | Product size (bp) | Reference |
|---|---|---|---|---|
| hly | Forward: GCGCAACAAACTGAAGCAAAGG<br>Reverse: CGATTGGCGTCTTAGGACTTGC | 60 | 106 | this study |
| inlA | Forward: GCACCAACGAAAGCCGGATA<br>Reverse: GTTGTAGGCGGTGTGTTCCC | 60 | 173 | this study |
| PlcA | Forward: TCCCAGAACTGACACGAGCA<br>Reverse: TTCACACTCGGACCATTGTAGTCA | 60 | 177 | this study |
| 16SrRNA | Forward: GAGCGCTGAAGGTTGGTACT<br>Reverse: TGTCTCAGTCCCAATGTGGC | 60 | 272 | this study |

**Table 2. The inhibitory effects of living bacteria, supernatant, and pH = 3.5 on *L. monocytogenes* growth after 24-hour incubation.**

| Treatment condition | Living bacteria | Neutralized CFS | Not neutralized CFS |
|---|---|---|---|
| IZD | 8 ± 0.5 | 6.6 ± 1.15 | 9 ± 0.2 |

**IZD - inhibition zone diameter.**
**CFS - cell free supernatant.**

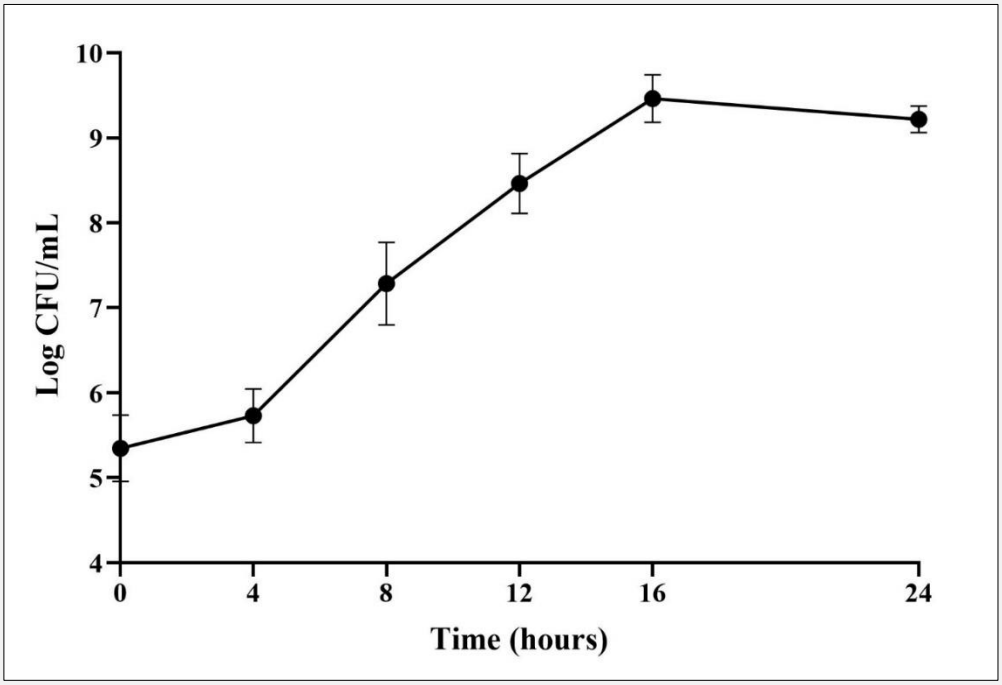


**Figure 1. The growth curve of *L. lactis.***

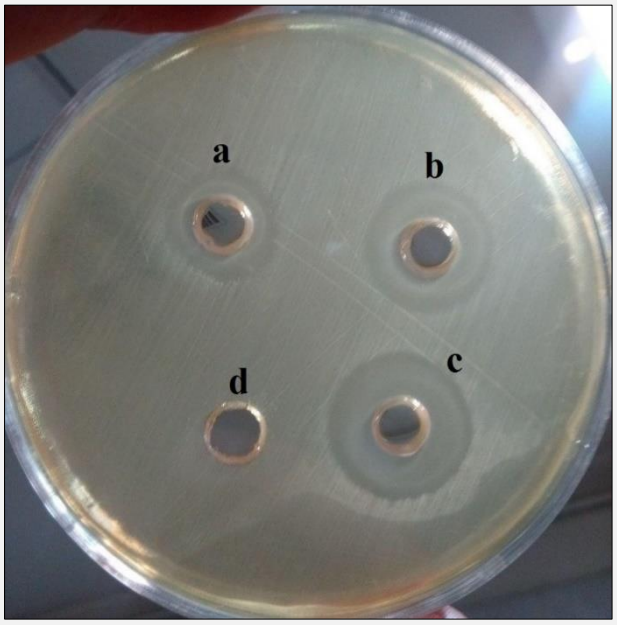


**Figure 2. Inhibition zone diameter (IZD) of *L. monocytogenes* in the presence of (a) neutralized supernatant, (b) living bacteria, (c) supernatant. MRS medium was used as negative control (d).**

was found to be increased by one-fold. Also, the expression of the *hly* gene after 24 hours treatment with CFS was found to be increased to 6-fold which had the highest reduction in expression compared to the other two genes. Effect of CFS *L. lactis* on *L. monocytogenes* at different times showed that the expression of the *pclA* gene was less affected compared to other genes. The expression alterations of virulence-related genes of *L. monocytogenes* including *hly*, *inlA*, and *plcA* genes in the presence of CFS are illustrated in Figure 4.

## DISCUSSION

*L. monocytogenes* is a food-borne pathogen that contaminates food equipment and devices [22]. Controlling *L. monocytogenes* in the food industry, one of the most important ways of transmitting this bacterium, has become a serious health care challenge. Creating appropriate sterilization techniques to limit the growth and distribution of these microorganisms in food factories and the high demand of consumers for the use of healthy foods with natural preservatives have shifted attention. Among the biological preservatives, most attention is given to lactic acid bacteria which produce several types of antimicrobial compounds including bacteriocin, nisin, and pediocine, showing anti-listeria activity [23, 24]. In this study, a bacteriocin-producing strain (*L. lactis* ATCC11454) was used. Our data showed that this strain was able to inhibit the growth of *L. monocytogenes* (ATCC74902). The diameter of the inhibition zone was varied from 8 to 12 mm. Besides, further molecular assessments revealed an alteration in the expression of virulence-related genes in treated *L. monocytogenes.* In our research, agar well diffusion method was applied to determine the sensitivity of *L. monocytogenes* to *L. lactis* and the IZD was measured in presence of living bacteria, its neutralized CFS, and in acidic CFS (pH = 3.5). Our results showed that the effect of acid supernatant was greater than living bacteria and the effect of living bacteria was greater than neutralized supernatant, where the diameter of the inhibition zone was decreased to about 8 ± 0.5 mm, which is consistent with the results of the Ogunbanwo and colleagues [25,26]. In a report, the antimicrobial effect of CFS of *L. fermentum* and *L. rhamnosus* on *listeria* bacteria isolated from raw milk (unpasteurized milk) of cattle by agar well diffusion method showed that the mean IZD was 21.10 and 21.08 mm for *L. fermentum* and *L. limonus* [27]. In another study, the inhibitory activity of *L. plantarum* and *Pseudococcus pentosaseus* isolated from dairy products on *L. monocytogenes* showed an IZD of 1 - 14 mm after 24 h treatment [28].

Evaluation of the inhibitory ability of 8 lactic acid bacteria on the growth of *L. monocytogenes* isolated from different sources such as raw milk, dairy equipment,

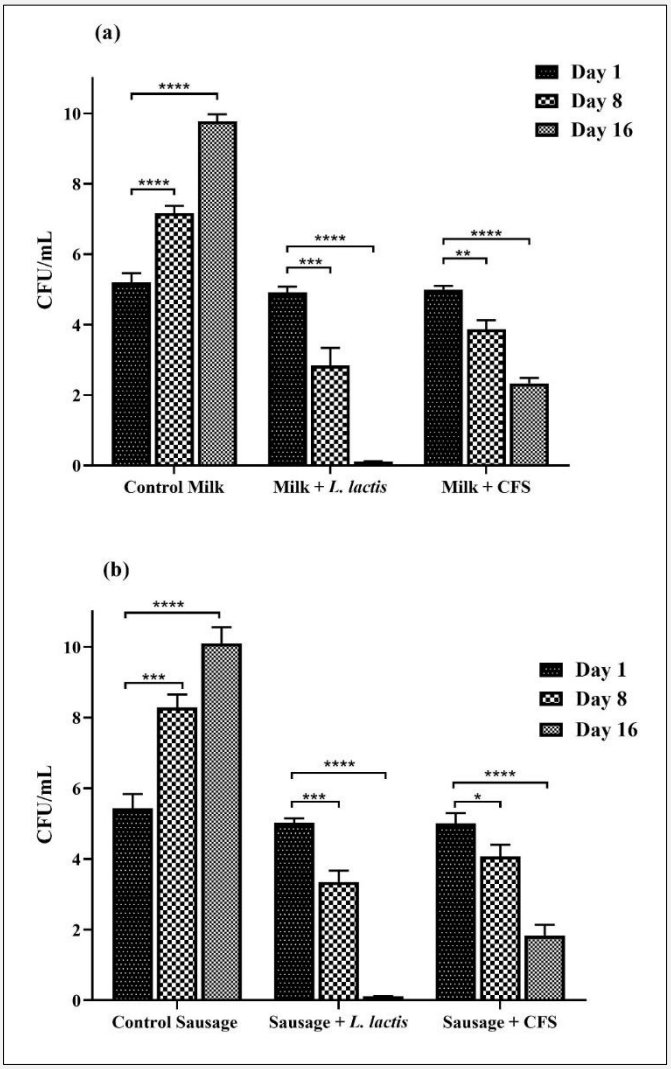


**Figure 3. The inhibitory effects on *L. monocytogenes* in (a) milk and (b) sausage containing of *L. lactis* or its CFS (mean ± SD). * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; by one-way ANOVA with Dunnett's multiple comparisons test (Control milk and sausages: milk and sausages were infected with *L. monocytogenes).***

and lymph nodes showed that *L. Lactis* were only effective against *L. monocytogenes* isolated from raw milk where the mean IZD was about 16 mm, more effective compared to our research [29].

The results of our work regarding the effect of *L. lactis* and its supernatant on the number of *L. monocytogenes* in infected milk showed *L. lactis* has a more significant effect on reducing the copy number of *L. monocytogenes* compared to supernatant and control milk. Similar to our results, antimicrobial activity of the supernatant of *L. lactis* on *Salmonella enterica*, *L. monocytogenes*, *Staphylococcus aureus*, and *E. coli* inoculated in pasteurized milk showed that *L. lactis* N8-r-Lecl due to the production of nisin and leucine has a strong antimicrobial ability which can be used in the storage and processing of pasteurized milk [30]. However, the effect of filtered and heated supernatant of 40 strains of *Lactococcus lactis* isolated from non-dairy sources on *L. monocytogenes* in the milk infected with the bacterium revealed that only 4 strains had an appropriate inhibitory effect [31].

Also, using the colony count method, the effect of *L. lactis* and its supernatant on the number of *L. monocytogenes* in infected sausages revealed that the *L. lactis* has a significant effect on reducing the number of bacteria compared to supernatant-treated and control sau-

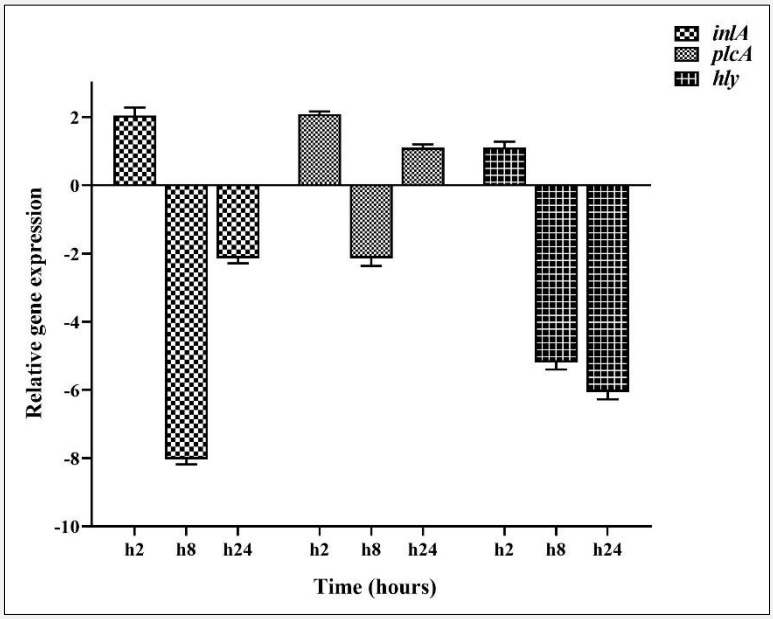


**Figure 4. The expression alterations of virulence-related genes of *L. monocytogenes* including *hly*, *inlA*, and *plcA* genes in the presence of CFS.**

sages. The anti-microbial effects of *L. croatus* 54M16 against *L. monocytogenes* during the production of fermented sausages in MRS medium revealed that *L. croatus* was able to inhibit the growth of the bacteria after 48 hours at 4°C or 5 days at 15°C, in its stationary phase of growth [32]. In another study, the effect of bacteriocin-producing lactobacillus plantarum on the growth rate of *L. monocytogenes* in the process of making fermented sausages showed a 1-log decrease in *L. monocytogenes* copy number after 19 days' treatment [33]. In the present study, *L. monocytogenes* bacteria was uncountable after 16 days of sausage treatment with *L. lactis*. In various studies, the maximum inhibitory effect of *L. lactis* or its supernatant has been reported at different hours after inoculation, varying from 2 days to 19 days [33-35]. According to the large volume of evidence, *L. lactis* and its supernatant are believed to be involved in the growth inhibition of *L. monocytogenes*; however, the actual molecular mechanisms, through which this bacterium applies its inhibitory activity, have remained unclear. In a study by Miranda and colleagues, the effect of *L. lactis* on the expression of genes associated with stress (gbu), osmotic pressure (gro EL), temperature (gadD2), and acid (sigB) in *L. monocytogenes* was investigated [36]. Their finding showed that the growth of nisin-producing *L. lactis* can alter the expression of stress-associated genes; however, the understanding of these mechanisms in food is difficult due to hard and strict protective methods. In this study, the role of *L. lactis* and its supernatant on the expression of virulence-associated genes of *inlA*, *Plc*, and *hly* in *L. monocytogenes* was investigated and the results showed that this bacterium and its CFS might lead to down-regulation of all three genes. Nonetheless, more studies are required to validate these finding and the results are suggested with caution.

In conclusion, in the present study, the inhibitory effect of *L. lactis* and its supernatant was first proven by the agar well diffusion method. The impact of *L. lactis* and its supernatant on the copy number of *L. monocytogenes* in infected milk and sausages were then investigated by colony count assay, wherein the results indicated a decrease in the number of *L. monocytogenes* in both milk and sausages. Finally, the inhibitory effect of CFS of *L. lactis* on the expression of pathogenic genes including *Plc*, *inlA*, and *hly* of *L. monocytogenes* was evaluated by RT-qPCR, which showed that supernatant reduced the expression of the *inlA* and *hly* genes. For further studies, it is suggested that isolation of *lactococcus* be performed from different sources and the effect of these isolates is proposed to be carried out on the expression of biofilm-forming genes of *L. monocytogenes* that play an important role in the bacterial survival in the environment. It also is better to assess the possible toxicity of *L. lactis* and nisin on human cells and animal models. The use of electron microscopy is recommended for a more

precise examination of the inhibitory mechanism of *L. lactis* on *L. monocytogenes* and biofilm formation.

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**Declaration of Interest:**
The authors declare that they have no competing interests.

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