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A comparative study between inhibitory effect of *L. lactis* and nisin on important pathogenic bacteria in Iranian UF Feta cheese

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Abstract

Introduction: In the present study, the inhibitory effect of nisin- producing *Lactococcus lactis* during co- culture and pure standard nisin were assessed against selected foodborne pathogenes in growth medium and Iranian UF Feta cheese. In comparison *L lactis*, not only proves flavor but also plays a better role in microbial quality of Iranian UF Feta cheese as a model of fermented dairy products.

Materials and methods: *L. lactis* subsp. *lactis* as nisin producer strain, *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* as pathogenic strains were inoculated in Ultra- Filtered Feta cheese. Growth curve of bacterial strains were studied by colony count method in growth medium and UF Feta cheese separately and during co-culture with *L. lactis.* Nisin production was determined by agar diffusion assay method against susceptible test strain and confirmed by RP- HPLC analysis method.

Results: Counts of *L. monocytogenes* decreased in cheese sample containing *L. lactis* and standard nisin, to 10^3 CFU/g after 7 days and it reached to undetectable level within 2 weeks. *S. aureus* counts remained at its initial number, 10^5 CFU/g, after 7 days then decreased to 10^4 CFU/g on day 14 and it was not detectable on day 28. *E. coli* numbers increased in both treatments after 7 days and then decreased to 10^4 CFU/g after 28 days. Despite the increasing number of *E. coli* in growth medium containing nisin, due to the synergistic effect of nisin and other metabolites produced by Lactococcus lactis and starter cultures, the number of *E. coli* decreased with slow rate.

Discussion and conclusion: The results showed, *L. monocytogenes* was inhibited by *L. lactis* before entering the logarithmic phase during co- culture. *S. aureus* was also inhibited during co- culture, but it showed less sensitivity in comparison with *L. monocytogenes.* However, the number of *E. coli* remained steady in co- culture with *L. lactis.* Also, we found that, in all cheese samples, *E. coli* decreased slowly after 28 days which may be due to the synergistic inhibitory effects of nisin and other metabolites produced by *L. lactis* and starter culture strains. These conditions are compatible to UF Feta cheese making processes. The usage of *L. lactis* is more effective in terms of pathogenic inhibitory in comparison with free nisin. Using *L. lactis* as an adjunct starter culture can assist microbial quality improvement and prevent important pathogens, which may survive during food processing, because of the production of beneficial metabolites.

Key words: *Lactococcus lactis*, Nisin, Preservation, Co- Culture, Pathogenic bacteria

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Introduction

Food protection against spoilage and pathogenic bacteria has always been a high priority for the public health. In addition, the unhealthy effects of processed food and the disadvantages of chemical food preservatives, encourage researchers to innovate new strategies for food safety (1). They also struggle to cope with the increasing demands of consumers for high quality nutritious food with improved organoleptic characteristics. Biopreservation, the use of microorganisms or their products for preserving food, is a new approach to food safety. Recent studies demonstrate that certain Lactic acid bacteria (LAB) are stabile to use as biopreservative. They can not only compete well for nutrients with pathogens, but also produce antimicrobial active metabolites. Moreover, they are harmless to humans (2 and 3).

LAB can improve quality of fermented products by inhibiting the pathogens, providing hygienic safety, extending shelf life and enhancing sensory properties (4). Food protection effect of LAB on pathogenic and spoilage bacteria is through the production of substances such as, organic acids, hydrogen peroxide, diacetyl, acetoin and also bacteriocins, which can act as bio- preservative (4 and 5) . Use of LAB especially bacteriocinogenic strains in the form adjunct culture is an appropriate substitute for preservatives in food fermentation processes to prevent and combat undesirable bacteria (2, 6, 7 and 8).

L. lactis is one of the most important members of LAB. *L. lactis* strains produce nisin, which has a broad spectrum inhibitory effect on Gram- positive bacteria (9). Several studies have shown that nisin could inhibit various Gram- positive bacteria such as *L. monocytogenes* in food (9 and 10). Among dairy products, cheeses especially soft ones such as UF Feta cheese are good media to transfer pathogens to human. Pathogenic bacteria such as *L. monocytogenes, E. coli* and *S. aureus* can survive during manufacturing soft cheeses such as Feta (11). A number of studies have reported the effects of nisin and nisin producing strain on the pathogenic bacteria in different kinds of cheese such as, Cheddar, Camembert and Gouda (6, 9 and 12). El- Gazzar et al. assessed antagonistic effect between *L. monocytogenes* and *Lactococci* during fermentation of products from ultrafiltered skim milk (14). Based on their results, more inactivation was observed in permeate than skim milk and retentate. However, no published reports are available that evaluate the effects of nisin and *L. lactis* on pathogenic bacteria on UF Feta cheese. In manufacturing Feta cheese, removal of water from milk by ultrafilteration leads to significant increase in concentration of microbial contaminants (13 and 14). The main purpose of this study was to investigate one industrial applicable method by adding one of safe known lactic acid bacteria (*L. lactis*) in starter culture of ultra- filtered condensed milk to produced fermented dairy products. Therefore, control and decrease of any bacterial pollution even Gram negative bacteria such as *E. coli* caused by inappropriate pasteurization or post contamination, in UF cheese by adding safe bio- preservative such as nisin or nisinogen strain (*L. lactis*), is great importance.

Materials and methods

Bacterial strains and media

All bacterial strains were obtained from the Persian Type Culture Collection (PTCC), Iran. *L. lactis* subsp. *lactis* PTCC 1336 was used as a nisin producer strain. *L. monocytogenes* PTCC 1301, *E. coli* PTCC 1399 and *S. aureus* PTCC 1431 were used as pathogenic strain bacteria. All stock cultures were maintained at- 80°C in skim milk and 20% glycerol. Working cultures of *L. lactis* were kept on de Man, Rogosa and Sharp (MRS) medium (HiMedia Laboratory, India) and pathogenic bacteria were maintained on BHI agar (Oxoid, Hampshire, England) at 4°C. *Micrococcus luteus* PTCC 1169 was used as an indicator strain for nisin bioassay and was maintained like pathogenic bacteria.

Growth curve study of *L. lactis*

Growth curve and pH profile of *L. lactis* were plotted. *L. lactis* growth was measured by colony count in MRS and M17 broth. The culture (24 h) of *L. lactis* was inoculated into the growth media to give a final concentration of 10^7 CFU/ml. They were incubated in rotary shaker incubator at 30°C, 100 rpm and the samples were taken every 4 hours. Colony count was carried out by pour plate method on MRS and M17 agar and incubation at 30°C for 48 h. Nisin production in the two media was also assessed and compared.

Determination of nisin activity produced by *L. lactis*

Sample preparation

Samples, from culture of *L. lactis*, were taken every 4 hours during 48 h. Then, the samples were adjusted to pH 2 by concentrated HCl (Merck, Germany), containing 0.1% v/v of Tween 20 (Sigma Chemical Co., St. Louis, Mo.), and were heated at 90°C for 5 min. Subsequently, they were centrifuged at 12,000 × g for 10 min. The collected supernatants were then filtered through a 0.22 μm membrane filter (Millipore® Corp., Bedford, MA), and were used for nisin activity quantification by agar well diffusion bioassay method (10 and 15).

Nisin activity quantification

Nisin determination was carried out by agar diffusion method (15). BHI medium (Merck), consisting of 0.75% agar (Bacto agar, Difco) and 1% volume per volume (v/v) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) was prepared. The medium was then inoculated with 1% v/v of a 24- h culture of *Micrococcus luteus* with an optical cell density of 1.7 at 600 nm to get approximately 10^8 CFU/ml of the medium. Four wells were bored on each plate using a sterilized borer and 50 μl of each supernatant sample was placed into each well in triplicate, and the fourth well was filled with blank (50 μl of 0. 02 N HCl). Before incubation at 37°C for 24 h, all plates were incubated overnight at 4°C for diffusion of nisin. The plates were examined for diameter of inhibition zones using a digital caliper (AACO, China).

In order to plot the standard curve of nisin, different concentrations of nisin were prepared. For this purpose, at first, a stock solution of nisin (1000 IU/ml) was prepared by dissolving 0.025 g of commercial nisin 10^6 IU/g (Sigma Chemical Co., St. Louis, Mo.) in 25 ml of sterile 0.02 N HCl. Then using 0.02 N HCl, various concentrations of standard nisin solutions (500, 400, 300, 200, 100, 50, 25, 10 and 5 IU/ml) were prepared. The standard curve was constructed by plotting diameters of inhibition zones versus the logarithm_{10} of nisin concentrations (16).

RP- HPLC analysis of nisin

In order to confirm nisin production, HPLC analysis was carried out. The cells of *L. lactis* were removed by centrifugation (10,000 × g, 30 min, 4°C) and nisin was precipitated by 30% ammonium sulfate (Merck, Germany) and kept overnight at 4°C while being stirred gently. Precipitated proteins were collected by centrifugation (12,000 × g, 30 min, 4°C), resuspended in 2 ml sterile solution of 0. 02 N HCl (Merck, Germany) and dialyzed for 24 h in Spectra/Por® no. 7 dialysis tubing (Spectrum laboratories Inc., USA, molecular weight cut off, 2000 Daltons). This fraction was concentrated by freeze-drying, resuspended in 1.5 ml of 0.02 N HCl sterile solutions, and filtered through a 0.22 μm membrane filter (Millipore® Corp. Bedford, MA).

The above preparation and a 2% standard nisin (Sigma Chemical Co., St. Louis) solution were further purified by gel filtration chromatography on Sephadex G-25 as described by Pirad et al. (17). After the gel filtration chromatography, fractions containing nisin (determined by bioassay method) were polled and dialyzed against 0. 02 N HCl with two changes of the solution and were further concentrated by freeze- drying. The lyophilized samples were injected into a Knauer HPLC unit (Model K- 1001, Knauer, Germany) equipped with an analytical reversed phase (RP) C18 column (EurosilBioselect, Knauer, Germany) for retention time measurement (18). Samples were eluted with the mobile phase consisting of 0.1% (v/v) trifluoroacetic acid (TFA) in a mixture of water (eluent A) and acetonitrile (eluent B) (Both reagents were HPLC grade, obtained from Merck, Germany). Samples were initially eluted with 100% A for 5 min, then with a linear gradient 0 – 50% B over 45 min, followed by a linear gradient to 100% B over 5 min maintained at 100% B for 7 min. The flow rate was maintained at 1 ml/min, absorbance was monitored at 215 nm using K- 2501 UV detector (Knaure, Germany) and the column was kept at a constant temperature of 35°C. Data analysis was performed using a chromatography software package (ChromGate, version 3.1, Knaure, Germany).

Determination of inhibitory effect of *L. lactis*

The effect of *L. lactis* on growth of each test strain was evaluated separately in MRS medium (HiMedia Laboratory, India). For this purpose, overnight cultures of each pathogenic bacterium and *L. lactis* were inoculated into MRS broth simultaneously to give the concentration of 10^5- 10^6 CFU/ml and 10^6- 10^7 CFU/ml, respectively. They were incubated in rotary shaker incubator at 30°C, 100 rpm and the numbers of bacteria were enumerated every 4 hours. For pathogenic bacteria the colony count was determined using pour plate method on appropriate selective medium after 24 h incubation at 37°C. *Listeria* selective agar (Difco, USA) was used for *L. monocytogenese* and Baird Parker agar (Oxoid, Hampshire, England) and EMB (Difco, USA) agar were used for *S. aureus* and *E. coli*, respectively.

Minimum inhibitory concentration (MIC) of nisin

The minimum inhibitory concentration (MIC) of nisin was assessed by broth dilution method (19). For this purpose, a stock solution containing 10,000 IU/ml of nisin was prepared by dissolving 0.1 g nisin in 10 ml 0.02 N HCl. Then it was diluted serially, from 5000 to 125 IU/ml, using BHI broth.

Tubes containing different dilutions of nisin were inoculated with an overnight culture of each pathogenic bacterium (10^6 CFU/ml) separately and incubated at 37°C, 30°C and 8°C. The growth was inspected visually and spectrophotometrically at 600 nm after 24, 48, 72 h and 1 week of incubation. The blank was the medium alone incubated under the same conditions. The MIC was defined as the lowest concentration of nisin that inhibited the growth of bacterium after the incubation period. The MIC determination repeated independently for 3 times, always with *Micrococcus luteus* (a sensitive strain to nisin) as positive control.

Cheese production

UF cheese was manufactured from retentate with 1% starter culture and 1% salt (provided by Bel- Sahar, roozaneh dairy company, Qazvin, Iran). Overnight culture of each pathogenic bacterium was inoculated to retentate to give concentration of 10^5 to 10^6 CFU/ml separately. For treatment containing *L. lactis,* the retentate was inoculated to reach 10^7 to 10^8 CFU/ml of *L. lactis*. In treatment with nisin, 300 IU/ml nisin was added to retentate after the inoculation of pathogenic bacteria. For each bacterium the positive control cheese was prepared by adding each pathogen without the addition of nisin or *L. lactis.* Subsequently, the retentate was poured in cups and after reaching pH 4.7 to 4.8, 0.2 ml rennet (provided by Bel- Sahar, roozaneh dairy company) per 100 ml retentate was added. The cheese cups were maintained at 8°C and analyzed during a period of 30 days (20).

Microbiological analysis

The samples of cheese were taken on days 1, 7, 14 and 28. A sample (2.5 g), taken from different parts of cheese, was pooled and homogenized with 22.5 ml of sterile sodium citrate (Merck, Germany) solution (9). The cheese samples were then serially diluted in sterile 2% sodium citrate. Bacteria counts were determined on duplicate plates of their selective media, as described earlier.

Nisin extraction and bioactivity in cheese

For extraction of nisin from cheese, acid extraction method was performed. Cheese samples (2.5 g) were mixed and homogenized with 10 ml of sterile 0. 02 N HCl and the pH was adjusted to 2 with 10 N HCl. Then samples were kept in boiled water for 5 min. After cooling and temperature adjustment to 20°C, the volume was adjusted to 12.5 ml and the samples were centrifuged at 10000 × g for 30 min at 4°C. Subsequently, the supernatant was transferred to sterile containers and maintained at 4°C for 30 min in order to solidify the fat phase. The supernatant was filtered through a 0.22 μm filter (Millipore® Corp., Bedford, MA). The pH of filtrates was adjusted to 5.5 with sterile 4 N NaOH (Merck, Germany), after which, they were kept at 4°C for 1 h before further experiments (6). Nisin activity was determined by agar diffusion method which was described earlier.

To make sure that the results are reproducible, each experiment was conducted at least two times in triplicates.

Results

Growth curve of *L. lactis* and nisin production

MRS and M17 are used as suitable media for lactic acid bacteria (10). In this study the growth of *L. lactis* and maximum nisin production were investigated in both media. The lag and log phase of *L. lactis* in MRS and M17 were approximately the same but maximum growth rate in MRS was slightly better (Fig. 1. a). However, *L. lactis* produce more nisin in MRS (Fig. 1. b).

The maximum nisin production in MRS was 477 IU/ml, whereas in M17 it was 245 IU/ml. Moreover, pathogenic bacteria were able to grow better in MRS medium than that of M17 (data not shown). Therefore, MRS was chosen for the rest of the experiments.

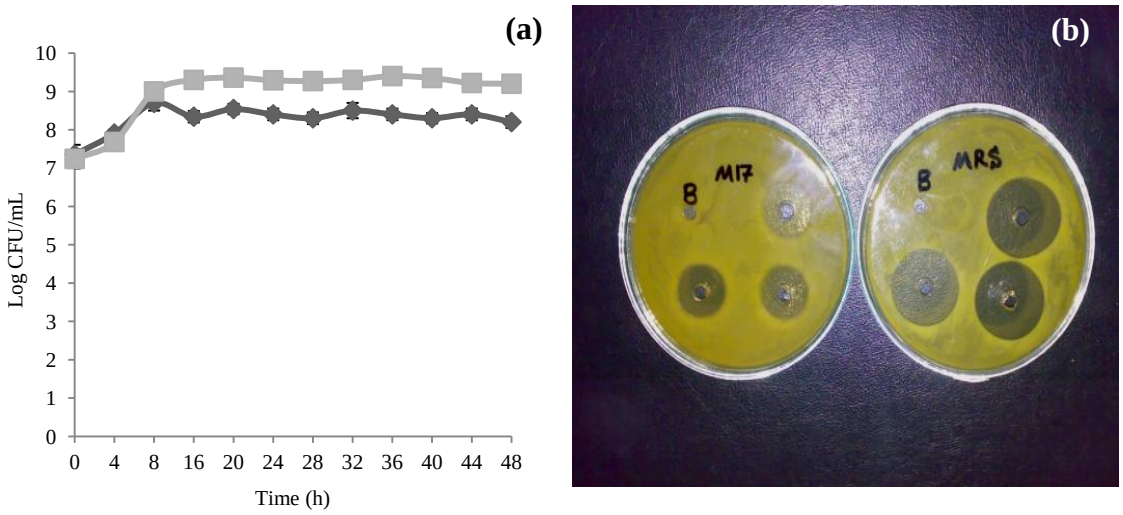

Fig. 1- Growth curve of *L. lactis* base on colony count in MRS (dotted line) and M17 (solid line) (a), Inhibition zones of supernatant from *L. lactis* growth in two media: MRS and M17 (b). B: Blank; *M. luteus* PTCC 1169 was used as indicator strain

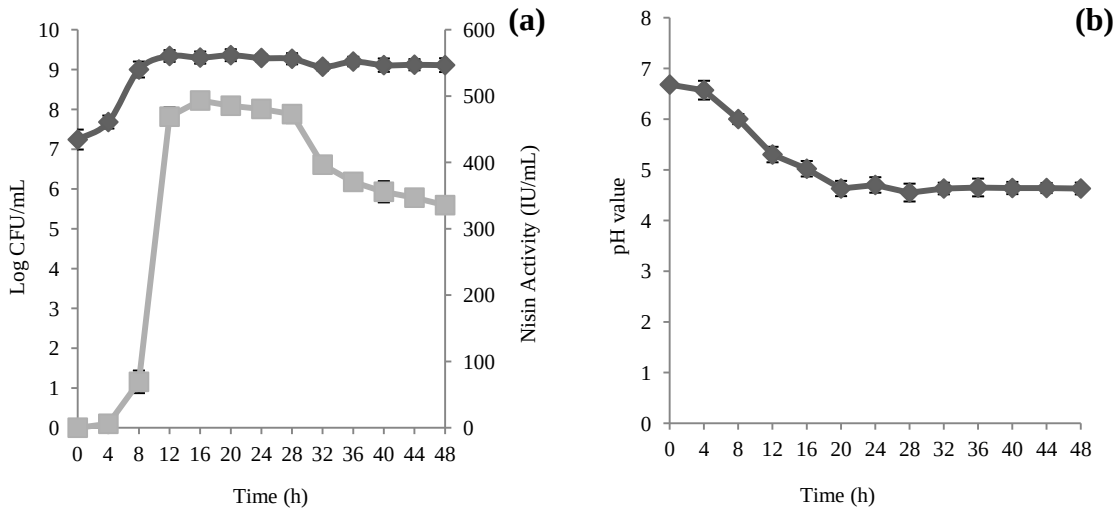

Fig. 2- Growth curve of *L. lactis* (dotted line) and curve of nisin production (solid line) (a); pH profile during *L. lactis* growth (b)

The number of *L. lactis* cells increased following a short lag phase after 4 h in MRS broth (Fig. 2. a). The highest absorbance was also observed between the 12th and 16th h of the growth. No nisin activity was observed at the first 4 h of *L. lactis* growth; however, nisin production started to escalate sharply and reached its highest level, 477 IU/ml, after 12 h parallel with the logarithmic phase of *L. lactis* growth. Subsequently, the growth stayed steady during the next 18 h. Nisin quantity then decreased gradually may be by increases of protease activity after 30 h. The pH value began to decline in parallel with bacterial growth and gradually reached 4.6 ± 0.2 after 48 h due to acid production from carbon sources of medium. Minimum pH value was observed after 20 h (Fig. 2b).

RP- HPLC analysis

For reconfirmation of nisin production by *L. lactis*, bioassay was followed by RF-HPLC. Samples of fermentation broth and standard nisin solution were eluted with the mobile phase consisting of Tri- fluoroacetic acid (TFA) in a mixture of water and acetonitrile. RP- HPLC analysis indicated that the purified peptide, produced by *L. lactis* and standard nisin has identical retention times (from 46.0 to 47.0 minutes) and these fractions had the same nisin activity (data not shown) (Fig. 3).

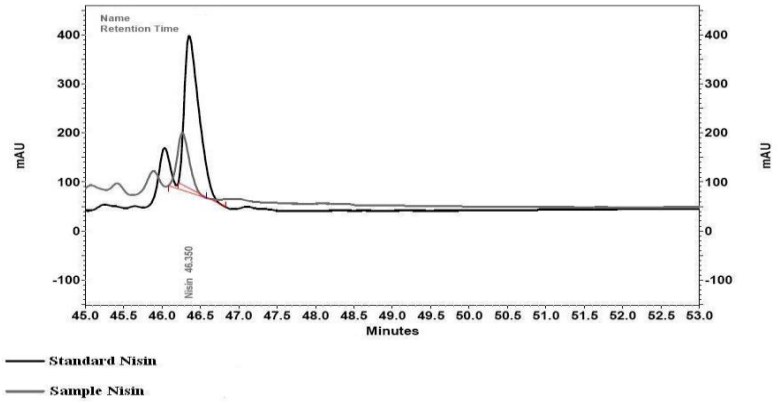

Fig. 3- RP- HPLC analysis of standard nisin and nisin purified from *L. lactis*. Black curve: Standard nisin; Gray curve: nisin purified from the culture medium

Inhibitory effects of *L. lactis* on pathogenic bacteria

L. monocytogenes was inhibited completely by *L. lactis* during co- culture within logarithmic phase in comparison with pure culture of *L. monocytogenes* (Fig. 4. a). In the case of *S. aureus*, its number started to increase in monoculture and decreased in its co- culture with *L. lactis* from 4.6 logs CFU/ml to 2 logs CFU/ml after 48 h (Fig. 4. b). However, it did not decrease completely before 48 h as it happened for *L. monocytogenes*.

In the case of *E. coli*, a slight inhibitory effect was observed but no reduction in its count occurred and the growth remained steady (Fig. 4. c). Number of *E. coli* in monoculture reached its peak after 20 h, whereas in co- culture, it maintained the same level and just showed a small change. Therefore, *L. lactis* had an inhibitory effect on *E. coli*, but it did not decrease the number of *E. coli* during 48 h.

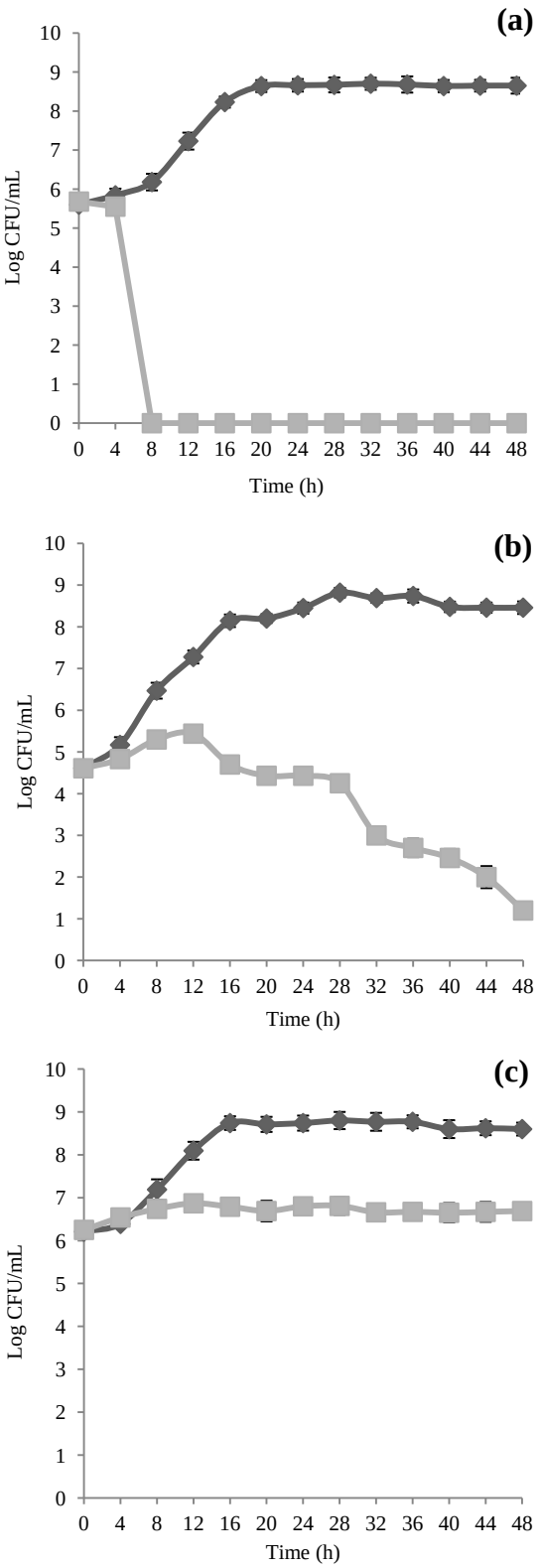

Fig. 4- Growth curve of bacteria during co- culture in MRS. dotted lines (- - - -) represent monoculture and solid lines represent co- culture in all graphs; *L. monocytogenes* growth curve (a), *S. aureus* growth curve (b) and *E. coli* growth curve (c)

Minimum inhibitory concentration (MIC) of nisin

MIC of nisin was compared at two different pH (6.8 and 4.7) from 24 h to 1 week. It was found that MIC of selected microorganisms was completely depended on pH and temperature. At 30°C and 37°C in culture medium (pH 6.8 to 7.2), the MIC of *L. monocytogenes* and *S. aureus* was 2000 IU/ml and 3000 IU/ml, respectively. Their MIC decreased significantly at 8°C to less than 250 IU/ml. Isoelectric pH in which rennet is added to cheese is 4.7 to 4.8. MIC of *L. monocytogenes* and *S. aureus* at this pH decreased to less than 500 IU/ml at 30°C and 37°C. In the case of *E. coli*, no inhibitory activity was observed after 72 h at any concentration between 125 and 5000 IU/ml (data not shown). *M. luteus* as positive control did not grow at any of these concentrations.

Microbiological analysis of cheese

Microbiological studies of cheese illustrated that, the number of *L. monocytogenes* decreased from 10^5 CFU/g to 10^3 CFU/g after 7 days and subsequently no *L. monocytogenes* was observed from day 14 in samples containing *L. Lactis*. After day 7, the viable count of *L. monocytogenes* in controlling cheese decreased by 2 log units. On day 28, no *L. monocytogenes* was found in controlling cheese. The trend of reduction in cheese with nisin was the same as controlling sample, although this drop was slightly less in samples containing nisin (Table 1).

The number of *S. aureus* remained at 10^5 CFU/g after 7 days in the 2 treatments (cheese with nisin and *L. Lactis*) and then reduced to undetectable level after 28 days. In controlling cheese, a slight reduction was observed; however, it was not inhibited completely during the study (Table 2).

Colony count of *E. coli* in cheeses increased in both treatments even in controlling cheese. Number of *E. coli* increased to 10^6 CFU/g in control and cheese with *L. lactis*, and to 10^7 CFU/g in cheese containing standard nisin. It declined gradually in both treatments and even in controlling cheese then after (Table 3).

Table 1- Inhibition of *L. monocytogenes* in cheese containing *L. lactis* and nisin (mean CFU/g ± SD)

Cheese samples	1st Day	7th Day	14th Day	28th Day
Control cheese	$8 \pm 0.05\times10^5$	$8 \pm 1.1\times10^3$	$1.5 \pm 0.35\times10^3$	ND*
Cheese with *L. lactis*	$8\pm 0.08\times10^5$	$1.6 \pm 0.14\times10^3$	ND	ND
Cheese with nisin	$8\pm 0.12\times10^5$	$7.6 \pm 0.35\times10^3$	$4.1 \pm 1.26\times10^3$	ND

*ND: Not Detectable, ≤10 CFU per sample

Table 2- Inhibition of *S. aureus* in cheese containing *L. lactis* and nisin (mean CFU/g ± SD)

Cheese samples	1st Day	7th Day	14th Day	28th Day
Control cheese	$7 \pm 0.35\times10^5$	$7 \pm 0.27 \times10^5$	$1.5 \pm 0.17\times10^5$	$3.5 \pm 0.35\times10^2$
Cheese with *L. lactis*	$7\pm 0.13\times10^5$	$4.5 \pm 0.28\times10^5$	$8.2 \pm 0.56\times10^4$	ND*
Cheese with nisin	$7\pm 0.21\times10^5$	$6.5 \pm 0.35\times10^5$	$9 \pm 0.28\times10^4$	ND

*ND: Not Detectable, ≤10 CFU per sample

Table 3- Inhibition of *E. coli* in cheese containing *L. lactis* and nisin (mean CFU/g ± SD)

Cheese samples	1st Day	7th Day	14th Day	28th Day
Control cheese	$6 \pm 0.13\times10^5$	$7.6 \pm 0.28\times10^6$	$5.5 \pm 0.14\times10^6$	$4 \pm 0.7\times10^4$
Cheese with *L. lactis*	$6 \pm 0.11\times10^5$	$6.2 \pm 0.49\times10^6$	$3.5 \pm 0.35\times10^6$	$2 \pm 0.7\times10^4$
Cheese with nisin	$6 \pm 0.22\times10^5$	$2.1 \pm 0.35\times10^7$	$6.6 \pm 0.35\times10^6$	$4.2 \pm 0.4\times10^4$

Determination of nisin activity in cheese

In cheese containing *L. lactis*, no substantial amount of nisin was detected. In cheese containing nisin (second treatment) there was a dramatic reduction in the amount of nisin during the first week after cheese making and it reached 24 IU/g and 10 IU/g at day 7 and 14, respectively. Then it further decreased to 5 IU/g at day 28 and remained steady thereafter.

Discussion and conclusion

L. monocytogenes, E. coli and *S. aureus* are bacteria of major concern. They can easily be transmitted to human through food (7). They can survive during processing and storage of fermented milk and cheese (7 and 11). In this study the inhibitory effect of *L. lactis* on these foodborne pathogens was investigated in Iranian UF Feta cheese. *L. lactis* plays the most important role in manufacturing fermented dairy products such as sour milk, cream, butter and different varieties of cheese and has been traditionally used as starter (4). Growth curve and nisin production of *L. lactis* in M17 and MRS media were distinguished. Both media are almost equal for *L. lactis* growth. However, nisin production in MRS broth was better than M17. In our strategies, the selected media must support not only growth of *L. lactis* and nisin production, but also growth of other pathogenic bacteria. Thus, MRS was chosen as an appropriate medium for *L. lactis* and its associated culture with pathogenic bacteria.

Nisin production of *L. lactis* was studied by bioassay and HPLC analysis. HPLC results confirmed that the nisin produced by *L. lactis* PTCC 1336 has the same retention time as standard nisin. The pH profile of *L. lactis* fermentation in MRS broth showed that production of acid starts immediately after lag phase. *L. lactis* preserves fermented food by hostile effects on other organisms through the conversion of the sugars to organic acids and consequently, lowering the pH, nutrients competition and also producing bacteriocins and other compounds (4).

As the results show, *L. monocytogenes* was inhibited by *L. lactis* before entering the logarithmic phase during co- culture. *S. aureus* was also inhibited during co-culture, but it showed less sensitivity in comparison with *L. monocytogenes*. However, the number of *E. coli* remained steady in co- culture with *L. lactis*. According to a study by Benkerroum and Sandin , *L. monocytogenes* was extremely sensitive to nisin at low pH and it was totally inhibited before 24 h at pH lower than 5 (21). Almore et al. reported the correlation between pH reduction and inhibition of *S. aureus* during co- culture with *L. lactis* in micro- filtrate milk (22). They reported that low pH had a notable effect on the reduction of this pathogen in parallel with other factors such as bacteriocin production. In addition, various studies indicated that LAB would play remarkable role in suppression of Gram-negative bacteria through the production of short chain fatty acids such as lactic acid, and other metabolites (23). Thus, nisin production, pH reduction and other produced metabolites can inhibit these bacteria during co- culture.

Based on MIC study, nisin revealed no inhibitory effect on *E. coli*. This is due to the cell membrane structure of Gram-negative bacteria (23 and 24). *L. monocytogenes* and *S. aureus* were more sensitive to nisin at pH 4.7 at 8°C. These conditions are compatible to UF Feta cheese making processes. Since the MIC of these bacteria at 8°C (the storing temperature of cheese) was less than 125 IU/ml, 300 IU/ml (7.5 mg/l) was chosen for cheese bio- preservation. The maximum level of nisin usage in the US determined by FDA is 250 mg/l (25).

In the second part of the study, the inhibitory effect of *L. lactis* on pathogenic bacteria in UF Feta cheese was also assessed. According to our results on MIC at 8°C and production of nisin in co-culture, two cheese treatments were designed by adding *L. lactis* and 300 IU/g nisin. *L. monocytogenes* was inhibited before day 14 of storage in samples containing *L. lactis*. This inhibition was confirmed by the results of Maisnier- patin et al. (12) study on inhibition of *L. monocytogenes* in Camembert cheese containing nisinogenic starters. According to their results, a dramatic reduction happened from 6 to 24 h and the reduction continued till the end of the second week. However, Listeria was able to restart its growth after 6 weeks. In our research in UF Feta cheese, *L. lactis* was able to decrease the number of *L. monocytogenes* to undetectable level after 2 weeks. This occurred sooner than cheese containing nisin and even controlling cheese. Higher number of *L. monocytogenes* in cheese containing nisin might be due to a slight inhibition of starter culture by nisin (12).

In the case of *S. aureus*, it was inhibited in treatments containing *L. lactis* and nisin before 30th day of cheese storage. Hamama et al. (7) studied the stability of *S. aureus* in Jben, a Moroccan cheese, in the present of

nisin- producing *L. lactis*. They found that, reduction in number of *S. aureus* depended on inoculum size. In lower inoculum (10^3 CFU/ml) it reached undetectable level earlier than higher inoculum (10^5 CFU/ml). The results did not show complete inhibition of *S. aureus* in higher inoculum size (10^5 CFU/ml) during their studies. In our studies, it dropped to undetectable level from 10^5 CFU/ml after 28 days.

The number of *E. coli* in all cheese samples Containing nisin increased during the first 2 weeks of storage which may be due to resistance of Gram- negative bacteria to nisin (24). In cheese containing nisin this was greater due to slight inhibition of starter cultures by nisin and *E. coli* continued growth one fold higher than other treatments. This is in agreement with the results of Ramsaran et al. (11) on Feta cheese. In contrast, it was observed that, in all cheese samples *E. coli* decreased gradually after 28 days which may be due to the inhibitory effect of metabolites of starter culture and *L. lactis* (6) . Reduction in the number of pathogenic bacteria was observed in all controlling cheese. This suggests the inhibitory effect of common starter cultures. Benkerroum and Sandine observed a reduction in *Listeria* count to control samples because of simultaneous impact of various inhibitory substances produced during the growth in cottage cheese (21).

In cheese containing *L. lactis*, no significant amount of nisin was detected by biological assay after one week. Study of Maisnier- Patin et al. (12) showed the maximum amount of nisin produced by nisin- producing culture on Camembert cheese was after 9 h and its concentration decreased gradually between 9 and 24 h, after which it declined dramatically during ripening. This confirms the results of our study, in which nisin is produced at logarithmic phase of growth. The biological activity of nisin in UF Feta cheese containing 300 IU/g nisin progressively declined to 1.7% during 28 days of storage. This inactivation might be induced by enzymatic system produced by starter culture.

Although starters can assist in safety of cheese, they cannot totally guarantee microbial quality of cheese. Ultrafiltration of milk brings about concentration of macromolecules and even retention and concentration of bacteria and their spores. If a pathogenic bacterium such as *Listeria* exists in ultra- filtered milk, it may have more time for propagation before the reduction of pH. Thus, addition of adjunct culture (*L. lactis*) and antibacterial substances such as nisin can enhance the microbial quality. This research showed that the usage of *L. lactis* is more effective in terms of pathogenic inhibitory in comparison with free nisin. Using *L. lactis* as an adjunct starter culture can assist microbial quality improvement and prevent important pathogens, which may survive during food processing, because of the production of beneficial metabolites.

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مقایسه اثر *لاکتوکوکوس لاکتیس* و نیسین برای مهار رشد باکتری‌های بیماری‌زا در پنیر فتا ایرانی

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چکیده

مقدمه: در مطالعه حاضر اثر کشت باکتری *لاکتوکوکوس لاکتیس* تولید کننده نیسین در کشت توام و نیسین استاندارد خالص بر باکتری‌های بیماری‌زای غذایی در محیط کشت و پنیر فتا ایرانی بررسی شد. در مقایسه *لاکتوکوکوس لاکتیس* نه تنها باعث بهبود طعم و مزه پنیر شد، بلکه نقش بهتری در بهبود کیفیت میکروبی پنیر اولترافیلتر فتا به عنوان یک مدل غذای لبنی تخمیری داشت.

مواد و روش‌ها: برای این منظور *لاکتوکوکوس لاکتیس زیرگونه لاکتیس* به‌عنوان تولید کننده نیسین و سویه‌های *لیستریا منوسیتوژنز، اشرشیا کلی* و *استافیلوکوک اورئوس* به‌عنوان سویه‌های استاندارد بیماری‌زا به پنیر اولترافیلتر تلقیح شدند. منحنی رشد هر یک از باکتری‌ها به شکل تنها، کشت توام با *لاکتوکوکوس لاکتیس* و همراه با نیسین رسم شد. تولید نیسین از روش انتشار در آگار بر علیه سویه حساس استاندارد اندازه گیری و با RF-HPLC تایید شد.

نتایج: نتایج نشان داد که تعداد *لیستریامنوسیتوژنز* در هر دو محیط حاوی نیسین و *لاکتوکوکوس لاکتیس* بعد از یک هفته از لگاریتم ۷ به ۳ و طی دو هفته به صفر رسید. *استافیلوکوکوس اورئوس* مقاومت بیشتری داشت و تعداد آن پس از ۲۸ روز به صفر رسید. در حالی که تعداد *اشرشیا کلی* در طی هفته اول روند افزایشی و سپس، تا روز ۲۸ از لگاریتم ۴ کمتر نشد. با وجود افزایش تعداد *اشرشیاکلی* در محیط کشت حاوی نیسین، تعداد آن در پنیر به علت اثر هم‌افزایی نیسین و دیگر متابولیت‌های تولیدی توسط *لاکتوکوکوس لاکتیس* و دیگر سوی‌های مایه پنیر نه تنها افزایش نیافت بلکه رو به کاهش نهاد.

بحث و نتیجه‌گیری: نتایج نشان داد که در محیط کشت و پنیر فتا تعداد *لیستریا منوسیتوژنز* در ابتدای فاز لگاریتمی توسط *لاکتوکوکوس لاکتیس* کاهش و *استافیلوکوکوس اورئوس* با وجود حساسیت کمتر به نیسین نیز مهار شد. تعداد *اشرشیا کلی* با وجود مقاومت به نیسین در کشت توام افزایش نیافت وحتی روندی کاهشی نشان داد. این پژوهش نشان داد که استفاده از *لاکتوکوکوس لاکتیس* به همراه سویه‌های آغازگر اثر مهار کنندگی بهتری از نیسین بر رشد و کنترل آلودگی باکتری‌های بیماری‌زا دارد. استفاده از *لاکتوکوکوس لاکتیس* به عنوان کشت همراه با سویه‌های مایه در افزایش کیفیت محصول موثر و مانع رشد باکتری‌های بیماری‌زای مهم می‌شود.

واژه‌های کلیدی: *لاکتوکوکوس لاکتیس*، نیسین، محافظت کننده، باکتری‌های بیماری‌زا

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