The combined action of the Nisin and Lactoperoxidase system activation on the microbiological quality of raw milk with special emphasis against *E.coli O157H7* in milk

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Summary

Escherichia coli O157:H7 were isolated from 90 bovine and ovine locally produced soft cheese samples and their identification were confirmed based on the cultural, biochemical reactions and serological properties, *E.coli* O157:H7 isolates obtained by plating on the Chromogenic agars. They were further tested serologically for the presence of both O157 and H7 antigenes using the agglutination test kit. The highest non-significant prevalence (P>0.05) level of *E.coli* O157:H7 was found in the ewe's soft cheese samples (37.77%) followed by the cow's soft cheese samples (31.11%). The antimicrobial potency of the Nisin against the sensitive *Lactobacillus* strain was lost after (10) minutes of heating at (80°C) while retaining (100%) of its antimicrobial potency after its exposure to the pasteurization time and temperature (63°C/ 30 min.). The highest antimicrobial potency of Nisin was achieved at neutral pH (100%) while 90% and 45% of its potency were retained under acidic (pH=3) and alkaline (pH=9) conditions respectively. Nisin had short bacteriocidal incubation period against the sensitive lactobacillus strain where its antimicrobial potency reduced after 48 hours and lost after 120 hours of refrigeration storage period. *E.coli* O157:H7 was insensitive to the action of Nisin while stressed *E.coli* O157:H7 by activation of lactoperoxidase system in pasteurized milk was susceptible to its action and such result gave an indication of the synergistic effect of Nisin with activated lactoperoxidase system.

Keywords: Nisin, *E.coli* O157:H7, Soft cheese, Microbiological quality.

Introduction

Milk is regarded as a good medium for the growth and multiplication of microbes due to its nutrient rich constitution that may lead to become unfit for human consumption (1). *Escherichia coli* O157:H7 was recognized as a virulent pathogen to human with a low infectious dose that caused food-borne illness in 1996 (2). The domestic ruminants such as cattle, sheep and goat are the natural hosts for *Escherichia coli* O157:H7, so the milk could be contaminated through the direct contact with both the farm environments and dairy cattle (3). Activation of lactoperoxidase system other than refrigeration is used for the stabilization of raw and pasteurized milk since the lactoperoxidase system helps to reduce the bacterial load that may lead to extend the shelf life of milk .The lactoperoxidase system could act synergistically in combination with other food preservation technique such as Nisin to control the growth of spoilage and pathogenic microorganisms in milk (4). Nisin is antibacterial peptide produced by Lactococcus lactis bacterium, and is used to extend the shelf life of many foods as food preservative by inhibiting the pathogenic and gram positive spoilage bacteria (5). The injures of the gram negative bacterial cells by the antibacterial action of lactoperoxidase system supporting the action of Nisin, where both inhibitors (lactoperoxidase system and Nisin) act on the cellular cytoplasmic membrane target. The lactoperoxidase system could increase the permeability of the cytoplasmic membrane to the action of Nisin that facilitates the antibacterial effect of Nisin (6).

Materials and Methods

Escherichia coli O157:H7 was isolated from 90 bovine and ovine locally produced soft cheese samples after 24 hours of incubation at 37 °C on the Chromogenic agar. The identification of *E.coli* O157:H7 was based on cultural, biochemical and serological characteristics *E.coli* O157:H7 isolates were further tested serologically for the presence of

both O157 and H7 antigenes using agglutination test. Stock solution of Nisin was prepared in HCl (0.02 mol, pH3) and its concentrations of 30 and 50 IU/ml were tested (7). The antimicrobial activity of Nisin against the both sensitive strain (*L. acidophillus* LA-K) and indicator organism (*E. coli* O157:H7) was determined by using a well diffusion assay (8). Sterile whole milk was inoculated with a fixed number of *E. coli* O157:H7 of 1×106 cfu/ml and then subjected to a stress condition by activation the natural lacoperoxidase system. The antimicrobial activity of Nisin was tested on the growth rate survival of stressed *E.coli* O157:H7 in pasteurized milk by using pour plating method on VRB agar and also by using a well diffusion assay method.

Results and Discussion

The laboratory studies of the cultural isolation revealed that 14 isolates (31.11%) were isolated from bovine (cows) soft cheese samples while 17 isolates (37.77%) were isolated from ovine (ewe's) soft cheese samples. Data revealed that there was no difference in the percentages of *E.coli* O157:H7 isolation between the above mentioned two types of soft cheese samples from Al-Rassafa districts as shown in (Table, 1).

Table, 1: The prevalence of *E. coli* O157:H7 in bovine and ovine soft cheese samples that were collected from AL-Rasaffa districts.

Source of cheese samples	No. of samples examined	No. of positive samples	% Positive samples
Cows	45	14	31.11
Ewes	45	17	37.77
Total	90	31	34.44

X_2=0.4, No differences between cow's and ewe's soft cheese samples.

The raw milk and locally produced dairy products (such as locally produced soft cheese) are regarded as a major vehicle for *E.coli* O157:H7 transmission to human by consumption such products (9 and 10). The current results of prevalence level (34.44%) was higher than those obtained by (11) who recorded that three isolates of *E.coli* O157:H7 were isolated from 15(20%) raw milk samples that were collected from rural districts surrounding Baghdad city while (12) reported the higher prevalence level (51.54%) of *E.coli* O157:H7 contamination in raw milk samples that were collected from the rural areas surrounding Baghdad city also was higher than those obtained by (13) who reported that 6 (15%) out of 40 raw milk samples that were collected from eight villages surrounding Baghdad city were positive for *E.coli* O157:H7 isolation. The antimicrobial potency of the Nisin against the sensitive *Lactobacillus* strain was lost after 10 min. of heating at 80°C while retaining 100% of its antimicrobial potency after its exposure to the pasteurization time and temperature (63°C/ 30 min.). The highest antimicrobial potency of Nisin was achieved at neutral pH 100% while 90% and 45% of its potency were retained under acidic (pH=3) and alkaline (pH=9) conditions, respectively, Nisin had short bacteriocidal incubation period against the sensitive lactobacillus strain where its antimicrobial potency reduced after 48 hours and lost after 120 hours of refrigeration storage (Table, 2).

Table, 2: The effect of different pH values, refrigeration storage periods and temperature of heating on the antibacterial activity of nisin 30 IU/ml against *L. acidophillus* LA-K.

pH values	Potency %	Refrigeration storage periods (hrs.)	Potency %	Heating Temp. °C/min	Potency %
3	90.9	24	100	63°C /30min	100
7	100	48	89.47	80°C /10min	0
9	45.4	120	0		

One of the important feature that affect Nisin activity, solubility and stability is the pH of its fluid. The solubility and stability characteristics were dramatically changed at high (pH=10) and the mechanism of decrease in the activity and stability of Nisin at different pH conditions is still unknown but could be due to denaturation or modification in the chemical structure or both of them (14). The heat stability of Nisin is very important feature in the food and dairy products industries as a preservative because many of heating temperatures are used in the food processing. The heat sensitivity of Nisin could be attributed to its chemical structure or the

structural changes that might cause Nisin to loss its efficacy (15). In developing countries the lactoperoxidase system has been used as an essential technique to improve the keeping quality and extending the shelf life of raw milk. Application of good hygienic practices and usage the non-thermal method or bioactive molecules with LPS could provide a natural method for raw milk preservation (16). The average mean log values (log cfu /ml) of total Coliform counts in raw whole milk samples that were stabilized by the activation of their natural lactoperoxidase system and subjected to the action of the Nisin and stored at ambient temperature (30°C) over the four time points of 0, 6, 24 and 48 hrs. are shown in (Table, 3). Under the conditions used, activation of the natural lactoperoxidase system significantly ($P<0.05$) decreased the total Coliform counts with time of milk storage at ambient temperature, where the average mean log value of the starting initial coliform counts in raw whole milk was significantly ($P<0.05$) reduced from 6.83±0.012 (69×10^5cfu /ml) at 0 hour to 5.73±0.010 (54×10^4 cfu/ml), 4.60 ±0.028 (40×10^3 cfu/ml) after 6 and 24 hrs., respectively and then increased significantly ($P<0.05$) to 5.11±0.052 (13×10^4 cfu /ml) after 48 hours at ambient storage. The average mean log value of the starting initial coliform counts in the control activated raw milk samples reduced significantly ($P<0.05$) from 6.83±0.012 (69×10^5cfu/ml) to 6.20±0.008 (16×10^5 cfu /ml) after exposure to the action of the Nisin at 0 hr. and to 4.66±0.014 (46×10^3 cfu/ml), 4.11±0.160 (13×10^3 cfu/ml) and 3.47±0.097 (3×10^3cfu /ml), after 6, 24 and 48 hrs. of exposure to the action of Nisin at ambient storage temperature, respectively. The highest antimicrobial effectiveness against the viable Coliform bacterial cells in raw milk samples was after 48 hrs. of ambient storage temperature. These data in line with several previous, studies (17 and 18) that reported that the activation of lactoperoxidase system delayed the growth of different groups of bacteria in raw milk and retard the milk spoilage for several days compared to that spoilage which could be achieved when a refrigeration used alone.

Table, 3: The action of Nisin (50IU/ml) in combination with activated Lactoperoxidase system (LPS) on the total Coliform counts (cfu/ ml) in raw milk at about 2-3 hrs and stored at ambient temperature (30°C).

Storage time (hours)	Coliform counts (log cfu/ml)	
	Activated LPS (control) Mean ± SE	Activated LPS with Nisin Mean ± SE
0 hr.	6.83 ±0.012 (69×10^5) Aa	6.20 ± 0.008 (16×10^5) Ab
6 hrs.	5.73±0.010 (54×10^4) Ba	4.66 ±0.014 (46×10^3) Bb
24 hrs.	4.60±0.028 (40×10^3) Ca	4.11 ± 0.160 (13×10^3) Cb
48 hrs.	5.11±0.052 (13×10^4) Da	3.47 ±0.097 (3×10^3) Db

LSD=0.13, Different capital letters in a column revealed significant differences (P<0.05) between hours of incubation. Horizontal different small letters revealed significant differences (P<0.05) between mean value of coliform counts.

Farmers, producers, retailers and consumers shared the responsibility for the reduction in the pasteurized milk shelf life. The storage temperature of pasteurized milk could affect its quality and safety and might lead microorganisms to multiply in pasteurized milk and reduce its shelf life which lead to economic losses (19). The average mean log values (log cfu / ml) of survival bacterial cells of stressed *E.coli* O157:H7 that subjected to the action of the Nisin and stored at ambient temperature over the three time periods of 6, 24 and 48 hrs. are shown in (Table, 4) .There was a significant ($P<0.05$) increase in the *E.coli* O157:H7 counts over the three time points of ambient storage temperature in the control milk samples that were neither stabilized by the activation of their lactoperoxidase system nor subjected to the action of the Nisin. The average mean log value of the starting initial count of *E.coli* O157:H7 in the control milk samples increased significantly ($P<0.05$) from 6.02±0.091 (1×10^6 cfu/ml) at 0 hr. to 7.34±0.026 (22×10^6cfu /ml), 8.11±0.044 (13×10^7cfu /ml) and to 9.30±0.024 (20×10^8cfu /ml) after 6, 24 and 48 hrs. of milk storage at ambient temperature, respectively. The time exposure of inoculated stabilized pasteurized milk to the

action of the Nisin at ambient storage temperature had a significant (P<0.05) influence on the viability loss of stressed *E.coli* O157:H7 from hours 6 to 48.The average mean log value of an initial count of 6.02±0.091 (1×10^6cfu/ml) at 0 hr. significantly (P<0.05) decreased to 5.87±0.057 (75×10^4 cfu/ml), 5.51±0.015 (33×10^4cfu/ml) and 4.95 ±0.027 (90×10^3 cfu/ml) survivor cells of *E.coli* O157:H7 after 6, 24 and 48 hrs. of exposure to the action of the Nisin at ambient storage temperature, respectively.

Table, 4: The effect of Nisin (50IU/ml) in combination with activated Lactoperoxidase system on the survival rate of *E.coli* O157:H7 (cfu/ml) in pasteurized milk that stored at ambient temperature (30°C).

Storage period (hours)	Counts of *E.coli* O157: H7 (log cfu / ml)	
	Control Mean ±SE	Combination of Nisin with activated LPS Mean ±SE
0 hr.	6.02 ± 0.091 (1×10^6) Aa	6.02 ± 0.091 (1×10^6) Aa
6 hrs.	7.34± 0.026 (22×10^6) Ba	5.87 ± 0.057 (75×10^4) Bb
24 hrs.	8.11 ± 0.044 (13×10^7) Ca	5.51 ± 0.015 (33×10^4) Cb
48 hrs.	9.30 ± 0.024 (20×10^8) Da	4.95 ± 0.027 (90×10^3) Db

LSD=0.21, Different capital letters in a column revealed significant differences (P<0.05) between hours of incubation. Horizontal different small letters revealed significant differences (P<0.05) between mean values of *E.coli* O157: H7 counts.

Exposure of stabilized milk to the action of the Nisin resulted in a reduction of viable count of *E.coli* O157:H7 from (1×10^6 cfu /ml) at 0 hour to (90×10^3 cfu /ml) after 48 hours of storage at ambient temperature. This result was in agreement with (13) who reported that exposure of stabilized milk to the action of the crude bacteriocin produced by the *L. acidophilus* LA-K resulted in reduction of viable count of 3.935 log cfu/ml of *E.coli* O157:H7 after 48 hours of milk storage at ambient temperature.

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الفعل التآزري للنيسين ونظام اللاكتوبيروكسيديز المفعل على النوعية الجرثومية للحليب الخام مع التاكيد ضد الايشريشيا القولونية *E.coli* 0157:H7 في الحليب

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الخلاصة

عزلت بكتيريا الاشيريشيا القولونية O157:H7 من 90 عينة من الجبن الطري للأبقار والاغنام، شخصت عزلات الايشيريشيا القولونية بالاعتماد على الخصائص الزرعية، البايوكيميائية والمصلية، ظهرت المستعمرات النموذجية لبكتيريا الايشيريشيا القولونية المعوية النزفية O157:H7 على الوسط الصباغي بلون بنفسجي، أجريت المزيد من الفحوصات المصلية على هذه العزلات للكشف عن المستضد الجسمي (O157) والمستضد السوطي (H7) باستعمال اختبار التلازن (تلازن اللاتكس السريع). أشارت الدراسات المختبرية الحالية للعزلات الزرعية إلى أنّ 31 (34.44%) عينة من اصل 90 عينة من كلتا عينات الأجبان الطرية المحلية للأبقار والأغنام موجبة لبكتيريا الايشيريشيا القولونية المعوية النزفية O157:H7. حيث كان التلوث عالي وبدون فروقات حيث كانت نسبة التلوث لعينات الجبن الطرية المحلية الصنع للأغنام (37.77) تليها عينات الاجبان الطرية للابقار (31.11%). الفعالية التثبيطية لمحلول النيسين و بتركيز30 وحد دولية / مللتر ضد بكتيريا *Lactobacillus acidophilus* LA-K الحساسة مستقر حرارياً واحتفظ على 10% من قوتة الفعالة بعد تعرضة للتسخين على درجة حرارة (63°م) و لمدة 30 دقيقة، في حين كان لتعريض محلول، في حين كان يتعرض محلول النيسين للـ (80°م) لمدة 10 دقائق تأثير كبير في فقدان قوته الفعالة الى (0%) وبصورة معنوية (P<0.05)، أثرت درجة حرارة خزن محلول النيسين بدرجة حرارة الثلاجة بصورة معنوية (P<0.05) في قوته الفعاله ضد سلالة البكتيريا الحساسة *Lactobacillus acidophilus* LA-K اذ كانت شدة الفعاليه التثبيطية بعد مدة خزن 48 ساعة في حين فقدت القوة التثبيطيه لمحلول النيسين (0%) بعد مرور 120 ساعة على خزنه في درجة حرارة الثلاجه. بكتيريا الايشيريشيا القولونية O157:H7 غير حساسة للفعل النيسين تعريض البكتريا للاجهاد في نظام الالكتوبروكسيديز المفعل في الحليب المبستر يجعلها أكثر حساسية لفعل النيسين وهذا دليل على ألفعالية التازرية لنظام الالكتوبروكسيديز المفعل مع النيسين.

الكلمات المفتاحية: النيسين، الايشيريشيا القولونية المعوية النزفية O157:H7، الجبن الطري، النوعية الجرثومية.