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Antibacterial Effects of Lactoperoxidase System and Combined with Bacteriocine Against *E. coli* O175:H7 in Raw Milk

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Abstract

The present study was planned to evaluated the antimicrobial potency of the LPS alone or in combination with the crude bacteriocin that produced by *L. acidophilus* LA-K against *E. coli* O157:H7 in pasteurized milk. 40 raw milk samples that were collected randomly at weekly intervals (5 samples/week) from Baghdad city and transport to laboratory for analyzer. The identification of *E. coli* O157:H7 isolates were confirmed based on biochemical reactions and both cultural and serological characteristics.

Pasteurized whole milk was experimentally inoculated with an initial count of 50×10^6 cfu/ml of *E. coli* O157:H7 and then subjected to the action of two different concentrations of crude bacteriocin after the activation of its LPS and storage at ambient and refrigeration temperatures over the five time points of 0.5 hr., 6 hrs., 24 hrs., 48 hrs. and 5 days of milk.

Our study showed that non-significant ($p < 0.05$) reduction in their counts was observed after 6 hours of milk storage at either ambient or refrigeration temperatures in control milk sample. While data revealed that significant ($p > 0.05$) differences in the average mean log values of the total *E. coli* O157:H7 counts were found between the control samples and those that exposed to the action of each of the two different concentrations of crude bacteriocin over the time.

The result show that crude bacteriocin that diluted (1/110)ml times in milk resulted in a decrease of viable count of *E. coli* O157:H7 of 4.723 cfu/ml and 0.815 log cfu/ml after 5 days of storage at ambient and refrigeration temperatures respectively, while an increase of crude bacteriocin concentration to diluted (1/15) ml times in milk resulted in a decrease of viable count of 3.326.589 log cfu/ml and 6.589 log cfu/ml after 48 hours and 5 days of milk storage at ambient temperature respectively, whereas resulted in a decrease of 0.863 log cfu/ml after 5 days of refrigeration storage.

Keywords: *Raw milk; Bacteriocin; Lactoperoxidase syste*

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Introduction

Milk and milk products are a major part of human food and play a role in the diet (Pal, 2014). Because it contain many nutrients, like: protein, vitamins, calcium, phosphorus, magnesium, zinc, etc. so that it necessary for healthful living of all age groups of humans and both sex (Das., *et al.* 2015). It's regard good media for the growth of microorganisms (Ledenbach and Marshall, 2009). Bacterial contamination of raw milk due to indicated inadequate packaging system and improper temperature control which favor microbial growth and metabolism and brings in undesirable changes (Sarkar, 2015).

The Gram-positive organisms can be present in raw milk, but they also may enter milk products at various points during production and processing (Singh., *et al*. 2011). One of the most significant food-borne pathogens that have gained increased attention in recent years is *E. coli* O157:H7. Illness that results from *E. coli* O157:H7 infection can be life threatening and susceptible individuals showed a range of symptoms including haemorrhagic colitis, hemolytic – uremic syndrome and thrombotic thrombocytopenic purpura (Caprioli., *et al*.2005).

Bacteriocins are peptides produced by some bacterial species exerting a bactericidal mode of action on susceptible strains (Elotmani., *et al*. 2003). Is active against Gram-positive bacteria; this substance is used as a food preservative for many years, especially in cheese and other milk products. the lactoperoxidase enzyme (LP), thiocyanate (SCN)) and hydrogen peroxide H_2O_2) (Elotmani and Assobhei 2003). LP system occurs naturally in bovine milk and in the saliva of human beings and animals (Elotmani and Assobhei 2003); and has inhibitory activity against Gram negative and Gram-positive bacteria.

Beside bacteriocin and/or in combination with the lactoperoxidase system (LPS) would stimulate this indigenous antibacterial system in raw milk and provide new opportunities for the control of pathogenic *E. coli* O157:H7, improving milk safety and considerably extend its shelf–life (FDA/WHO,1991 and FSANZ, 2002). Application of bacteriocins with LPS may increase inactivation of *E. coli* O157:H7 which were normally insensitive to bacteriocins because their cellular targets were shielded by an outer membrane (Masschalck., *et al*. 2000). The aim of this study antibacterial effects of lactoperoxidasae and combined with bacteriocin against *E. coli* O175:H7 in raw milk.

Material and Methods

Sample collections

Fourth raw milk samples were collected randomly at weekly intervals (5 Samples/week) in sterile 250 ml plastic bags from different villages surrounding Baghdad city and transport to laboratory for analyzes. *Escherichia coli* O157:H7 was isolated from milk using Eosin Methylene Blue agar (EMB agar) by spread plate method. Colonies with green metallic sheen were selected and sub cultured in Nutrient agar at 37˚C for 24 hours and then refrigerated at 4˚C (Ravindran., *et al*. 2016) and use biochemical test and chromogenic agar.

Extraction of bacteriocin

The crude bacteriocin was obtained from the bacteriocin producing strains Lactobacillus acidophilus LAK K (CHR-Hansen (USA) Procured from School of Animal Sciences LSU Ag center (Louisiana State University) by DR. Najim Hadi Najim which was grown in MRS broth under anaerobic condition at 37°C for 24 hours the supernatant fluid was separated from cells by centrifugation at 5000 rpm for 30 min. Late the supernatant was collected and pH was adjusted to 6.8-7 with sterile in NaOH to exclude the effect of organic acids was filtered through a syringe filter with pore size of 0.45 µm , then heating for 10 min at 70°C to inactivate antibacterial peptides (protease) and to destroy cells and were stored at 4°C in a refrigerator (Abd and Ali 2015).

Activation of lactoperoxidase system

Milk sample was preserved according to (soomro., *et al*. 2012) procedure the activation of lactoperoxidase system (LPS) with 20 mg/liter strength of each activator I (sodium thiocyanate) and with 66.6 ml activator II (hydrogen peroxide). Activation of LPS was after 3 hours of morning milk by addition of sodium thiocyanate and hydrogen peroxide, after one minute of thorough mixing.

The antibacterial activity of activated lactoperoxidase system in combination with bacteriocin against *E. coli* O157:H7 in pasteurized milk

Raw milk samples that were screened to be free from *E. coli* O157:H7 contamination were experimentally pasteurized at 63°C for 30 minutes in a water bath unit in the laboratory. pasteurized milk samples were then cooled to ambient (25°C) temperature and lactoperoxidase system was activated by treating one liter of pasteurized milk with 20 ml of stock solution of activator I (sodium thiocyanate) and with 6.66 ml of stock solution of activator II (H_2O_2).

The lactoperoxidase activated pasteurized milk was experimentally inoculated with a fixed number of *E. coli* O157:H7 (50×10^7 cfu) and then the efficacy of different levels of crud bacteriocin either 10 times (1/10) or 5 times (1/5) against the same microorganism in pasteurized milk were tested with three replications. In addition to that, the lactoperoxidase activated pasteurized milk samples were treated in the same conditions as mentioned above but without bacteriocin (using sterile peptone water 0.1% wt./v instead of bacteriocin) to serve as a control with three replications and stored at refrigeration (5°C and 25°C) temperature for 0.5 hr, 6 hrs, 24 hrs, 48 hr sand 5 days.

Statistical Analysis

Data were followed to Statistical Analysis System (SPSS, 2008) and the significant differences were determined at ($p < 0.05$).

Result and Discussion

The antimicrobial potency of the LPS alone or in combination with bacteriocin against *E. coli* O157:H7 in milk

The present study showed that no significant ($p > 0.05$) reduced from 7.697(50×10^6 cfu/ml) to 7.568 ($3610^6 \times$ cfu/ml) after 6 hours of milk storage at ambient temperature, but in contrast their counts increased significantly ($p < 0.05$) to 9.590 (39×10^8 cfu/ml) and to 9.193 (16×10^8 cfu/ml) over the time points of 24 hours and 48 hours of milk storage at ambient temperature respectively while reduced significantly ($p < 0.05$) to 8.688 (49×10^7 cfu/ml) after 5 days of storage at ambient temperature that activation of LPS of the control pasteurized milk that inoculated with *E. coli* O157:H7 counts (Table 1).

The result showed that high a significant (p >0, 05) between that bacteriocin dilution level of either 10 times (1/10) or 5 times (1/5) in the viable counts of stressed *E. coli* O157:H7 after each exposure time from 0.5 hrs. To 5 days of milk storage at ambient temperature. the average mean log value of an initial count of 7.697 (50×10^6 Cfu/ml) in the control milk samples, there were significant ($p > 0.05$) reductions to 7.490, 7.379, 6.201, 4.875 and 2.767 log Cfu/ ml survivors of *E. coli* O157:H7 cells after 0.5 hr., 6 hrs., 24 hrs 48 hrs and 5 days of exposure to the crude bacteriocin that diluted 10 times (1/10) in stabilized milk and stored at ambient temperature respectively. While The average mean log value of an initial count of 7.697 (50×10^6 cfu/ml) in the control stabilized milk was reduced to 7.225, 5.930, 5.182, 3.935 and 0.666 log cfu/ml after exposure to the crude bacteriocin that diluted 5 times (1/5) in same time and storage (Table 1).

Period of storage at ambient temperature (25°C)	Counts of *E. coli* O157:H7 (log cfu/ml)		
	Activation of LPS	Activation of LPS +Bacteriocin	
	Control	(1/10) ml	(1/5) ml
	Mean ± SE	Mean ± SE	Mean ± SE
0.5 hour	7.697 ± 0.025 Ab	7.490 ± 0.029 Aa	7.255 ± 0.054 Aa
6 hours	7.568 ± 0.037 Ab	7.379 ± 0.105A Aa	5.930 ± 0.195 Cb
24 hours	9.590 ± 0.006 Aa	6.201 ± 0.140 Bb	5.182 ± 0.099 Cb

48 hours	9.193 ± 0.024 Aa	4.875 ± 0.028 Bc	3.935 ± 0.176 Cc
5 days	8.688 ± 0.345 Ab	2.767 ± 0.767 Bd	0.666 ± 0.666 Cd

Table 1: *Effect of different concentrations of crude bacteriocin in combination with activated LPS on the survival rate of E. coli O157:H7 (Log cfu/ml) in pasteurized milk stored at ambient temperature (25ᵟ).*

L.S.D = 0.60

-Different small letters in a column revealed significant differences ($p > 0.05$) between hours of incubation .

-Horizontal different capital letters revealed significant differences ($p > 0.05$) between the mean values

Antimicrobial potency of the LPS alone or in combination with bacteriocin against *E. coli* O157:H7 in pasteurized milk stored at refrigeration temperature

Our study show that average mean log value of the starting initial count of *E. coli* O157:H7 was non significantly ($p < 0.05$) reduced from 7.690 (49×10^6 cfu/ml) to 7.531 (34×10^6 cfu/ml) after 6hours of milk refrigeration storage, but their counts increased significantly ($p > 0.05$) to 7.984 (96×10^6 cfu/ml) and to 8.940 ($8710^7 \times$ cfu/ml) over the time points of 24 hours and 48 hours of milk refrigeration storage respectively while reduced significantly ($p > 0.05$) to 7.804 (64×10^6 cfu/ml) after 5 days of milk refrigeration storage. An –Hung., *et al*. (1995) and Arias., *et al*. (2000) showed the ability of *E. coli* O157:H7 to survive in different environmental conditions including refrigeration as well as Richert., *et al*. (2000) observed the survival of *E. coli* O157:H7 at refrigeration temperature of 4°C . Daher (2013) showed the ability of *E. coli* O157:H7 to survive and grow well under refrigeration temperature of 4°C.

The result showed that high a significant ($p > 0.05$) in the viable counts of stressed *E. coli* O157:H7 after 6hours of exposure to the bacteriocin in different level of either 10 times or 5 times at refrigeration storage, but non-significant ($p > 0.05$) reduction was observed after each exposure time of 24 hrs., 48 hrs. and 5 days of milk refrigeration storage for the two different concentrations of crud bacteriocin (Table 2). From the average mean log value of an initial count of 7.397 (25×10^6 cfu/ml) after 0.5hr of exposure to bacteriocin that diluted 10 time, there were significant ($p > 0.05$) reduction to 6.851, 6.662, 6.634 and 6.582 log cfu/ml survivors of *E. coli* O157:H7 after 6 hrs., 24 hrs., 48 hrs and 5 days of exposure to the crude bacteriocin that diluted to 10 times and stored at refrigeration temperature respectively. The same trend of viability loss results were obtained when the crude bacteriocin diluted 5 times in milk, where the average mean log value of an initial count of 7.146 (14×10^6 cfu/ml) after 0.5hr of exposure to bacteriocin that diluted 5 times was reduced significantly ($p > 0.05$) to 6.599, 6.190, 6.105 and 6.283 log cfu/ml survivors of *E. coli* O157:H7after 6 hrs, 24 hrs., 48 hrs. and 5 days of exposure to the crude bacteriocin and stored at refrigeration temperature respectively (Table 2). Crude bacteriocin that diluted 10 times in milk resulted in a decrease of viable count of *E. coli* O157:H7 of 0.815 log cfu/ml after 5 days of refrigeration storage (i.e from 7.397 at 0.5 hr. to 6.582 log cfu/ml at 5 days). An increase of crude bacteriocin concentration to double strength when diluted 5 times in milk resulted in a decrease of viable count of *E. coli* O157:H7 of 0.863 log cfu/ml after's days of refrigeration storage (i.e from 7.146 at 0.5 hr. to 6.283 log cfu/ml at 5 days).

The use of bacteriocins in heat processed foods may reduce the thermal processes intensity and improve both the nutritional and organoleptic properties of the food and minimize the heat processing cost (Deegan., *et al*. 2006). Bacteriocins from LAB are proteinaceous compounds which have inhibitory effect against closely related species and other gram positive bacteria (Cocolin., *et al*. 2007). Pores in the membrane of the sensitive cells were formed by bacteriocins and resulting in the leakage of cellular materials (McAuliffe., *et al*. 2001). Generally many bacteriocins of LAB were active against Gram-positive bacteria but Gram- negative bacteria were little insensitive to bacteriocin (Todorov., *et al*. 1999). The differences in resistance between Gram-positive and Gram-negative bacteria was attributed to difference in the cell envelopes of these bacteria (Andersson 1986). The sensitivity of Gram-positive bacteria can be attributed to the susceptible nature of lipoteichoic acid in their cell envelop which is absent in Gram-negative bacteria (Albano., *et al*. 2007).

Although the nature of the Gram-negative cell wall restricts the activity of LAB bacteriocins, but bacteriocins may be used in combination with other treatment to increase their effectiveness (Deegan., *et al*. 2006).

A bacteriocin alone in milk is not likely to ensure satisfactory and safety, this is of particular significance with regards to Gram-negative pathogenic bacteria that are protected by the presence of an outer membrane but when the outer membrane is impaired by agent such as LPS, the outer membrane is disrupted rendering Gram-negative bacteria to be sensitive to the action of bacteriocin (Deegan.,. *et al*. 2006). The test organism *E. coli* O157:H7 was resistant to the action of crude bacteriocin that was produced by L. acidophilus LA-K and no inhibition clear zone was detected after its treatment with bacteriocin by agar diffusion method (Khudhier, 2011 and Daher, 2013). An overall conclusion on the basis of this investigation pointed out that a combination of bacteriocin with the activation of LPS acted synergistically against *E. coli* O157:H7 in pasteurized milk i. e more effective in destroying cells of target bacteria than either of them alone. Activation of the LPS inflected sub lethal injury or damage of the cell wall and cell membrane (cell envelop) or changes in membrane permeability of Gram- negative survivors which became susceptible to the action of bacteriocin. When the outer membrane of gram- negative cells was injured by activation of the LPS, the permeation of bacteriocin to the cytoplasmic membrane was facilitated.

Periods of refrigeration storage at (5°C)	Counts of *E. coli* O157:H7 (log cfu/ml)		
	Activation of LPS	Activation of LPS +Bacteriocin	
	Control	(1/10) ml	(1/5) ml
	Mean ± SE	Mean ± SE	Mean ± SE
0.5 hour	7.690 ± 0.115 Ab	7.397 ± 0.522 Aa	7.146 ± 0.504 Aa
6 hours	7.531± 0.166 Ab	6.851± 0.626 Bb	6.599 ± 0.650 Bb
24 hours	7.984 ± 0.191 Ab	6.662 ± 0.682 Bb	6.190 ± 0.596 Bb
48 hours	8.940 ± 0.251 Aa	6.634 ± 0.620 Bb	6.105 ± 0.496 Bb
5 days	7.804 ± 0.204 Ab	6.582 ± 0.342 Bb	6.283 ± 0.026 Bb

Table 2: *Effect of different concentrations of crude bacteriocin in combination with activated LPS on the survival rate of E. coli O157:H7 (Log cfu/ml) in pasteurized milk stored at refrigeration temperature (5ć).*

L.S.D = 0.60

-Different small letters in a column revealed significant differences (p > 0.05) between hours of incubation.

-Horizontal different capital letters revealed significant differences (p > 0.05) between the mean values.

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