International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Volume 3, Issue 3, March - 2016

Research Article

SOI: http://s-o-i.org/1.15/ijarbs-2016-3-3-34

Efficacy of the Bacteriocin in combination with hydrogen peroxide (H_2O_2) for reduction *E.coli* O157:H7 and increase the shelf life of raw milk

Zina Saab Khudhir*

Department of Vet Public health, College of Veterinary Medicine, Baghdad University, Iraq
*Corresponding author: *zinasaabe@yahoo.com*

Abstract

Isolates of *E.coli* O157:H7 were isolated from 20 cow's and Buffalo's (10 each) locally produced soft cheese samples that were collected randomly at weekly intervals from the retail markets inside the Baghdad city. Their identification were confirmed based on the cultural, biochemical and serological properties .The current study revealed that 7(35%) out of 20 cow's and Buffalo's soft cheeses samples were positive for *E.coli* O157:H7 the highest significant ($p<0.05$) prevalence of *E.coli* O157:H7 was found in cow's soft cheese samples (40%) followed by Buffalo's soft cheese samples (30%). The current research was planned to investigate the quality of raw milk that preserved by combination of crude bacteriocin with H_2O_2 with special emphasis against *E.coli* O157:H7. The highest significant ($p<0.05$) reduction in the average log values of total aerobic bacterial counts were found in all raw milk samples that subjected to the action of crude bacteriocin with 0.05% H_2O_2 compared to the other H_2O_2 concentrations (0.02%and 0.04%) and the control after 48hrs of milk storage at ambient temperature. The synergistic antimicrobial activity of the crude bacteriocin in combination with 0.05% H_2O_2 had a significant ($p<0.05$) influence against *E.coli* O157:H7 in pasteurized milk .The average log value of an initial count of 5.98log cfu/ml at 0 hrs significantly ($p<0.05$) reduced to 5.361 and 5.079 log cfu/ml survivor cells of *E.coli* O157:H7after 24 hrs and 48 hrs of exposure to the action of crude bacteriocin in combination with 0.05% H_2O_2 at ambient storage temperature respectively. An overall conclusion on the basis of the current results pointed out that milk can be preserved by using a combination of crude bacteriocin with 0.05% H_2O_2 against both the spoilage and the pathogenic bacteria such as *E.coli* O157:H7in tropical countries such as Iraq.

Keywords: soft cheese, microbiological quality of raw milk, crude bacteriocin, hydrogen peroxide (H_2O_2), *E.coli* O157:H7.

Introduction

E.coli O157:H7 is one of the emergent pathogens that are recognized as food borne diseases in 1996 (Li *et al*., 2011). Katani *et al* ., (2015) and Bonardi *et al* ., (2015) reported that *E.coli* O157:H7 caused bloody diarrhea and hemolytic uremic syndrome with renal failure in humans but asymptomatic in ruminants ,hydrogen peroxide (H_2O_2) is regarded as an effective and affordable simple method that can be used in the tropical countries for extending milk shelf life since milk is produced in small quantities by a large of small holders leading to both milk collection and delivery to the dairy processing plant time consuming (Odoi, 2003). The bactericidal action of H_2O_2 against the gram negative bacteria has been recorded in both the water and food systems (Liao and Sapers., 2000).This antimicrobial action could be attributed to its ability to form the hydroxyl radical which can damage the DNA and the membrane constituents of the target bacteria (Juven and pierson.,1996).Crude bacteriocin in combination with H_2O_2 provide new opportunities as a hurdle technology for controlling the spoilage and the pathogenic bacteria, extending milk shelf life

and improving the safety of milk (Osullivan *et al*., 2003). The current study was planned to investigate the synergistic antibacterial activity of using different concentrations of H_2O_2 in combination with crude bacteriocin as an attempt to preserving raw milk at ambient temperature and increasing its shelf life in Iraq which is considered as one of the tropical countries.

Materials and Methods

Twenty soft cheese samples that manufactured from cow's and buffalo's inside the farmer homes in rural areas were collected from retail markets in Baghdad city. Identification of *E.coli* O157:H7 isolates were carried out by cultural characteristics on chromagar, biochemical tests (gram stain , Indole, motility and KCN) and serological tests for both the somatic (O) and flagellar (H) antigen . (Vecchi and Drago., 2006).

Microbiological analysis:

Eleven grams portion from each soft cheese sample was taken aseptically and transferred into a sterile stomacher plastic bag containing 99ml of sterile warm aqueous solution of sodium citrate (2%wt/v). The contents were homogenized for 5 minutes by a stomacher to provide a dilution of 10^{-1}then tenfold decimal serial dilutions (up to 10^{-6}) were prepared using a sterile peptone water (0.1%wt/v) as a diluent and 0.1 ml from each appropriate dilution was spread on the chromagar for the isolation of *E.coli* O157:H7 after aerobic incubation at 37°C for 24 hrs (Najim *et al* .,2012).Tenfold serial dilution (10^{-7} to 10^{-9}) were prepared from MRS broth using the sterile peptone water as a diluent and pour plated onto the MRS agar and incubated anaerobically inside the anaerobic jar at 37°C for 48 hours. *Lactobacillus acidophilus* LA-K colonies were purified by three consecutive streaking on MRS agar and then test tubes containing 10ml of MRS broth were inoculated with lactobacillus acidophilus LA-K and incubated anaerobically at 37 °C for 24 hrs.

Preparation of crude bacteriocin:

The crude bacteriocin was obtained from the bacteriocin producing strain *Lactobacillus acidophilus* LA-K which was grown in MRS broth under anaerobic condition at 37 °C for 24 hrs and the supernatant fluid was separated from cells by centrifugation at 10000 rpm for 15 min.

The supernatant was collected and the pH was adjusted to 7 with sterile 1N NaOH and filtered through a syringe filter with pore size of 0.22µm , then heating for 5 min at 70 °C to prevent inactivation of antibacterial peptides by protease and killed cells and then stored at 4 °C in a refrigerator according to the Method of food microbiology protocols (2001) .

Preparation the Hydrogen Peroxide (H_2O_2) (30% w/v) for reduction of *E.coli* O157:H7 counts in pasteurized milk in combination with crude bacteriocin:

Three concentrations of H_2O_2 (0.02, 0.04 and 0.05%) were prepared as described by Dirar (1967) and added separately in combination with (50µl) of crud bacteriocin to pasteurized whole milk samples that cooled and inoculated with fixed number of *E.coli* O157:H7 ($1x10^{-6}$) cfu/ml .The inoculum was prepared by transferring five colonies of *E.coli* O157:H7from overnight old cultured (18-24) hours on nutrient agar to a tube containing 5ml of sterile nutrient broth and the count of approximately 1×10^{-6} cfu / ml was determined after aerobic incubation for 24 hours at 37°C. The bacterial counts were confirmed by preparing serial tenfold decimal dilutions of an inoculum in sterile peptone water and pour plated (Khudhir., 2011). All milk samples and the control were tested for any reduction in the *E.coli* O157:H7 counts after storage at ambient temperature (30 °C) for 0, 6 and 24 hrs with three replications.

Total aerobic bacteria as indicator for the quality of raw milk samples:

The total aerobic bacterial count that was used as an indicator for the quality of the raw milk samples was measured before and after preservation by the crude bacteriocin with H_2O_2 and regularly measured over the points of 0, 24 and 48hrs of storage. The count of survivor cells was monitored by using the pour plating method using nutrient agar and incubated at 37° C for 24hrs.The visible colonies were counted after the aerobic incubation as cfu/ml.

Statistical Analysis:

Means of data at (95% differences level) were analyzed using one way analysis of variance (ANOVA) .Software SPSS Version (18) was used to compare bacterial counts (cfu/ml) that transformed into log10 in the combination of crude bacteriocin and H_2O_2 applications. All results were reported as means values in the tables.

Results

Morphological, biochemical and serological characteristics of *E.coli* O157:H7are shown in table-1.*E.coli* O157:H7 was gram negative rods and positive for both the indol and motility tests but was unable to grow in the potassium cyanide (KCN) broth. Typical *E.coli* O157:H7 colonies were appeared on chromgenic agar as mauve in color. Presumptive *E.coli* O157:H7 isolates were subcultured on the nutrient agar for serological tests and showed agglutination as an indicator for the presence of both O157and H7antigenes.

Table.1 Numbers of positive *E.coli* O157:H7 isolates with their cultural biochemical and serological characteristics:

Number and source of cheese samples		Positive isolates	cultural and biochemical characteristics				Serological characteristics	
			Gram stain	Chrom-agar	Indole test	Motility test		
Cows	10	4	Gram negative rods	Mauve colony	Red ring	Motile	H antigen	**+ve**
Buffalos	10	3				KCN test No growth	O antigen	**+ve**
Total	20	7						

+ve reaction = Agglutination

The current study revealed that 7(35%) out of 20 bovine soft cheese samples were positive for O157:H7.The highest significant ($p<0.05$) prevalence of *E.coli* O157:H7 was found in cow's soft cheese samples (40%) followed by buffalo's soft cheese samples (30%) as shown in table 2.

Table 2: The prevalence of E. coli O157:H7 in cow's and buffalo's soft cheese samples that were collected from Baghdad city

Source of cheese samples	Number of positive samples	The percentage of positive samples (%)
Cow's	4/10	40
Buffalo's	3/10	30
Total	7/20	35

The effect of crude bacteriocin in combination with different concentrations of H_2O_2 on the total aerobic bacterial counts in raw milk stored at ambient temperature is shown in table-3.The total aerobic bacterial count as an indicator test for the hygienic milk quality was monitored over the three time periods of 0 hr, 24hr and 48hrs of milk storage at ambient temperature. There was a significant ($p<0.05$) increase in the total aerobic bacterial counts over the above mentioned three time periods in all the control milk samples where the counts increased significantly ($p<0.05$) from the starting initial count of 7.697 log cfu/ml at 0hrto 7.707 and 8.393 log cfu/ml after 24 hrs and48hrs of milk storage respectively .There were a significant ($p<0.05$)differences in the average log values of total aerobic bacterial counts between the control and the others that treated with a combination of crude bacteriocin with different concentrations of H_2O_2 for each time period. The highest significant ($p<0.05$) reduction in the average mean values of total aerobic bacterial counts were found in all raw milk samples that treated with a combination of crude bacteriocin and 0.05% H_2O_2 compared to the other H_2O_2 concentrations (0.02% and 0.04%) over the three time periods of milk storage. The average log values of total aerobic bacterial counts were significantly ($p<0.05$) reduced from initial count of 7.697 log cfu/ml to 6.518 log cfu/ml immediately after the addition of the crude bacteriocin in combination with 0.05% H_2O_2 at 0 hrs while their counts were significantly ($p<0.05$) reduction to 6.204 and 6.079 log cfu/ml after 24hrs and 48hrs of milk storage respectively as shown in table -3.

Table- 3-The effect of crude bacteriocin in combination with different concentrations of H_2O_2 on the total aerobic bacterial counts (log10CFU/ml) in raw milk.

Total aerobic bacterial counts (log10cfu/ ml)				
Milk storage (hours)	**Control Mean ± SE**	**Combination of crude bacteriocin with the different concentrations of H_2O_2 Mean ± SE**		
		0.02%	0.04%	0.05%
0 hr.	7.697±0.015 Aa	7.591±0.013 Cb	7.447±0.033 Cc	6.518±0.009 Cd
24 hrs.	7.707± 0.009 Ba	7.531±0.026 Bb	7.322±0.039 Bc	6.204±0.0192 Bd
48hrs.	8.393±0.033 Ca	7.342±0.040 Ab	7.146±0.045 Ac	6.079±0.035 Ad

LSD =0.03

-Different capital letters in column revealed the significant differences($p<0.05$) between the incubation hrs

-Horizontal different small letters revealed the significant differences ($p<0.05$) between mean values of bacterial count.

The average log values of survival *E. coli* O157:H7 cells in pasteurized milk that subjected to the action of both the crude bacteriocin and H_2O_2 (0.05%) over the three time periods of 0 hrs, 24 hrs and 48 hrs are shown in table -4. There was a significant ($p<0.05$) increase of *E.coli* O157:H7counts over the three time period of storage in the control milk samples. The average log values of the starting initial count of *E.coli* O157:H7 (5.98log cfu/ml) in the control milk samples increased significantly ($p<0.05$) to 7.132 and 7.443 log cfu/ml after 24 hrs and 48hrs of milk storage at ambient temperature respectively. The antimicrobial activity of the crude bacteriocin in combination with(0.05%) H_2O_2 had a significant ($p<0.05$) effect against *E.coli* O157:H7 .The time of exposure of inoculated pasteurized milk to the action of both the crude bacteriocn and H_2O_2 (0.05%) at ambient storage temperature had a significant ($p<0.05$) influence on the viability loss of *E.coli* O157:H7 from hours 24 to 48. The average log values of an initial count of 5.98 log cfu/ml at 0 hr significantly ($p<0.05$) reduced to 5.361 and 5.079 log cfu/ml survivor cells of *E. coli* O157:H7 after 24 to 48 hours of exposure to the action of crude bacteriocin in combination with H_2O_2 (0.05%) at ambient storage temperature respectively.

Table-4- The effect of crude bacteriocin in combination with the (0.05%) of H_2O_2 on the *E. coli* O157:H7 count (log10/ml) in pasteurized milk

Time (hours)	**Count of *E.coli* O157: H7 (log10/ml) Combination of crude bacteriocin**	
	Control Mean ±SE	**Control with (0.05%) of H_2O_2 Mean ±SE**
		0.05%
0hr	5.98 ± 0.050 Aa	5.98 ± 0.050 Aa
24hrs	7.132±0.060 Ba	5.361±0.034 Bb
48hrs	7.443±0.030 Cb	5.079±0.118 Cc

LSD =0.01

-Different capital letters in column revealed the significant differences($p<0.05$) between the incubation hrs

-Horizontal different in the small letters revealed the significant differences ($p<0.05$) between mean values of the *E.coli* O157:H7 count.

Discussion

E.coli O157:H7 colonies that were examined for the shape and color were appeared with mauve color on the selective chromogenic agar. Further tests were used for identification of *E.coli* O157:H7 isolates such as gram stain, indole, motility and un able to grow in the potassium cyanide broth and the isolates subcultured on the nutrient agar for serological testes for both (O157 and H7 antigens) as shown in table -1. Out of 20 locally produced soft cheese samples that were examined for the prevalence of *E.coli* O157:H7 the highest prevalence of *E.coli* O157:H7 was found in the cows soft cheese samples 4/10 (40%) followed by buffalos soft cheese samples 3/10 (30%) as shown in Table -2. In this investigation the combinational of antibacterial agents (crude bacteriocin and hydrogen peroxide H_2O_2) are promising for usage in raw and pasteurized milk. Numerous antibacterial agents have been used in the raw and dairy products to prevent the food borne illness and to prolong the shelf life of raw milk .Such antibacterial agents can minimize the microbial load and inhibiting the growth of pathogens that contaminated the products during the processing, handling and transportation. The combinational of antibacterial agents (crude bacteriocin and hydrogen peroxide H_2O_2 on the total aerobic bacterial counts is shown in table3.There were a significant differences ($P<0.05$) between the control and each of crude bactericin and H_2O_2 at (0.05%) concentrations on the total aerobic bacteria counts (log10CFU/ ml) in raw milk for each sampling time the total counts were 6.518±0.009, 6.204±0.0192 and 6.079±0.035 at 0,24 and 48 hrs of storage time respectively .Today consumers demented for health safe products having a good quality and with long shelf life Abutbul., *et al* 2004). The combinational interaction of antimicrobials has a great interest specially the antimicrobial agent from the different (animal, plants and microbial) sources that which has a different effects on the bacterial membrane, because the combination of the two antimicrobial agents could enhance the activity of each other that what called (synergism). Additive food grade bacteriocin such as nisin, is commercially available for producers in a powdered form that is legal for use in raw milk and dairy products. Bacteriocin will not change the flavor or color of the both raw milk and other dairy products which is important for specialty dairy makers it is also considered a Generally regarded as safe (GRAS) and a natural additive (Danisco. 2006). *E. coli*, O157:H7, *L. monocytogenes, Salmonella* and *S. aureus* are the emerging pathogens that have been implicated in the soft cheese associated with human illness (Altekruse., *et al* 1998). *E. coli* O157:H7 is a gram negative non spore forming rod, produces a powerful toxin that causes bloody diarrhea and can lead to kidney failure and death in young children immunosuppressive and old peoples (Duffy, *et al*. 2006). It is most often implicated in the consumption of raw milk and dairy products, undercooked beef and unpasteurized milk (CDC., 2001). There are so many difficulties in cooling system and storage and transportation of raw milk in Iraq because of the high cost of energy and equipment's theses may lead to lowering in the hygienic quality of raw milk before reaching to dairy plants that will leads to spoilage of raw milk therefore this investigation deals with the evaluation of the efficacy of two food grad agents to preserve and/or extend the shelf life of raw and pasteurized milk. The synergistic actions or (hurdle technology) of crude bacteriocin and different concentrations of hydrogen peroxide effect in the quality of raw and pasteurized milk that should be explored in order to determine potential advantages in the keeping quality and extended the shelf life when applied to various dairy products. The *E. coli*, O157:H7 counts in pasteurized milk samples declined significantly ($P<0.05$) when combined action of crude bacteriocin with the 0.05% H_2O_2 to less than detectable numbers that observed by the control for each sampling time, the results demonstrates that The *E. coli*, O157:H7 counts in the control sample (5.98 ± 0.050) at 0 hr decreased significantly ($P<0.05$) to 5.361±0.034log cfu/ml and 5.079±0.118 after 24 and 48hrs respectively. Saha *et al*., (2003) reported that The H_2O_2 did not present any health hazard while the microbial quality of raw milk that treated with H_2O_2 increased significantly when compared with untreated milk, it was concluded that the concentrations of 0.04% to 0.05% H_2O_2 is enough to preserve raw milk up to 24 hrs .From the results that above mentioned the combination of crude bacteriocin with H_2O_2 is more effective to inhibit the growth of spoilage and pathogenic microorganism in the both the raw and pasteurized milk under climatic condition such as in Iraq also addition of 0.05% H_2O_2 to raw and pasteurized milk is enough to preserving them for up to 48 hours. The mixtures of crude bacteriocin and hydrogen peroxide make the inhibitory concentrations were lower than when used for the individual compounds this feature can dissolved the problem associated with development microbial resistance to single antimicrobials agents. Combining these antimicrobial agents can converts their individual static inhibitory effects into bactericidal effects.

References

Abutbul, S.; Golan-Goldhirsh, A.;Barazani, O.and Zilberg, D. (2004). Use of *Rosmarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp).Aquaculture 238:97-105.

Altekrus, S.F.; Babagaleh, T.B.; Mowbray, J.C.; Bean, N.H., and Potter, M.E. (1998) .Cheese associated outbreaks of human illness in the United States, 1973-1992.J. Food Prot. 61:1405-1407.

Bonrdi,S.;Alpigiani,L.;Tozzoli,R.;Vismarra,A.;Zecca, V.;Grppi, C; Bacci,C.; Bruini,I and Brindani, F.(2015).Shiga toxin producing *E. coli* O157:H7,0126 and O111 in cattle feces and hides in Italy .Vet.Recopen.,2:e00061.

Centers for Disease Control and Prevention. (2001). Surveillance for Food borne-Disease Outbreaks, United States, 1993-1999.CDC Surveillance Summaries, March 17, 2000; 49/Ss-1.

Danisco.(2006). Product description-PD 214210-1.0EN Nisaplin.In.

Dirar, H. A. (1967).The use of H_2O_2 in controlling *Pseudomonas fragi* in Milk.M. Sc. Thesis, University of Khartoum.

Duffy, G.; Cummins, E., Nally, P.; O'Brien, S. and Butler, S. (2006). A review of quantitative microbial risk assessment in the management of E. coli O157:H7 on beef. Meat Science 74:76-88.

Juven, B.J. and M.D. Pierson. (1996). Antibacterial effects of hydrogen peroxide and methods for its detection and quantitation. J. Food Prot. 59(11):1233-1241.

Katani,R.;Cote,R.;Garay,J.A.;Li.;Arthur,T.M.;Debroy, C.;Mwangi,M.M and Kapur, V.(2015).Complete genome sequence of SS52,a strain *E.coli* O157:H7 recovered from super shedder cattle genome announce., 3(2).

Khudhir ,Z. S.(2011). Antibacterial activity of L. acidophilus bacteriocin against *E. coli* O157:H7 in raw milk. PhD thesis, College of Veterinary Medicine, University of Baghdad.

Li, D., Feng; Y., Zhou, L.; Ye, Z., Wang, J. and Ying, Y. (2011).Label-free capacitive immunosensor based on quartz crystal Au electrode for rapid and sensitive detection of *Escherichia coli* O157:H7. Analytica Chimica Acta 687(1): 89-96.

Liao, C. and G.M. Sapers. (2000). Attachment and growth of Salmonella chester on apple fruits and in vivo response of attached bacteria to sanitizer treatments. J. Food Prot. 63(7):876-883.

Najim,H.N.; Abdul, A.A, and Zina, S.K.(2012). Confirmed coliform test in laboratory Manual for milk testing .Department of Veterinary Public Health, College of Veterinary. Medicine, Baghdad University 1st ED.148-149.

O'Sullivan, L.; Ryan, M. P.; Ross, R. P., and Hill, C. (2003). Generation of food-grade lactococcal starters which produce lantibiotics lacticin3147 and lacticin 481. Applied and Environmental Microbiology, 69,3681–3685.

Odoi, A.(2003). Effectiveness and affordability of hydrogen peroxidein milk preservation under tropical conditions. Milchwisenschaft, 58 (1-2):65-67.

Saha, B. K.; Ali, M. Y.; Chajraborty, M.; Islam, Z. and Hira, A. K. (2003). Study on the preservation of raw milk with hydrogen peroxide for rural dairy farms. Pakistan, J. of Nutrition. 2(1):36- 42.

Sergio, A.; Fernando, J. M.; Aida, A. and Raul, R. (2001). Food Microbiology Protocols .Food Microbiol. Bio., 14:141-146.

Vecchi, E. Deand Drago, L.(2006) *Lactobacillus sporogenes* or *Bacillus coagulans*: misidentification or mislabelling? International Journal of Probiotics and Prebiotics, 1(1): 3-10.

Access this Article in Online	
	Website: www.ijarbs.com
	Subject: Dairy Sciences
Quick Response Code	

How to cite this article:
Zinasaab Khudhir. (2016). Efficacy of the Bacteriocin in combination with hydrogen peroxide (H_2O_2) for reduction *E.coli* O157:H7 and increase the shelf life of raw milk. Int. J. Adv. Res. Biol. Sci. 3(3): 265-270.