European Journal of Nutrition & Food Safety

13(9): 22-30, 2021; Article no.EJNFS.80581
ISSN: 2347-5641

Effect of Lactoperoxidase Enzyme System in Improving Shelflife of Raw Milk from Dairy Farms in Khartoum State, Sudan

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJNFS/2021/v13i930446

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/80581

Original Research Article

Received 15 October 2021
Accepted 20 December 2021
Published 22 December 2021

ABSTRACT

Lactoperoxidase enzyme (LP) is a natural component in milk with broad spectrum of antimicrobial activity applications in *in vivo* and *in vitro*. LP system (LPS) was developed to eliminate the growth of bacterial pathogens and augment the shelf life of raw milk. The aim of this study was to evaluate the effect of activation of LPS on the growth and survival of bacterial pathogens and preserving raw milk quality. A checklist was developed and filled in ten farms at the time of milking to detect the hygienic measures in these farms. Two liters of milk were collected in sterile containers and warmed to 37°C/15min. LPS was activated by dissolving 40mg and 30mg of sodium thiocyanate and sodium percarbonate, respectively, in one liter of milk and followed by thorough mixing. 50ml from this liter of milk was added to two new containers and designated as group B and C. Moreover, group C was supplied with *E. coli* (10^6-10^7 cfu/ml). While milk in group D, LPS was not activated, instead milk was only supplied with *E. coli* (10^6-10^7 cfu/ml). Group A was considered as the control group (neither activated nor supplied with *Escherichia coli*). Tenfold serial dilution was performed to determine the bacterial total viable count (TVC) and milk clotting time (per seconds)

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in each group for 2, 4, 6, 8 and 10 hrs. The TVC and the milk clotting time in each group were statistically expressed as pairs between the groups (A versus B; C versus D) with significance probability of P≤0.05. The checklist analysis reflected the bad hygiene measures in the dairy farms. For TVC, in 2hrs incubation; no significant difference was observed between group A versus group B. While group C versus group D showed high significant difference. After 4hrs incubation, TVC showed prominent significance difference between the control compared to group B. While group C versus group D showed no statistical significance differences. At 6hrs time period, a significant difference was observed between the compared groups. However, no significance differences were observed between the groups after 8hrs and 10hrs of incubation. For clotting time, in 2hrs incubation, a significant difference between the compared groups was observed. After 4hrs incubation, a significant difference between the control compared to group B was prominent, while group C versus group D showed no statistical differences in clotting time. However, no significant differences were observed between the groups after 6, 8 and 10hrs of incubation in the clotting times. Taken together these results indicated that the LPS system was efficient until 6hrs and could prolong milk shelf life and prevent milk clotting.

Keywords: Lactoperoxidase enzyme; Sodium thiocyanate; Sodium percarbonate TVC; raw milk; clotting time.

1. INTRODUCTION

Lactoperoxidase (LP) enzyme is a member of mammalian heme peroxidases with a broad spectrum of antimicrobial activity applications in *in vivo* and *in vitro* [1]. LP system (LPS) was developed to eliminate the growth of bacterial pathogens from foods and augment the shelf life of foods such as cow milk [2,3] as it possesses a bactericidal and bacteriostatic agents activities [3,4]. For instance, the peroxidases present in cow milks are suitable for multiple manipulations such as raw and pasteurized milk conservation and foodstuffs preservation [5-9]. Thus the system has been used as a natural biopreservative with generally recognized as safe status [10].

Lactoperoxidase, hydrogen peroxide and thiocynate are considered as natural components in cow milk in concentrations required to inhibit bacterial growth in milk [6]. Lactoperoxidase concentration in milk differs based on the season, diet, breeding and lactation period [6]. The level may reach about 30 mg/liter and the high concentration was recorded few days post-partum [11,12]. Thiocyanate and hydrogen peroxide concentrations levels differ based on the animals feeding regime and derived mainly from the enzymatic hydrolysis of glucosides and detoxification of cyanide.

The collection, storage and transportation of milk are the most critical points for milk bacterial contamination. Therefore, refrigeration of milk would safely prolong milk shelf life. However, in some rural regions the limited facilities in these regions would hamper milk refrigeration. Therefore, LP system can be a useful mean to increase the shelf life of milk in these regions despite that the antimicrobial efficiency of the system would be lost after two hours post milking [13]. Moreover, some reports demonstrated that, the addition of some compounds such as thiocyanate and hydrogen peroxide in traces would restore and prolong the antimicrobial activity of the enzyme system [14-17].

The LPS is a non-specific system for protecting and conserving the lactating mammary gland and extending the milk shelf life [18]. The system comprises of multiple series of biochemical reactions including the lactoperoxidase enzyme, thiocyanate ion a rising from hepatocytes metabolism and hydrogen peroxide a rising from cellular metabolism [19,20]. These three significant components are essential to enhance and activate the LPS. The final products of these components comprises hypothiocyanic acid and hypothiocyanate ion. These products were shown possessing antimicrobial activities [21,9,3].

Some other compounds such as boric acid, hypochlorides, formalin and hydrogen peroxide were previously examined to preserve and prolong milk shelf life [22]. However these compounds demonstrated toxicity, thus, were illegal additives unless used as preservatives in laboratories or as disinfectants [23]. Recently a study by Lara-Aguilar and Samuel [24] showed that lactose oxidase could be used to developed

enzyme-based preservation technologies for application where cold chain access is limited. However the LPS is recommended as good chemical preservative system for milk since it demonstrates no side effects for humans if given at low traces or concentrations [5]. Thus in this study, we aimed to evaluate the effect of activation of LP-system on the growth and survival of bacterial pathogen using *Escherichia coli* as a model in raw cow milk. In addition, the keeping quality (KQ) of the raw milk was also examined.

2. MATERIALS AND METHODS

2.1 Checklist

As milk production hygiene is complementary to the effective application of the Lactoperoxidase system of milk preservation, a checklist was developed and filled in the field at the time of milk sample collection for ten milking farms.

2.2 Collection of Milk Samples

Milk samples were collected from different traditional and modern dairy farms located in Khartoum State. One liter of milk was collected from each farm in sterile containers and immediately tested for antibiotics residues.

2.3 Antibiotics Residues Analysis

This test was performed using unisensor Milk kit (KIT035), BELGIUM that used to detect Ampicillin, Sulfadimethioxin or Tetrayclines in raw milk. The test was performed to guarantee that the milk samples were free of antibiotics. Milk samples that demonstrated negative results for the residues were used in the study. The milk samples containers were placed in icebox with icepacks and immediately transported to the laboratory of College of Veterinary Medicine University of Bahri for analysis.

2.4 Laboratory Analysis

The milk sample (one liter) was initially subjected to bacterial load using total bacterial count to elucidate the initial microbial load. The milk sample was divided into four sterile tubes each with 50ml milk and the tubes were labeled as A, B, C and D. Tube A was considered as the control group and the other tubes were considered as the test tubes with different treatments.

2.5 Pretreatment of Milk Samples

The activation of the lactoperoxidase system was carried out within two hours from the time of milking. The milk samples were warmed to 37°C for 15 min to activate the LPS immediately as described by CAC [25]. Firstly, 40mg of sodium thiocyanate (NaSCN) was added per liter of milk. The milk was then mixed to ensure an even distribution of the SCN- and plunged for about 1minute with a clean plunger is normally satisfactory. Secondly, 30mg of sodium percarbonate (hydrogen peroxide) was added to the same liter of milk. The milk was then stirred for another 2-3 minutes to ensure that the sodium percarbonate was completely dissolved and the hydrogen peroxide is evenly distributed in the milk. Sodium thiocyanate and sodium percarbonate were added in the order stated above. The enzymatic reaction starts in the milk when the hydrogen peroxide (sodium per carbonate) is added, and completes within about 5 minutes from the addition of H_2O_2; thereafter, no hydrogen peroxide will be present in the milk. Fifty ml from this liter of milk (with sodium thiocyanate and sodium percarbonate) was added to each of tube B and C. Moreover, tube C was supplied with *E. coli* (10^6-10^7 cfu/ml). While tube D was not activated with sodium thiocyanate and sodium percarbonate, instead was only supplied with *E. coli* (10^6-10^7 cfu/ml). Fig. (1) demonstrated the process of the preparation of the control and the treated milk samples used in this study.

2.6 Bacteriological Tests

Analysis of bacteriological quality of the milk (total viable count and alcohol test) was carried out to ensure that good hygienic standards are not neglected. Since the effects of the system are predominantly bacteriostatic, an initial high bacterial population in the milk can still be revealed by such tests.

2.6.1 Determination of Total Viable Count (TVC)

For TVC, 100µl from each milk sample, from the control group and the treated groups (B, C and D groups), was mixed with 900µl of sterile 0.1% peptone water (Merck, Germany). Tenfold serial dilution was performed in 0.1% peptone water and the appropriate dilutions were spread onto nutrient agar plates (Merck, Germany). The counting of the growing colonies in each plate

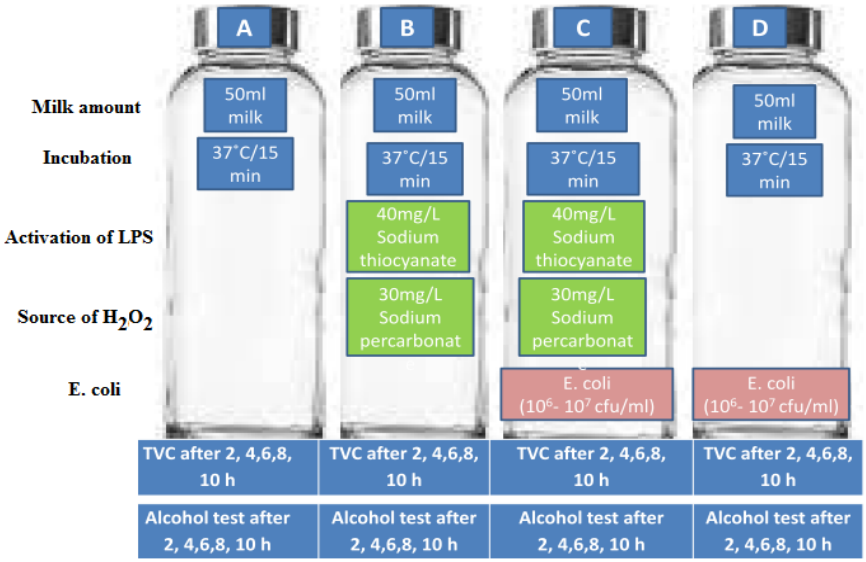

Fig. 1. Showed the steps and the methods of preparation and activation of lactoperoxidase enzyme system (LPS) in milk samples

was performed after 2, 4, 6, 8 and 10hrs for each group. The plates were incubated at 37°C for 48hrs. The resulting colonies were counted and expressed as colony forming units per ml (cfu ml^{-1}) for each sample. For each time period the experiment was performed three times (triplicates).

2.6.2 Alcohol test

One ml of milk from each group was added to equal volumes of 5% alcohol (ethanol). The development of milk clotting was noted and referred to the end of shelf life. Alcohol test was performed at 2, 4, 6, 8 and 10hrs for each group in triplicates.

2.7 Statistical Analysis

Data of present study were analyzed statistically using factorial design. The analysis of variance (ANOVA) tests were carried out by using the general linear model procedure of the Statistics (Version 8.0). The Least Significant Difference (LSD) was used to determine the differences between the means with probability≤0.05

3. RESULTS AND DISCUSSION

A checklist was introduced to the farms during milking and samples collection. The result of the checklist was demonstrated as percentage for each question. Table (1) showed the percentages (%) of the answers delivered by the farms senior milking man or the owner of the farm. The results of the checklist clearly demonstrated the worse hygienic measures in the dairy farms.

The collected milk samples used in the study were tested free of antibiotics. The presence of antibiotics in milk might impact the survivability *E. coli* used in the study. The bacterial colonies (cfu) obtained from the bacterial viable count in each group was statistically expressed as pairs between the groups. For instance, the control group (A) was versus group (B) as one pair and group (C) was versus group (D) as one pair. The results in each time period were statistically expressed as means and standard deviation errors with the standard error means with significance probability of P≤0.05. As shown in table (2), pair 1 in the first two hours showed no significant differences between the control group versus group B. However, the mean in the control group was higher than that in the activated group. The lower mean in the activated group might be due to the start of the activation of the system that had bacteriostatic effect that resulted in reduced mean in this group [26,22]. In pair 2, group C versus group D showed high significant difference (P 0.004). The activated system beside addition of *E. coli* showed significant reduced mean. This might be attributed to the system activation that inhibited bacterial multiplication [26,22]. After four hours of incubation, in pair 3 and pair 4, the former demonstrated significant difference of the control compared to group B (P 0.012), while the latter

showed no statistical significant differences. However, the number of the growing colonies in activation of the system beside addition of bacteria (in pair 4) showed decreased number of growing colonies. At 6hrs time period, in pair 5 and pair 6, the mean of the activation of the system in group B versus the control group (A) and the mean of group C versus that in group D both demonstrated statistical significant difference of P 0.043 and P 0.001, respectively. This result indicated that the system was still efficient at this time period since the means where the system activated showed reduced means values of cfu. The results also coincided with previously published work [27,26].

Table 1. Evaluation of hygienic measures in the investigated dairy farms

Question	%	%	%
Are there identification marks	(20)Yes	(80) No	
Are there records in the farm	(30)Yes	(70) No	
Veterinary supervision	(50) always	(20) sometimes	(30) not exist
Removal of manure	(70) weekly	(30) monthly	(0) others
Are there special cloth during milking	(20)Yes	(80) No	
Do milk men wear gloves during milking	(0)Yes	(100) No	
Do milk men wash their hands and arms before milking	(20)Yes	(80) No	
If the answer is Yes:			
a. Do they dry their hands	(100)Yes	(0) No	
b. Are wounds and injuries covered	(100)Yes	(0) No	
Are there health cards for milk men	(0)Yes	(100) No	
Place of milking	(90) Hangars	(10) parlour	(0) other
Milking method	(90) manual	(10) machine	
Is hair clipped from udders	(30)Yes	(60) No	(10) sometimes
Udder cleanliness before milking	(70)Yes	(10) No	(20) sometimes
Teat pre-dipping	(10)Yes	(90) No	
Teat post-dipping	(10)Yes	(90) No	
Are the teats dried before milking	(10)Yes	(90) No	
Use of milk laxatives	(90)Yes	(10) No	
Is the milk tested by the eye	(100)Yes	(0) No	(0) sometimes
Conduction of periodic mastitis test	(10)Yes	(90) No	
Milking of healthy cows first	(60)Yes	(20) No	(20) sometimes
Are the infected cows isolated	(40)Yes	(60) No	
In case of infection, is feeding calves forbidden	(40)Yes	(60) No	
In case of infection, is the manure removed regularly	(60)Yes	(40) No	
a. In case of YES, is milk disposed of safely?	(80)Yes	(20) No	
What is the place of disposal of infected milk	farm land (40)	Pots (40)	Thrown(20)
When udder inflammation is detected, are antibiotics administered	(80)Yes	(20) No	
Use of udder ointment in drying cows	(60)Yes	(40) No	
Use of udder ointment in treatment	(90)Yes	(10) No	
Giving medication in an appropriate dose	(70)Yes	(30) No	
Is there an infection with mastitis	(40)Yes	(60) No	
Are there any problem with hooves	(30)Yes	(70) No	
Is there a hoof bath available	(0)Yes	(100) No	
Are there placenta retention conditions?	(40)Yes	(70) No	
Are cows with chronic mastitis culled	(80)Yes	(20) No	
Milk storage: types of milk storage containers	(90) stainless	(10) others	
Cleaning of storage containers and utensils			
a. use of soap or detergent for washing	(80)Yes	(20) No	
b. boiled water is used for rinsing the utensils	(10)Yes	(90) No	
Frequency for washing and cleaning the storage containers:			
a. before and after milking	(100)Yes	(0) No	
b. only after milking	(0)Yes	(100) No	
Water sources:	(90) municipal	(10) well	(0) other

Table 2. The mean (cfu) of TVC between the pairs in the different time periods

Pairs and times		Mean/cfu	Standard deviation	Standard Error Mean	Significance (p≤0.5)
Pair 1	2hrs control	227	39.50949	22.81082	
	2hrs thio	105.6667	22.14347	12.78454	0.107
Pair 2	2hrs thio +bact	2416.6667	518.10552	299.12836	
	2hrs only bact	24466.6667	3250.1282	1876.46239	0.004
Pair 3	4hrs control	227	39.50949	22.81082	
	4hrs thio	115.3333	25.73584	14.85859	0.012
Pair 4	4hrs thio+ bact	15400000	2771281.292	1600000	
	4hrs 0nly bact	24033333.33	1101514.109	635959.4676	0.061
Pair 5	6hrs control	2416.6667	518.10552	299.12836	
	6hrs thio	756.6667	237.13569	136.91035	0.043
Pair 6	6hrs thio+ bact	72333333.33	27098585.45	15645375.6	
	6hrs only bact	287000000	19157244.06	11060440.02	0.001
Pair 7	8hrs control	24466.6667	3250.1282	1876.46239	
	8hrs thio	23733.3333	2814.84162	1625.14957	0.853
Pair 8	8hrs thio+bact	16333333333	3700450423	2136456048	
	8hrs only bact	28066666667	2315887159	1337078075	0.076
Pair 9	10hrs control	2706666.667	342685.4729	197849.55	
	10hrs thio	2533333.333	497426.7115	287189.4458	0.204
Pair 10	10hrs thio+ bact	2.65667E+11	22546248764	13017082793	
	10hrs only bact	2.90333E+11	16743157806	9666666667	0.257

thio: (Sodium thiocyanate and Sodium Percarbonate were added to activate the system. bact: Bacteria was added

Table 3. The mean of clotting time/seconds between the pairs in the different time periods

Pairs and times		Mean/Seconds	N	Standard Deviation	Standard Error Mean	Significance (p≤0.5)
Pair 1	2hrs control clotting	243.3333	3	20.81666	12.0185	
	2hrs thio clotting	382.3333	3	22.74496	13.13181	0.016
Pair 2	2hrs thio+ bact clotting	120.3333	3	9.50438	5.48736	
	2hrs only bact clotting	41	3	11.53256	6.65833	0.023
Pair 3	4hrs control clotting	179.3333	3	22.00757	12.70608	
	4hrs thio clotting	293	3	24.87971	14.36431	0.035
Pair 4	4hrs thio+ bact clotting	66.6667	3	12.58306	7.26483	
	4hrs only bact clotting	20	3	10	5.7735	0.07
Pair 5	6hrs control clotting	60.6667	3	23.86071	13.77599	
	6hrs thio clotting	153.3333	3	36.29509	20.95498	0.116
Pair 6	6hrs thio+ bact clotting	33	3	8.18535	4.72582	
	6hrs only bact clotting	19.6667	3	9.50438	5.48736	0.252
Pair 7	8hrs control clotting	20	3	10	5.7735	
	8hrs thio clotting	30.3333	3	8.5049	4.91031	0.43
Pair 8	8hrs thio+ bact clotting	11.3333	3	4.04145	2.33333	
	8hrs only bact clotting	7.6667	3	2.51661	1.45297	0.159
Pair 9	10hrs control clotting	14.3333	3	5.1316	2.96273	
	10hrs thio clotting	38	3	7.2111	4.16333	0.073
Pair 10	10hrs thio+ bact clotting	10.3333	3	5.50757	3.1798	.
	10hrs only bact clotting	6	3	3.4641	2	0.069

thio: (Sodium thiocyanate and Sodium Percarbonate were added to activate the system. bact: Bacteria was added

When the time extended to 8hrs (pair 7 and pair 8) and 10hrs (pair 9 and pair 10) there was no significant differences between the pairs despite the means values of the activated system were reduced compared to the non-activated pairs.

Alcohol stability test was used as a best indicator for milk freshness since the test showed reliable and consistent results [26, 28]. It was shown that the extended storage period provided significant impact on alcohol test of the milk samples of the different treatments [26]. In this study the treated milk samples were incubated and tested after 2, 4, 6, 8 and 10 hrs for clotting with 5% alcohol. As shown in Table (3) the mean of the clotting time per seconds in pair 1 in the first two hours

showed significant difference between the control group (A) versus group B (P 0.016). Also in pair 2 the clotting in group C versus group D showed significant difference (P 0.023). After four hours of incubation the clotting time per seconds, in pair 3 and pair 4, the former demonstrated significant difference of the control versus group B (P 0.035), while the latter (pair 4) showed no statistical significant differences. However, the clotting time in activation of the system beside addition of bacteria (in pair 4) was higher. When the time extended to 6hrs (pair 5 and pair 6), 8hrs (pair 7 and pair 8) and 10hrs (pair 9 and pair 10) there were no significant differences in clotting time between the pairs despite the means values of clotting of the activated system were higher compared to the non-activated pairs. These results indicated that the milk shelf life would be extended to four hours without clotting. These results coincided in parts with previous work of Fanta et al. [26] and Ndambi et al. [22].

4. CONCLUSION AND RECOMMENDATIONS

In this study the activation of LPS in raw milk would potentially reduce the bacterial load and prolong the shelf life of the raw milk. The LPS showed reduced bacterial viable count till 6hrs of incubation and preserve milk from clotting for at least 4hrs of incubation. Evaluation of dairy farms reflected the unfavourable conditions of hygienic measures. The results of this study raised the awareness on the importance of LPS to be used by rural smallholder dairy farms and pastoral production system for improving the preservation and prevention of milk spoilage. However, the potential use of LPS as a bio preservative is still little technically and scientifically less evaluated, thus conducting further studies on LP safety and preservation is required.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ACKNOWLEDGEMENTS

Authors would like to thank Professor Adil MA Salman College of Veterinary Medicine, University of Bahri, Sudan for performing the statistical analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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