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Research Article

Effects of Activated Lactoperoxidase System on Microbiological Quality of Raw Milk

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Abstract

The poor microbiological quality of raw cow milk in Turkey is a major concern. It has been speculated that less activation of naturally present lactoperoxidase system in the milk is the reason for its poor microbiological quality. Hence, the objective of this study was to investigate the effects of activation of the lactoperoxidase (LP) system on microbiological quality of the raw milk. The milk samples collected from a dairy farm were analyzed in the laboratory by dividing into two equal parts as activated (experimental) and control group. The experimental group was activated by treatment with equal concentration of sodium thiocyanate and hydrogen peroxide (20:20 mg/kg) whereas the control sample remained unactivated. All samples were stored at 4°C during 12 h. The microbial load in all the samples was quantitatively determined at 0, 3, 6, 9 and 12 h. The quantitative changes in each microbial species in both growth were recorded and statistically analyzed. The initial count of total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast number were 7.10, 5.14, 6.42, 5.93 and 4.31 log cfu/mL, respectively, and at the end of 3 h the counts were 0.43, 2.23, 1.09, 0.93 and 0.37 log cfu/mL, respectively, were lower than controls. Significant ($P<0.05$) differences were observed for microbial count of activated and control samples except in case of lactic acid bacteria. The results of this study indicate that the addition of thiocyanate and hydrogen peroxidase to the milk activated lactoperoxidase enzyme already present in the milk and slowed down the microbiological growth, especially of the reducing proteolytic *Pseudomonas* spp. On comparison, the results for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast were statistically significant ($P<0.05$) and no significant change was observed in case of lactic acid bacteria.

Keywords: *Lactoperoxidase system, Thiocyanate, Milk quality, Microbiology*

Laktoperoksidaz Sistem Aktivasyonunun Çiğ Sütün Mikrobiyolojik Kalitesi Üzerine Etkileri

Özet

Türkiye'de çiğ inek sütünün mikrobiyolojik kalitesinin düşük olması, bir sorun olarak önemini korumaktadır. Sütte doğal olarak bulunan laktoperoksidaz sistemin yetersiz aktivasyonunun düşük mikrobiyolojik kalitenin nedeni olduğu ileri sürülmektedir. Bu nedenle, bu çalışmanın amacı, çiğ sütün mikrobiyolojik kalitesi üzerine laktoperoksidaz (LP) sisteminin aktivasyonu etkisini araştırmaktır. Bir mandıradan toplanan süt örnekleri aktive edilmiş (deneysel) ve kontrol grubu olarak iki eşit parçaya ayrılmış ve laboratuvarda analiz edilmiştir. Aktive edilmiş grubta, süt örnekleri eşit konsantrasyonda (20:20 mg/kg) sodyum tiyosiyanat ve hidrojen peroksit ile muamele edilmiş, kontrol numunesine ise aktivasyon işlemi yapılmamıştır. Tüm örnekler 4°C'de 12 saat süresince muhafaza edilmiştir. Bütün örneklerde toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae, laktik asit bakteri ve maya yükü, muhafazanın başlangıç, 3., 6., 9. ve 12. saatlerinde niceliksel olarak sayılmıştır. Soğuk muhafaza sırasında her türde meydana gelen niceliksel değişimler kaydedilmiş ve sonuçlar istatistiksel olarak analiz edilmiştir. Başlangıç toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya yükü, sırasıyla 7.10, 5.14, 6.42, 5.93 ve 4.31 log kob/ml olarak belirlenmiştir. Soğuk muhafazanın 3. saatinde, toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya sayıları, aktive edilmiş olan örneklerde, kontrol örneklerine göre, sırasıyla, 0.43, 2.23, 1.09, 0.93 and 0.37 log kob/mL daha düşük çıkmıştır. Aktive edilmiş ve kontrol örneklerinin mikrobiyal sayıları arasında, laktik asit bakterileri dışında, önemli farklılıklar ($P<0.05$) gözlenmiştir. Bu çalışmanın sonuçları, süte ilave edilen tiyosiyanat ve hidrojen peroksitin, sütte mevcut bulunan laktoperoksidaz enzimi ile birlikte çalışmasıyla, mikrobiyal gelişmeyi yavaşlattığını, özellikle proteolitik *Pseudomonas* türlerinde azalmaya sebep olduğunu göstermektedir. Sonuçlar karşılaştırıldığında, toplam mezofilik aerobik bakteri, psikrotrofik bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya sayılarındaki değişimler istatistiksel olarak önemli ($P<0.05$) bulunurken, laktik asit bakteri yükünde anlamlı bir değişiklik tespit edilmemiştir.

Anahtar sözcükler: *Laktoperoksidaz sistem, Tiyosiyanat, Süt kalitesi, Mikrobiyoloji*

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INTRODUCTION

Milk contains important nutrients, including proteins, carbonhydrates, unsaturated fats, minerals, and vitamins, hence it satisfies people's nutritional needs [1]. However, it is observed to be a food source, which likely exposes humans to major pathogenic microorganisms [2]. Some microorganisms present in the milk adversely affect the quality of milk and dairy products during process of collection, storage, and transportation [3]. This leads to food poisoning and causes economic losses [4]. Studies conducted in Turkey revealed that milk and dairy products are contaminated with enteric bacteria such as *Escherichia coli* [5]. Therefore, monitoring the bacterial load in milk is extremely important for maintaining the good quality of product and to ensure food safety and good human health.

In Turkey, the milk produced is of low microbiological quality, which is a serious issue. In the past decade, advanced production and processing technologies have been extensively used. However, studies related to the milk quality during its processing indicate that the microbiological quality of milk may be poor in Turkey and other countries [6,7].

Although, cold storage inhibits the growth of some microorganisms, like lactic acid (LA) bacteria, psychrotroph bacteria can cause souring of milk at low temperatures. Psychrotroph bacteria is a microflora with proteolytic and lipolytic enzyme activities and can grow dominantly well in cold conditions [8]. This situation leads to the growth of microorganisms, causing deterioration of the milk and dairy product.

Milk contains lactoperoxidase (LP) system a naturally occuring enzymatic system that inhibits the microbial growth in the milk. This system is effective against many gram-positive and gram-negative bacteria. Gram-negative bacteria are more sensitive to the lactoperoxidase system than the gram-positive ones [8]. The LP system consists of three major components, including lactoperoxidase enzyme, thiocyanate ion (SCN-) and hydrogen peroxide (H_2O_2). The LP system becomes completely active when these three components come together [9]. The enzyme in the presence of H_2O_2, catalyzes oxidation of thiocyanate (SCN-) to form an intermediate product hipothiocyanate, which exhibits bactericidal or bacteriostatic effect on the bacteria [10]. The formed intermediate product exerts antibacterial effect by oxidation of essential sulfydryl groups of the enzymes or proteins present in the bacteria. Although the milk proteins too have a small number of sulfydryl groups, however these are not oxidized by hypothiocyanate [3].

Depending both on the type of bacteria and animal characteristics, the lactoperoxidase enzyme is naturally present in the milk obtained from all the mammals [8]. Hydrogen peroxide is formed by lactic acid bacteria of the milk microflora [11]. The amount of thiocyanate may change depending on several factors such as species, genus and lactation period of the animals. Especially, the amounts of thiocyanate in milk depend on the feeds consumed by the dairy cows [12]. Nowadays, naturally-feeding husbandry produce milk with significantly less amounts of thiocyanate than the roughage-feeding cows. Thus, the decrease in the amounts of thiocyanate in milk, may cause insufficient activation of the lactoperoxidase system [13]. Therefore, several researchers suggested that LP system could be sufficiently activated by externally adding thiocyanate to the raw milk to improve its microbiological quality [1,8,14]. Even Food and Agriculture Organization (FAO) and World Health Organization (WHO) recommend the use of lacto-peroxidase system to control the microbiological quality of raw milk in some regions with poor hygienic standards [13].

The objective of this study was to investigate the effects of activated lactoperoxidase system on the microbiological quality of raw milk sample collected from dairy farms. We determined the effect of LP system only on few microorganisms such as mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast.

MATERIAL and METHODS

Sample of Raw Cow Milk

A total of 15 L of fresh raw cow milk was collected in the morning from dairy farms located in Çatalca, İstanbul. The collected stock was equally distributed in sterile sampling boxes in a thermobox at 4°C. The samples were brought to the laboratory on the same day and quickly subjected to the microbiological analysis.

Activation of the Lactoperoxidase System

One liter of raw cow milk was divided equally into two equal parts, each of 500 mL, under aseptic conditions. One part was used as a control, whereas the other part was subjected to the activation process. For activation of lactoperoxidase system, 20 mg/kg NaSCN (FlukaAnalytical 71938-250G, St. Louis, USA) and 20 mg/kg H_2O_2 (Merck 1.07210.1000, Darmstadt, Germany) were added to the experimental fraction and the samples were stirred thoroughly and further stored at 4°C for 12 h. The milk samples were then microbiologically examined for the presence of microflora including total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria (LAB) and yeast at different time intervals of 0, 3, 6, 9 and 12 h.

Microbiological Analysis

Each sample, 1 mL, was homogenized in 9 mL sterile physiological saline solution, pH 7.0 (Merck) according to

the Guidelines of ISO 6887-1 [15] by serial dilutions. Different microorganisms were grown on their specific selective media. Plate Count Agar (LAB149, Lancashire, UK) for total mesophilic aerobic bacteria [16] and psychrotroph bacteria [17], Pseudomonas Agar (Merck 1.07620.0500, Darmstadt, Germany) including C-F-C Selective Supplement (Merck 1.07627.0001, Darmstadt, Germany) for *Pseudomonas* spp.[18], Violet Red Bile Glucose Agar (LAB088, Lancashire, UK) for Enterobacteriaceae [19], Man-Rogosa-Sharpe Agar (MRS) (Merck 1.10660.0500, Darmstadt, Germany) for lactic acid bacteria [20] and Yeast Glucose Chloramphenicol Agar (Merck 1.16000.0500, Darmstadt, Germany) for yeast [21] were used. For growth of total mesophilic aerobic bacteria, psychrotroph bacteria, and lactic acid bacteria, 1 mL of each decimal serial dilutions was transferred onto the corresponding sterile plates, then appropriate media were poured into each plate and incubated at 30°C for 72 h, at 7°C for 5-7 d and at 37°C for 48 h, respectively. As for *Pseudomonas* spp., Enterobacteriaceae, and yeast, 0.1 mL of each dilutions were spread over the prepared plates and then incubated at 25°C for 48 h, at 37°C for 24 h and at 25°C for 5 d, respectively. After incubation, the grown colonies in the plates were counted and recorded as cfu/mL for each decimal serial dilution.

Statistical Analysis

All experiments were repeated thrice by conducting them on different days and the results were converted into logarithmic values. Statistical analyses were performed by SPSS ver. 22.0 (SPSS INC., USA). The comparison of the effects of lactoperoxidase system on microbiological quality in the experimental group was evaluated by one-way ANOVA ($P<0.05$). The statistical significance of differences between the group means was analyzed by Duncan's test.

RESULTS

The results in *Table 1* show the comparison between the bacterial count in raw cow milk sample in control and activated milk sample [treated with equal concentration of sodium thiocyanate and hydrogen peroxide (20:20 mg/kg)]. At 0 h, the initial counts of total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria, and yeast were found to be 7.10, 5.14, 6.42, 5.93, 6.92 and 4.31 log cfu/mL, respectively, in both samples. At the end of 3 h of cold storage of the activated raw milk, the log-reduction measurements for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria, and yeast were found to be 0.50, 2.09, 1.04, 0.97, 0.39, and 0.45 log cfu/mL, respectively. On comparison, the results for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast were statistically significant ($P<0.05$) and no significant change was observed in case of lactic acid bacteria.

DISCUSSION

In this study, we investigated and compared the microbiological quality of the nonactivated control and lactoperoxidase activated raw milk at different time intervals. We found that certain bacteria in LP activated milk were decreased in count as compared to the control raw milk.

Table 1. *Microbial counts in control and LP activated raw milk during cold storage (log cfu/mL)*
Tablo 1. *Soğukta muhafaza sırasında kontrol ve LP aktive edilmiş sütlerdeki mikroorganizma sayıları (log kob/mL)*

Microorganism	Group	Storage Periods (hour)				
		0.	3.	6.	9.	12.
TMAB	CON	7.10±0.23^{a}	7.03±0.21^{a}	6.94±0.16^{a}	7.00±0.22^{a}	7.14±0.19^{a}
	ACT	7.10±0.23^{a}	6.60±0.20^{b}	6.67±0.20^{b}	6.66±0.21^{b}	6.70±0.26^{b}
PSI	CON	5.14±0.13^{c}	5.28±0.15^{c}	5.34±0.12^{bc}	5.59±0.14^{ab}	5.78±0.15^{a}
	ACT	5.14±0.13^{a}	3.05±0.24^{c}	3.28±0.26^{c}	3.44±0.21^{bc}	3.71±0.26^{b}
PSE	CON	6.42±0.28^{a}	6.47±0.33^{a}	6.52±0.26^{a}	6.58±0.30^{a}	6.67±0.30^{a}
	ACT	6.42±0.28^{a}	5.38±0.47^{b}	5.34±0.30^{b}	5.33±0.26^{b}	5.42±0.23^{b}
ENT	CON	5.93±0.27^{a}	5.89±0.32^{a}	6.00±0.29^{a}	6.09±0.28^{a}	6.14±0.29^{a}
	ACT	5.93±0.27^{a}	4.96±0.24^{b}	4.84±0.23^{b}	4.86±0.23^{b}	4.80±0.20^{b}
LAB	CON	6.92±0.40^{a}	6.75±0.23^{a}	6.78±0.24^{a}	6.89±0.24^{a}	6.98±0.32^{a}
	ACT	6.92±0.40^{a}	6.53±0.20^{a}	6.39±0.29^{a}	6.66±0.17^{a}	6.45±0.33^{a}
Yeast	CON	4.31±0.19^{a}	4.23±0.19^{a}	4.43±0.16^{a}	4.34±0.22^{a}	4.42±0.24^{a}
	ACT	4.31±0.19^{a}	3.86±0.22^{b}	3.60±0.20^{b}	3.87±0.24^{b}	3.85±0.27^{b}

^{abc} *The differences between the means shown with same superscript in the same row is not significant ($P>0.05$);* ***TMAB:*** *Total Mesophilic Aerobic Bacteria;* ***PSI:*** *Psycrotrophic Bacteria;* ***PSE:*** *Pseudomonas spp.;* ***ENT:*** *Enterobacteriaceae;* ***LAB:*** *Lactic Acid Bacteria;* ***CON:*** *Control Group;* ***ACT:*** *LP Activated (Experimental) Group*

The bacteria, which can grow in mesophilic and aerobic conditions and can be saprophytic or pathogenic, are one of the most important indicators of the microbiological quality of milk and milk products [6]. Our study pointed that in the raw milk activated with LP system, which was kept in cold storage at 4°C, there was a change in the total number of mesophilic aerobic bacteria at the end of 3 h. The initial total mesophilic aerobic bacteria count (at 0 h) of 7.10 log cfu/mL was decreased to 0.43 log cfu/mL at the end of 3 h and this difference was statistically significant ($P<0.05$). This change remained constant until the end of 12 h of cold storage. In contrast, no change was observed in the total number of mesophilic aerobic bacteria in the control-raw milk (non-activated) at the end of 3 h, and it remained at an average value of 7.10 log cfu/mL. Further, there were no significant changes in the control group (non-activated) during the 12 h cold storage. Thus, our results suggested that external addition of thiocyanate increased activation of LP system in the raw milk, which reduced the microbial count and increased the quality of milk. Similar results have also been reported by other studies, where the activation of lactoperoxidase system significantly reduced the total count of mesophilic aerobic bacteria in raw milk [1,8,22]. One of these studies, added different quantities of thiocyanate and hydrogen peroxide to the raw milk and performed microbiological analysis at 24 h and reported reduction between 26% and 45% in all three activated groups. While another study performed the activation of LP system using raw cow milk at ambient temperature [23,24], and reported a reduction of 0.23 log cfu/mL in the activated group and an increase of 0.84 log cfu/mL in the control group at 7 h.

Cold storage of raw milk and dairy products is a prerequisite for the dairy industry. This is also done at farms and in processing plants to reduce the deterioration caused by mesophilic microorganisms. However, it cannot prevent the spoilage of raw milk by psychrotroph micro-organisms [25]. Our study obtained similar results for psychrotroph bacteria as were observed for mesophilic aerobic bacteria. We observed that their total counts in the LP activated raw milk were reduced nearly to 2 log cfu/mL in the first three h. Furthermore, same results were observed at 6 and 9 h and a tendency to rise in numbers was seen at 12 h of cold storage. No significant change was observed in the control-raw milk. The results indicate that activation of the lactoperoxidase system occurs within the first three h. A study reported that activation of the lactoperoxidase system in raw milk is quite effective against psychrotroph bacteria, since it increases the shelf life of cold stored milk for several days [26]. As reported by another study, the initial psychrotroph bacterial load in the raw cow milk was 5.5×10^3/mL, and in the activated group, it reduced to 2.6×10^6/mL on 6th day. While in the control group, the count remained the same until the end of the 2nd day [27]. The results of these studies indicate that activation of lactoperoxidase system showed both bactericidal and bacteriostatic effect on psychrotroph bacteria.

Pseudomonas species are the most common psychrotroph microorganisms that cause spoilage of raw milk. The most important features of these bacteria are their ability to grow in the cold refrigerator conditions [28]. Generally, *Pseudomonas* spp. are inhibited by the process of pasteurization. However, before the heat treatment, if they are present in high counts in the raw milk, they release heat resistant hydrolytic enzymes, which remain active and cause deterioration during storage of pasteurized product [29]. Especially, when *Pseudomonas* spp. produce a protease enzyme that breaks down the milk protein casein imparting a grayish color and bitter taste to the milk. Furthermore, the lipases produced by these bacteria hydrolyze milk fat to release low molecular weight fatty acids, which give bitter taste and sour and soapy flavor to raw milk [30]. In our study, during cold storage at 4°C the initial *Pseudomonas* spp. load in raw milk was noted as 6.42 log cfu/mL, while there was a slight increase in the control group, it reached 6.67 log cfu/mL at 12 h. Lactoperoxidase system activation caused 1.04 log cfu/mL reduction in *Pseudomonas* spp. count in milk within the first three h and later remained constant (with no increase) in the cold storage. According to a study, lactoperoxidase system activation shows both bactericidal and bacteriostatic effect on *Pseudomonas* spp. in milk [31]. In agreement with our study, Zapico *et al.*[32] reported that the raw goat milk after LP system activation showed 1.69 log cfu/mL reduction in *Pseudomonas* spp. count during the first 24 h. In a study by Saad *et al.*[33], after 2 h of the treatment, there was no reduction in *Pseudomonas* spp. count in the activated group. However, significant differences were observed when compared with the control group. Thus, our results differed from those of Saad *et al.*[33], since they reported that the activation of lactoperoxidase system has a bactericidal effect on *Pseudomonas* spp.

Enterobacteria virtually infect human and all animals as well as the environment all over the world. Within this family, *E. coli* is increasingly becoming a pre dominant form worldwide [34]. Enterobacteriaceae is a family of gram-negative, non-spore forming bacteria and is one of the most important bacterial group known to humankind [35]. The most common pathogens that belong to this family are the coliforms [36]. Coliforms are also known as hygiene indicators, which produce acid and gas and disrupt milk proteins by fermenting lactose to cause rapid deterioration of milk [37]. In our study, we examined the effect of activated lactoperoxidase system on the Enterobacteriaceae load in milk and compared it with the nonactivated raw milk. Initially, the number of Enterobacteriaceae was determined as 5.93 log cfu/mL and further no significant increase was observed in the control group during cold storage. While Enterobacteriaceae count in the lactoperoxidase system activated raw milk was reduced to 0.97 cfu/mL log in the first three h and this reduction was found to be statistically significant ($P<0.05$). During subsequent storage period, there was no significant change in the count. Similarly,

Erginkaya *et al.*[38] reported that the activation of the LP system caused a decrease in the Enterobacteriaceae load at 4℃. Likewise, Niguse and Seifu [24] also reported that the coliform count, in the activated milk sample was 1.73 log cfu/mL lower than in the control milk sample up to seven h of storage at ambient temperature.

Lactic acid bacteria play an important role in the development of desirable characteristics of fermented dairy products; and they are intentionally added to milk for production of these products [39]. However, they may also cause deterioration in the raw milk and certain dairy products [40]. They are particularly responsible for the usual souring of milk [41]. In our study, lactoperoxidase system activation did not show any significant impact on the lactic acid bacteria load *(Table 1)*. Odabaşı *et al.*[42], in their study regarding preparation of white cheese, reported that the LP system slows down the growth of lactic acid bacteria. Similarly, Ndambi *et al.*[43], reported that during incubation lactoperoxidase system activation reduced lactic acid formation by 12.50% in yogurt as compared to the control group. Thus indicating that LP system had bacteriostatic effect on the lactic acid bacteria. In our study, due to the short storage time, we could not observe the bacteriostatic effect of lactoperoxidase system on growth of these bacteria.

Yeasts are an essential component of the microflora of lactic acid production and important cause of spoilage of yogurt and fermented milk products, where the low pH provides a suitable environment for their growth [28]. High yeast load may indicate a lack of hygiene during the process of milking and storage [44]. We determined the effect of lactoperoxidase system on the yeast in the raw cow milk. During cold storage, initial yeast load was 4.31 log cfu/mL and no significant change was observed in the control group throughout the storage period. After three h, the lactoperoxidase system activated raw milk stored in cold showed a count of 3.86 log cfu/mL with 0.45 log reduction ($P<0.05$). Atamer *et al.*[45] reported that LP system has inhibitory effect on the yeast-mold growth. Another study on the raw sheep milk revealed that the yeast and mold load of 0.71 log cfu/mL in activated samples were low than control samples at 7°C at 6 h of activation [8].

Our study revealed that the Lactoperoxidase system activation led to a significant reduction in the number of aerobic mesophilic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast. This reduction occurred within the first three h. The length of this time depends on intermediate products formed as a result of oxidation of thiocyanate by the presence of equal concentration of hydrogen peroxide. The time may be extended by increasing the concentration of hydrogen peroxide and thiocyanate. However, this may slow down the activity of the starter culture in further processed products [42].

According to Jooyandeh *et al.*[46] lactoperoxidase system activation is more effective against gram negative bacteria than gram positive bacteria because gram positive bacterial cell wall is more resistant to hypothiocyanite ions.

In general, hydrogen peroxide can alone be used for the preservation of milk. However, in order to show the antibacterial effect, hydrogen peroxide should be incorporated up to 800 mg/kg in the raw milk. This amount is much higher than the amount used for activation of lactoperoxidase system [47]. Since we used very low amount, the reduction in bacterial load reported of our study cannot be correctly connected to hydrogen peroxide.

In summary, we found that external addition of thiocyanate and hydrogen peroxide to the raw cow milk to improve activation of the Lactoperoxidase system, a natural antimicrobial defense system, reduced the initial microbial load and slowed down the growth of aerobic mesophilic total bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast in the first three h after the treatment. In future, feeding the cow with thiocyanate rich feed can be tried, which in turn will increase the thiocyanate content of raw milk and will improve its microbiological quality.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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