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Effect of Lactoperoxidase System on Keeping Quality of Raw Cow's Milk in Thailand

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Abstract: In this study, the effect of the Lactoperoxidase (LP) system on antimicrobial activity, aerobic count, alcohol test and lactoperoxidase activity were determined in raw cow milk collected locally in Chiang Mai, Thailand, during summer season (March-April 2006). The active LP system was found to greatly increase the Keeping Quality (KQ) of all milk samples (both untreated and *Escherichia coli* treated samples). In addition, in the presence of the LP system, the decrease in microbial contamination was observed at the rate of 87% (untreated milk sample, treatment B) and 78% (*E. coli* treated milk sample, treatment C), respectively. Present results clearly demonstrate that the LP system can serve as an alternative method to control the microbial growth in cow milk.

Key words: Lactoperoxidase system, cow milk, *Escherichia coli*, microbial contamination

INTRODUCTION

The Lactoperoxidase (LP) system is widely known as a naturally antimicrobial system present in raw milk. The system has been shown to be active against both Gram-positive and Gram-negative microorganisms (Naidu, 2000; Marks *et al*., 2001). In brief, three components are required for the LP activation: the lactoperoxidase enzyme, thiocyanate and hydrogen peroxide. The LP enzyme is abundant in raw milk whereas thiocyanate and hydrogen peroxide are present at low concentrations. When activated, lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide generating several short-lived oxidation products in which two important products namely hypothiocyanite ($OSCN^-$) and hypothiocyanous acid (HOSCN), exhibit antibacterial activity (Kussendrager and van Hooijdonk, 2000).

In developed countries, the microbiological quality of raw milk is safeguarded during collection, storage and transportation via refrigeration. In contrast, this practice becomes an obstacle especially in rural areas of most developing countries due to limited facilities. The application of the LP-system has been proposed to be a useful procedure in preserving raw milk quality. Although the natural LP system in raw milk loses its effect within 2 h of the milking, its antimicrobial activity can be reactivated by addition of thiocyanate and hydrogen peroxide (IDF, 1994; Barrett *et al*., 1999). As a result, the shelf life of raw milk can be significantly prolonged by this means (FAO, 1999). In this study, we aim to evaluate the effect of the 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 is also examined.

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MATERIALS AND METHODS

Pretreatment of Milk Samples

Raw cow's milk samples were collected from the dairy farm of the Department of Animal Science, Faculty of Agriculture, Chiang Mai University between March and April 2006. Prior to any further experiment, the milk samples were warmed to 37°C for 15 min to activate the LP system immediately.

Inoculation of Milk Samples with *E. coli*

Milk samples were aseptically divided into four bottles of 200 mL each. Sample A was used as untreated raw milk control; sample B was subjected to activation of the LP system; whereas sample C and D were initially inoculated with *E. coli* ATCC 25922 (~10^6-10^7 cfu mL^{-1} and only sample C was subsequently introduced the LP system activation.

Activation of the LP System

The activation of the LP system (Sample B and C) was carried out by addition of 40 mg L^{-1} of sodium thiocyanate (Fluka, Switzerland) as a source of thiocyanate anion (SCN^-). After 1 min of thorough mixing, the samples were added with 30 mg L^{-1} of sodium percarbonate (Riedel-de Haën, Germany) as a source of hydrogen peroxide (IDF, 1988; Dajanta, 2003) and incubated at 37°C. The samples were then drawn from each milk sample every 2 h (from 0 to 8 h) to determine Total Viable Count (TVC) and LP activity whereas the alcohol test was determined every hour. The experiment was conducted in triplicate.

Alcohol Test

The milk samples were added with equal volumes of 75% alcohol (neutralized). The development of the milk clotting was noted and referred to the end of shelf life.

Determination of Total Viable Count

One milliliter of each milk sample was mixed with 9 mL of sterile 0.1% peptone water (Merck, Germany). Ten fold serial dilutions were then performed in 0.1% peptone water and 1 mL of appropriate dilutions were spread onto PCA plates (Merck, Germany) which were then incubated at 37°C for 48 h. The resulting colonies were counted and expressed as colony forming units per milliliter (cfu mL^{-1}) of sample.

Lactoperoxidase Activity Assay

The LP activity assay was determined using the modified IDF method (IDF, 1994) in which the ABTS (2,2'-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) was used as a substrate. The assay mixture consisted of 2 mL of ABTS source (2 mM ABTS, 0.2 mM H_2O_2, 0.1 M phosphate buffer pH 6.0) and 0.05 mL of diluted milk samples. Absorbance was measured at 412 nm after 2 min incubation using a spectrophotometer (Biomate 5, Unicam Co., Ltd., England) adjusted at 25°C. One unit of LP activity was defined as amount of the enzyme that catalyzes 1 μmol of substrate per minute under experimental conditions.

RESULTS AND DISCUSSION

Lactoperoxidase Activity

The activity of the LP enzyme was assayed every 2 h during the storage time (Table 1). Although there seemed to be some slightly fluctuations during the time tested, the LP activity of the four samples tended to decrease after 8 h of incubation.

Table 1: Lactoperoxidase activity in raw milk samples during incubation at 37°C for 8 h

Treatments	Lactoperoxidase activity (U mL^{-1}) analysis time (h)				
	Initial	2	4	6	8
Uninoculated raw milk					
Sample A (inactivated)	$14.01\pm1.27^{NS,a}$	$11.95\pm1.33^{NS,b}$	$14.37\pm0.51^{B,a}$	$11.20\pm0.24^{C,b}$	$8.54\pm0.50^{AB,c}$
Sample B (active LP)	$14.01\pm1.27^{NS,ab}$	$10.96\pm0.11^{NS,c}$	$16.11\pm0.58^{A,a}$	$12.69\pm1.17^{B,bc}$	$11.03\pm2.84^{A,c}$
***E. coli* inoculated raw milk**					
Sample C (active LP)	$14.01\pm1.27^{NS,ab}$	$11.13\pm0.30^{NS,c}$	$13.18\pm0.39^{C,b}$	$14.48\pm0.36^{A,a}$	$9.35\pm0.04^{AB,d}$
Sample D (inactivated)	$14.01\pm1.27^{NS,a}$	$10.93\pm0.21^{NS,b}$	$10.16\pm0.30^{D,bc}$	$9.63\pm0.46^{D,c}$	$7.79\pm0.20^{B,d}$

The values shown are mean±SD of three replicates. Means followed by the different capital and small letter(s) within the same column and row, respectively are significantly different, $p\leq 0.05$ (analyzed using Duncan's multiple range test), NS: Not significant ($p>0.05$) for any treatment

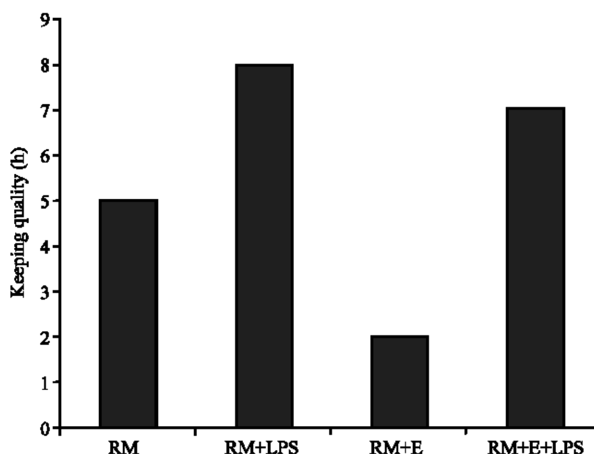

Fig. 1: Keeping quality (h) of milk samples incubated at 37°C: Raw milk control (RM), activated LP-system raw milk (RM+LPS), *E. coli* inoculated raw milk (RM+E) and *E. coli* inoculated raw milk and activated LP-system (RM+E+LPS)

Effect of the LP System on Keeping Quality

According to Barrett *et al*. (1999), the alcohol stability can be used as a good indicator of KQ due to its reliable and consistent results. As shown in Fig. 1, the KQ of untreated raw cow milk (sample A) was 5 h and in the presence of *E. coli* (sample D), the KQ was decreased to 2 h. In contrast, in samples B and C where the LP system was activated, the KQ values became higher to 8 and 7 h, respectively. Our data have confirmed that introduction of the LP system can extend the KQ parameters similar to those reported by Marks *et al*. (2001).

Effect of the LP System on the Growth and Survival of Microorganisms in Raw Milk

To further explore the possible use of the LP system whether it could serve as an alternative to cooling in Thailand, we designed the microbial-contaminated experiment in which the bacterium *E. coli* was used as a spoilage model. Milk samples, after milking, are rich in nutrients and thus tend to spoil easily by indigenous microorganisms under inappropriate conditions. Therefore, apart from normal milk samples, we also inoculated *E. coli* in the milk samples in order to accelerate the spoiling rate of the milk. The effect of the LP system on microbial population was then monitored between 0 and 8 h for both inoculated and uninoculated milk samples. As shown in Fig. 2, initial total viable counts of samples A and B (uninoculated samples) were 9.12×10^4 cfu mL^{-1} whereas those of samples C and D including the inoculation of *E. coli* at the beginning were 1.74×10^7 cfu mL^{-1}. Under storage

Table 2: Microbial cell count (cfu mL^{-1}) of raw milk samples after incubation at 37°C for 8 h

Treatments	Initial	Inactive LP system (8 h)	Active LP system (8 h)	Percent reduction[a]
Uninoculated raw milk	9.12×10^4	4.47×10^8	5.62×10^7	87
E. coli inoculated raw milk	1.74×10^7	7.76×10^8	1.74×10^8	78

[a]: Percent reduction in viable count was calculated as follows: 100 × (viable count in the control milk at 8 h-viable count in the LP activated milk at 8 h)/(viable count in the control milk at 8 h)

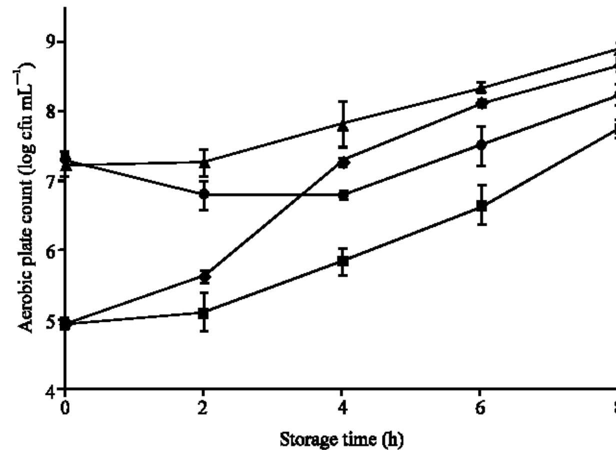

Fig. 2: Total viable count of microorganisms in the active and inactive LP system raw milk samples incubated at 37°C: Raw milk control (◆), activated LP-system raw milk (■), *E. coli* inoculated raw milk (▲) and *E. coli* inoculated raw milk and activated LP-system (●)

condition (from 0-8 h), although the total viable counts of the four milk samples reached their peak at the end of the experiment, their pattern during the storage was slightly different. In addition, the microbial count of sample D which was inoculated with *E. coli* was much higher throughout the period as expected. On the other hand, the microbial count of samples B and C where the LP system was activated tended to be lower from the beginning to the end of the experiment (Fig. 2). Interestingly, it was clearly observed that, when the LP system was introduced (samples B and C), the microbial counts were lower than those of the counterpart samples (sample A and D) throughout the experiment (Table 2).

The LP system has been shown to inhibit various Gram-positive and Gram-negative bacteria (reviewed in Naidu, 2000). Its activity has shown to be either bacteriostatic or bactericidal effect on sensitive strains depending on environmental conditions and bacterial species. According to El-Agamy *et al*. (1992), the LP system is bactericidal to Gram-negative bacteria and bacteriostatic to Gram-positive bacteria. Farrag *et al*. (1992) reported the bactericidal effect against *E. coli* O157:H7 at 4°C, while at 30°C the growth of pathogen was only inhibited for 12 h. In addition, it has been shown that the LP system could inhibit the growth of *E. coli* and *L. innocua* at 20°C for 24 h but did not cause any inactivation after this period (Garcia-Graells *et al*., 2000).

CONCLUSION

This study, part of a programme to improve the KQ of raw cow's milk quality in Thailand, clearly showed that the LP system exhibited antimicrobial effect in raw cow milk. The active LP system decreases the microbial population in raw milk samples (both untreated and *E. coli* treated experiments) under storage conditions determined. Present results also show that the LP system

affects the KQ of the raw milk and thus can be used to extend its shelf life. It is therefore possible to use the LP system as an alternative means to preserve raw milk quality during transportation or in areas with lack of cooling facility.

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