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*Published in:*  
Open Journal of Animal Sciences

*Link to article, DOI:*  
[10.4236/ojas.2020.103024](https://doi.org/10.4236/ojas.2020.103024)

*Publication date:*  
2020

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Dashe, D., Hansen, E. B., Kurtu, M. Y., Berhe, T., Eshetu, M., Hailu, Y., Waktola, A., & Shegaw, A. (2020). Preservation of Raw Camel Milk by Lactoperoxidase System Using Hydrogen Peroxide Producing Lactic Acid Bacteria. *Open Journal of Animal Sciences*, 10(3), 387-401. <https://doi.org/10.4236/ojas.2020.103024>

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# Preservation of Raw Camel Milk by Lactoperoxidase System Using Hydrogen Peroxide Producing Lactic Acid Bacteria

Dakalo Dashe<sup>1</sup>, Egon Bech Hansen<sup>2</sup>, Mohammed Yusuf Kurtu<sup>1</sup>, Tesfemariam Berhe<sup>1</sup>, Mitiku Eshetu<sup>1</sup>, Yonas Hailu<sup>1</sup>, Amsalu Waktola<sup>1</sup>, Adane Shegaw<sup>1</sup>

<sup>1</sup>School of Animal and Range Sciences, Haramaya University, Dire Dawa, Ethiopia

<sup>2</sup>Division for Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

Email: mitikuguya@yahoo.com, dfdaki@gmail.com

**How to cite this paper:** Dashe, D., Hansen, E.B., Kurtu, M.Y., Berhe, T., Eshetu, M., Hailu, Y., Waktola, A. and Shegaw, A. (2020) Preservation of Raw Camel Milk by Lactoperoxidase System Using Hydrogen Peroxide Producing Lactic Acid Bacteria. *Open Journal of Animal Sciences*, 10, 387-401. <https://doi.org/10.4236/ojas.2020.103024>

**Received:** April 10, 2020

**Accepted:** June 8, 2020

**Published:** June 11, 2020

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## Abstract

This study was conducted to investigate the effect of lactic acid bacteria (LAB) activated lactoperoxidase system (LPs) on keeping quality of raw camel milk at room temperature. Camel milk samples were collected from Errer valley, Babile district of eastern Ethiopia. The level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for activation of LPs was optimized using different levels of exogenous H<sub>2</sub>O<sub>2</sub>. Strains of LAB (*Lactococcus lactis* 22333, *Weissella confusa* 22308, *W. confusa* 22282, *W. confusa* 22296, *S. Infatarius* 22279 and *S. lutetiensis* 22319) with H<sub>2</sub>O<sub>2</sub> producing properties were evaluated, and *W. confusa* 22282 was selected as the best strain to produce H<sub>2</sub>O<sub>2</sub>. Storage stability of the milk samples was evaluated through the acidification curves, titratable acidity (TA), total bacterial count (TBC) and coliform counts (CC) at storage times of 0, 6, 12, 18, 24 and 48 hours. The LP activity and the inhibitory effect of activated LPs were evaluated by growing *E. coli* in pasteurized and boiled camel milk samples as contaminating agent. Results indicated that the *W. confusa* 22282 activated LPs generally showed significantly ( $P < 0.05$ ) slower rates of acidification, lactic acid production and lower TBC and CC during the storage time compared to the non-activated sample. The H<sub>2</sub>O<sub>2</sub> producing LAB and exogenous H<sub>2</sub>O<sub>2</sub> activated LPs in pasteurized camel milk significantly reduced the growth of *E. coli* population compared to non-activated pasteurized milk. Overall, the result of acid production and microbial analysis indicated that the activation of LPs by H<sub>2</sub>O<sub>2</sub> producing LAB (*i.e.* *W. confusa* 22282) maintained the storage stability of raw camel milk. Therefore, it can be concluded that the activation of LPs by biological method using H<sub>2</sub>O<sub>2</sub> producing LAB can substitute the chemical activation method of LPs in camel milk.

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## Keywords

Camel Milk, Lactoperoxidase System, Lactic Acid Bacteria, Preservation

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### 1. Introduction

Camels are important source of livelihood for millions of people living in the arid and semi-arid areas of many parts of the world, providing food, cash income and transport and have significant cultural values to the pastoral communities [1]. Camels are mainly kept for milk production and can produce milk for a longer period of time even during dry season [2] [3]. Camel milk has been traditionally consumed raw or in the form of fermented milk at household level for years with only limited amount being sold [4]. The changes in life styles such as fast-growing population, intensified growth of small towns and commercialization of pastoral products in the lowlands increased the demand for camel milk [5]. This condition opened market opportunities for the pastoral communities and peri-urban milk producers [6] [7].

However, camel milk is usually transported without cooling facilities for long distances to reach to the consumers or processing point which increased concerns over the microbiological quality of the milk [8]. Microbial growth is a major concern of public health as some can potentially cause milk-borne illness [9]. Milk with high levels of microbial contamination is not safe for direct consumption or it cannot be processed into different dairy products [10]. Therefore, prevention of quality loss through inhibition of bacterial growth during collection, transportation and storage of raw milk is of paramount importance. Several preservation techniques including cooling, heat treatment, acidification and addition of chemicals have been used at different levels from production to processing to prevent growth of spoilage and pathogenic microorganisms in foods [11]. Cooling of fresh milk during collection and transportation is widely used in most parts of the world especially in the developed countries [12].

In the areas where cooling facilities are unavailable to preserve raw milk due to economic and technical reasons, the International Dairy Federation [13] and Joint Food and Agricultural and World Health Organizations [14] had developed a method to increase the storage stability of the milk. The method is based on activating natural antibacterial system in raw milk which consists of lactoperoxidase (LP), thiocyanate ( $\text{SCN}^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The LPs are commonly activated by exogenously increasing the concentrations of the thiocyanate and  $\text{H}_2\text{O}_2$  [15]. However, consumer awareness and concern regarding chemical additives and the demand for safe foods have led to find alternatives in food preservation [16]. In this regard, an emerging preservation technique is demanded via activating the LPs by lactic acid bacteria (LAB). Lactic acid bacteria are capable of producing several metabolites including organic acids,  $\text{H}_2\text{O}_2$  and bacteriocins, which have antagonistic effect to a wide range of microorgan-

isms [17].

Hydrogen peroxide producing LAB was reported to inhibit growth of spoilage and pathogenic microorganisms [18]. This effect, however, might be due to the hydrogen peroxide produced or by the activated LPs from the production of  $H_2O_2$  in the milk. Previous work [19] indicated some strains of LAB (*Lactococcus lactis* 22333, *W. confusa* 22308, *W. Confusa* 22282, *W. confusa* 22296, *S. Infatarius* 22279 and *S. lutetiensis* 22319) isolated from camel milk can produce  $H_2O_2$ . However, the effect of these strains on the LPs activation properties is not evaluated. Besides, there is no information on the use of the  $H_2O_2$  producing LAB to activate the LPs and extend the keeping quality of raw camel milk. Therefore, this study was conducted to evaluate the effect of LAB activated LPs on the storage stability of raw camel milk at ambient temperature.

## 2. Materials and Methods

### 2.1. Collection of Milk Samples

The milk samples were collected from Errer valley, Babile District of eastern Ethiopia, about 30 km from Harar city. Errer valley is located at 9° 14'N latitude and 42° 14'E longitude at an altitude of 1300 - 1600 m.a.s.l. The milk was collected in clean and sterilized plastic containers from four households of five lactating camels at different parities and stage of lactations. The samples were pooled and packed under icebox and transported to Haramaya University Dairy Technology Laboratory. Milk samples were collected three times for each experiment and analysis was done in duplicates. The milk samples to be preserved with LPs were activated within 2 hrs of collection, as the indigenous antimicrobial activity in the freshly drawn milk is usually used up within 2 - 3 hrs due to suboptimal levels of the thiocyanate ion and hydrogen peroxide in the milk according to Codex Alimentarius Commission.

### 2.2. Determination of Thiocyanate Concentration

The thiocyanate concentration naturally present in camel milk samples was determined by spectrophotometer (3605, Jenway) at an absorbance of 460 nm, after deproteinisation of the milk with trichloroacetic acid (TCA) (Sigma-Aldrich T6399) and addition of ferric nitrate to form a ferric complex (orange to orange-red). Four millilitres (4.0 ml) of milk was mixed with 2.0 ml of 20% TCA solution. The mixture was mixed well and allowed to stand for 30 minutes. The solution was filtered through a filter paper (Whatman No. 40). One and half (1.5 ml) of the clear filtrate was then mixed with 1.5 ml of the ferric nitrate reagent and the absorbance was measured at 460 nm using spectrophotometer. The thiocyanate concentration was calculated from a standard curve prepared using known concentrations of sodium thiocyanate (Alfa Aesar 33388) [13].

#### 2.2.1. Growth of Lactic Acid Bacteria and Quantification of Hydrogen Peroxide

Strains of LAB producing  $H_2O_2$  (*Lactococcus lactis* 22333, *W. confusa* 22308, *W.*

*confusa* 22282, *W. confusa* 22296, *S. infatarius* 22279, *S. lutetiensis* 22319) were obtained from Technical University of Denmark (DTU), Copenhagen. The strains were originally isolated from camel milk from Babile area of eastern Ethiopia and were characterized by their H<sub>2</sub>O<sub>2</sub> production properties [19]. The strains were maintained on de Mann Rogosa Sharpe (MRS) agar and allowed to grow on Prussian blue (PB) agar, in MRS broth (Sigma-Aldrich 6966) and in pasteurized camel milk for detection and quantification of the H<sub>2</sub>O<sub>2</sub> produced.

The test organisms previously grown on MRS agar were also inoculated into MRS broth at room temperature for 72 hours and centrifuged (Sigma® 3-30KS) at 10,000 rpm for 15 minutes at 4°C. Proteinase K enzyme (Sigma-Aldrich P6556) (5 mg/ml) was added to cell free extract solution to exclude the antimicrobial effect of bacteriocins, and pH was adjusted to 7.0 by means of 0.1N NaOH to reduce the effect organic acids. The supernatant was filtered through 0.2 mm pore size cellulose acetate filter. Twenty-five millilitres (25 ml) of supernatant of broth cultures of the test organisms was measured into a 100 ml flask to which 25 ml of dilute H<sub>2</sub>SO<sub>4</sub> was added. This solution was then titrated with 0.1N Potassium permanganate (KMnO<sub>4</sub>). Each millilitre of 0.1 N KMnO<sub>4</sub> used was assumed to be equivalent to 1.701 mg of H<sub>2</sub>O<sub>2</sub>. The decolorization of the sample was regarded as the end point. The volume of H<sub>2</sub>O<sub>2</sub> produced was calculated according to AOAC [20].

$$\%H_2O_2 = \frac{\text{ml KMnO}_4 \times N \text{ KMnO}_4 \times \text{M.E}}{\text{ml H}_2\text{SO}_4 \times \text{Volume of sample}} \times 100$$

where: KMnO<sub>4</sub> = volume of KMnO<sub>4</sub> used (ml), N KMnO<sub>4</sub> = concentration of KMnO<sub>4</sub> used (Normality), M.E = equivalence factor, ml H<sub>2</sub>SO<sub>4</sub> = volume of H<sub>2</sub>SO<sub>4</sub> used.

### 2.2.2. Preparation of Inoculum and Determination of Acidification Curves

Fresh camel milk was divided into glass containers of 30 ml and boiled for 30 minutes at 90°C. The samples were cooled to room temperature and inoculated with single colonies of LAB from agar plates. The bacterial cultures were incubated at 30°C for 18 hrs and the mother culture was sealed and frozen at -20°C until used [19].

Fresh camel milk samples of 200 ml were pasteurized in autoclavable bottles at 64°C for 30 min. The samples were cooled to room temperature and inoculated with 1% of the mother culture. The acidification curves were followed using *iCinac* (Alliance Instruments, Frepillon, France). Calibrated and disinfected *iCinac* (pH) probes were inserted into the milk samples to ferment in a water bath at room temperature for 48 hours while the instrument continuously measures pH for every minute [19].

### 2.3. Effect of Lactoperoxidase System Activation on the Storage Stability of Camel Milk

An optimization experiment for the level of exogenous H<sub>2</sub>O<sub>2</sub> to activate LPs was

conducted using TBC and titratable acidity, considering 30 ppm to be used as a positive control in this experiment. *Weissella confusa* 22282 was selected for activation of the LPs due to its better H<sub>2</sub>O<sub>2</sub> production in MRS broth and reduction in acidification rate in pasteurized camel milk. Mother culture of the strain was prepared by inoculating the strain into MRS broth for 72 hours at 30°C and activating culture was prepared by growing the culture from the MRS broth in skim milk at a rate of 100 mg/L at 22°C for 16 hours [21]. The culture was inoculated into raw camel milk at rate of 1% and shelf life of the milk samples was evaluated through the pH (acidification curves), TA, TBC and CC at storage times of 0, 6, 12, 18, 24 and 48 hours. Raw milk with no addition and 30 ppm H<sub>2</sub>O<sub>2</sub> were used as negative and positive controls respectively (Table 1).

The TBC was done by pour plate method using standard plate count (SPC) agar (Sigma-Aldrich 70152). Plates with colonies ranging from 30 - 300 were counted and expressed as colony forming units per millilitre (cfu/ml) according to IDF [22]. The titratable acidity was determined by titrating the milk sample with 0.1 N NaOH (Himedia MB09) using a phenolphthalein indicator to an end-point of faint pink colour [23]. Coliform count was done by pour plate method using violet red bile agar (VRBA) (Sigma-Aldrich 70188). Plates with 15 to 150 cfu/mL were used for determining total coliform counts [22].

#### 2.4. Growth of *Escherichia coli* in Lactoperoxidase System Activated Camel Milk

The activity of lactoperoxidase in the milk and the effect of activated LPs on *Escherichia coli* were evaluated by growing the bacteria (*E. coli* ATCC 25922) in pasteurized and boiled camel milk samples. *Weissella confusa* 22282 (1%, V/V) and exogenous H<sub>2</sub>O<sub>2</sub> (30 ppm) were used as a source of H<sub>2</sub>O<sub>2</sub> to activate the LPs in both pasteurized and boiled milk samples (Table 2). Changes in *E. coli* population

**Table 1.** Effect of LPs activation on the storage stability of camel milk.

Treatments	Descriptions	Storage time (h)
T <sub>1</sub>	Raw milk	0, 6, 12, 18, 24 and 48
T <sub>2</sub>	Raw milk + <i>W. confusa</i> 22282 culture (1%, V/V)	0, 6, 12, 18, 24 and 48
T <sub>3</sub>	Raw milk + 30 ppm H <sub>2</sub> O <sub>2</sub>	0, 6, 12, 18, 24 and 48

**Table 2.** Lactoperoxidase activity and effect of activation of LPs on *E. coli* in camel milk.

Treatments	Description	Storage time (h)
T <sub>1</sub>	Pasteurized milk	0, 6, 12, 18, 24 and 48
T <sub>2</sub>	Pasteurized milk + <i>W. confusa</i> 22282 culture (1%, V/V)	0, 6, 12, 18, 24 and 48
T <sub>3</sub>	Pasteurized milk + 30 ppm H <sub>2</sub> O <sub>2</sub>	0, 6, 12, 18, 24 and 48
T <sub>4</sub>	Boiled milk	0, 6, 12, 18, 24 and 48
T <sub>5</sub>	Boiled milk + <i>W. confusa</i> 22282 culture (1%, V/V)	0, 6, 12, 18, 24 and 48
T <sub>6</sub>	Boiled milk + 30 ppm H <sub>2</sub> O <sub>2</sub>	0, 6, 12, 18, 24 and 48

were evaluated at storage times of 0, 6, 12, 18, 24 and 48 hours at room temperature.

Temperatures of 63°C/30 minutes and 80°C/15 seconds were used for pasteurizing and boiling the milk samples, respectively. The *E. coli* (ATCC 25922) culture were obtained from Haramaya University Pathology Laboratory and maintained on MacConkey agar plates at 32°C for 24 hours. Working cultures was prepared by transferring a single colony of *E. coli* from MacConkey agar into sterile Tryptone Soy Broth (TSB) (Sisco 24392 (TM 018)) to incubate for 24 hours at 37°C. Contaminating inoculum of the *E. coli* was prepared by transferring 0.5 ml of the working culture into 100 ml sterile TSB for 24 hours at 37°C [21]. The contaminating culture was then aseptically added to all the test samples at a rate 0.25% [24]. The respective test samples were then inoculated with 1% (V/V) *W. confusa* 22282 cultures and 30 ppm H<sub>2</sub>O<sub>2</sub> in both pasteurized and boiled milk samples and left at room temperature for the period of 48 hours (Table 2). Cultures from the milk samples were plated on MacConkey agar and incubated at 32°C for 24 hours [25].

## 2.5. Statistical Analysis

General linear model (GLM) procedure of SAS version 9.0 was employed to determine the significance between treatment means at a particular storage period. Mean separations were done using least significant difference (LSD) for variables whose F values were significantly different. Significant differences were calculated at 5% significance level. Descriptive statistics was used to calculate means and standard deviations of chemical compositions and amount of H<sub>2</sub>O<sub>2</sub> produced.

## 3. Results and Discussion

### 3.1. Thiocyanate Concentration of Camel Milk

The natural thiocyanate content in the present study was 22.34 ± 5.11 mg/L that falls within the ranges reported earlier (9.74 to 32.9 mg/L) [26] and (9.7 to 36.4 mg/L) [27] and higher than the level found (6.04 mg/L) [28] from Erer valley of eastern Ethiopia. Natural thiocyanate content of 7.38 was found from cow milk in Kombolchadistrict, eastern Hararghe of Ethiopia [29].

The higher thiocyanate content in the present study compared to the finding from the same area [28] might be due to differences in analytical measurements, feeding system and environmental conditions. Several factors could affect the thiocyanate concentration of milk such as age of the animal, health of the animal, species of animal, breed, lactation stage and nutritional condition among which the kind of feed supplied plays a major role [30]. Thiocyanate content might also vary among season of the year where the level in summer was higher than the thiocyanate concentration in winter [31]. Camels in the Erer valley of Babile area spend during the day outdoors browsing different types of plants including herbaceous plants, shrubs, shoots, cacti and different types of acacia



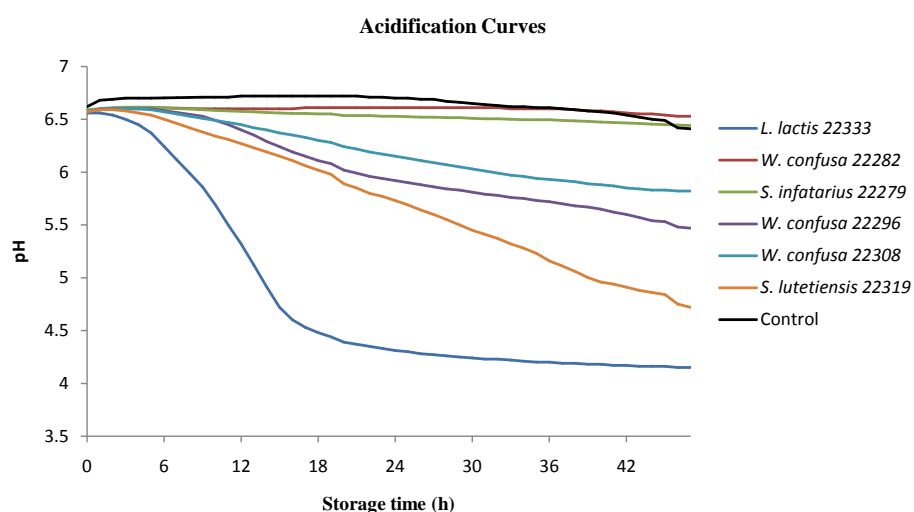
trees. Acacia trees and shrubs are expected to have high contents of cyanogenic glycosides which are a precursor of thiocyanate in plants [32].

The SCN value in the current study is higher than the concentration (15 ppm) required for the activation of the LPs according to Codex Alimentarius Commission [33]. The present finding is therefore showed that LPs in camel milk can be activated by natural SCN content in the milk with the addition of only desired amount of  $H_2O_2$  [27].

### 3.2. Production of Hydrogen Peroxide by Lactic Acid Bacteria

The result of the acidification for the  $H_2O_2$  producing strains of LAB indicated that *W. confusa* 22282 and *S. infantarius* 22279 are slow acidifying strains while *L. lactis* 22333 showed fast acidification in pasteurized camel milk at room temperature. *L. lactis* 22333 attained a pH of 4.3 at around 24 hours of storage while the pH in the *W. confusa* 22282 and *S. infantarius* 22279 treated samples shown no change (pH > 6.4) until the end of the storage period. The control treatment (pasteurized milk without LAB) started to drop at about 32 hours of storage and become below pH 6.4 at the end of the storage (Figure 1). This indicated that camel milk treated with *W. confusa* 22282 and *S. infantarius* 22279 had better stability compared to the control samples.

Similarly, the result of the  $H_2O_2$  quantification by titration showed that *W. confusa* 22282 produced significantly ( $P < 0.05$ ) the highest amount of  $H_2O_2$  ( $302.10 \pm 20.55$ ) in the MRS broth. *Lactococcus lactis* 22333 produced significantly ( $P < 0.05$ ) the least amount of  $H_2O_2$  ( $84.37 \pm 24.53$ ) in MRS broth (Table 3). All the strains except *L. lactis* 22333 were observed to grow yielding a deep blue colour around the colonies on PB agar due to reaction of  $H_2O_2$  to hexacyanoferrate (III) and iron (III) [34]. The overall results of  $H_2O_2$  indicated that *W. confusa* 22282 was the promising strain to use for activation of the antimicrobial system in raw camel milk.



**Figure 1.** Acidification curves for  $H_2O_2$  producing LAB strains in pasteurized camel milk ( $21^\circ C - 23^\circ C$ ).



**Table 3.** Production of H<sub>2</sub>O<sub>2</sub> by LAB in MRS broth (Mean ± SD).

Strains of LAB	Concentration of H <sub>2</sub> O <sub>2</sub> (mg/L)
<i>L. lactis</i> 22333	84.37 ± 24.53 <sup>e</sup>
<i>W. confusa</i> 22382	302.10 ± 20.55 <sup>a</sup>
<i>W. confusa</i> 22296	260.82 ± 25.29 <sup>b</sup>
<i>W. confusa</i> 22308	220.45 ± 19.63 <sup>c</sup>
<i>S. lutetiensis</i> 22319	174.64 ± 13.08 <sup>d</sup>
<i>S. infatarius</i> 22279	255.38 ± 12.86 <sup>cb</sup>

The production of H<sub>2</sub>O<sub>2</sub> by LAB in MRS broth in the current study is higher than the results reported by earlier researchers [35] [36]. Hydrogen peroxide yield of 0.4279 mg/L in MRS broth from *L. Lactis* [35], and *Leuconostoc mesenteroides* produced higher quantity of H<sub>2</sub>O<sub>2</sub> (24 mg/L) in normal MRS broth at 30 °C for 48 hours of incubation period [36]. However, higher concentration of 350 mg/L of H<sub>2</sub>O<sub>2</sub> from *L. lactis subsp. lactis* suspended in 0.5% (w/v) glucose plus 0.5% (w/v) lactate (pH 7.0) and incubated for 5 hours at 37 °C under aeration was also reported [37].

### 3.3. Effect of Activation of LPs by *Weissella confusa* 22282 on Storage Stability of Camel Milk

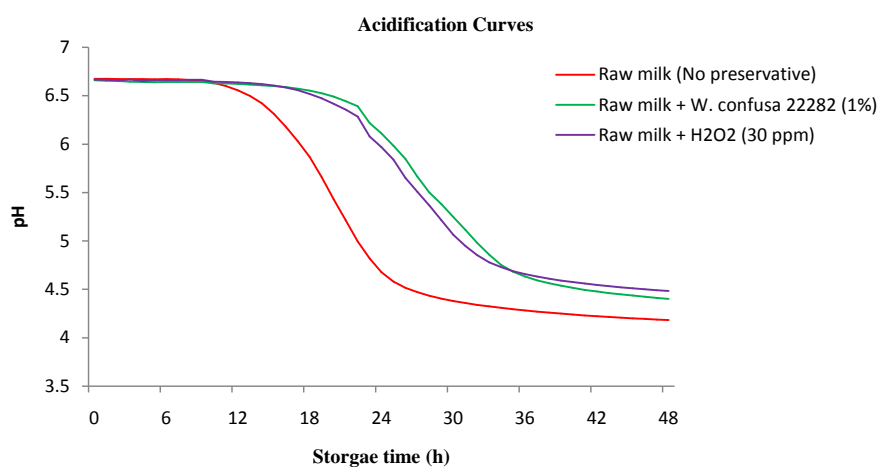
The activation of LPs by *W. confusa* 22282 and exogenous H<sub>2</sub>O<sub>2</sub> in raw camel milk significantly (P < 0.05) reduced the rate of acidification, TBC and CC. The values of pH (Figure 2) and lactic acid percentage (Table 4) in the activated samples for 18 hours of storage were within the acceptable level of pH and lactic acid of fresh camel milk, respectively. This could be due to retarded microbial growth as a result of antimicrobial properties of the LPs in activated samples. Decrease in lactic acid production was observed in LPs activated camel milk samples [27] [28] and other finding reported that there is less production of lactic acid in the LPs activated milk samples compared to the control sample because of the inhibitory nature of LPs [38].

The TBC and CC were significantly (P < 0.05) reduced in the treated samples compared to the non-activated control sample at 12, 18, 24 and 48 hours of storage. The TBC count in the LAB activated and H<sub>2</sub>O<sub>2</sub> activated LPs samples were decreased by 1.61 and 1.66 log units, respectively compared to the non-activated sample at 18 hours of storage. On the other hand, TBC in the exogenous H<sub>2</sub>O<sub>2</sub> activated LPs decreased below the initial value for up to 12 hours and then slightly increased toward the end of storage period. The LAB activated LPs sample shown nearly constant rate in bacterial reduction throughout the storage period compared to the H<sub>2</sub>O<sub>2</sub> activated milk indicating that LAB can constantly produce H<sub>2</sub>O<sub>2</sub> and other inhibitory components which have antagonistic effect on the microbial population. This finding can be supported by other findings [39] that the activation of the LPs may induce a longer-lasting bacteriostatic

**Table 4.** Effect of activation method of LPs on lactic acid development (%), TBC and CC ( $\log_{10}$  cfu mL<sup>-1</sup>) (Mean  $\pm$  SD) in raw camel milk.

Treatments	Storage time (h)					
	Initial	6	12	18	24	48
<b>Lactic acid</b>						
T <sub>1</sub>	0.12 $\pm$ 0.01 <sup>a</sup>	0.14 $\pm$ 0.01 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>a</sup>	0.22 $\pm$ 0.01 <sup>a</sup>	0.34 $\pm$ 0.02 <sup>a</sup>	0.63 $\pm$ 0.03 <sup>a</sup>
T <sub>2</sub>	0.12 $\pm$ 0.01 <sup>a</sup>	0.12 $\pm$ 0.01 <sup>b</sup>	0.14 $\pm$ 0.01 <sup>b</sup>	0.16 $\pm$ 0.01 <sup>b</sup>	0.20 $\pm$ 0.01 <sup>b</sup>	0.41 $\pm$ 0.04 <sup>b</sup>
T <sub>3</sub>	0.12 $\pm$ 0.01 <sup>a</sup>	0.12 $\pm$ 0.01 <sup>b</sup>	0.13 $\pm$ 0.01 <sup>b</sup>	0.15 $\pm$ 0.00 <sup>b</sup>	0.21 $\pm$ 0.01 <sup>b</sup>	0.48 $\pm$ 0.02 <sup>c</sup>
<b>TBC</b>						
T <sub>1</sub>	5.12 $\pm$ 0.48 <sup>a</sup>	5.58 $\pm$ 0.48 <sup>a</sup>	6.48 $\pm$ 0.59 <sup>a</sup>	7.29 $\pm$ 0.80 <sup>a</sup>	8.42 $\pm$ 0.72 <sup>a</sup>	9.67 $\pm$ 0.04 <sup>a</sup>
T <sub>2</sub>	5.12 $\pm$ 0.48 <sup>a</sup>	5.17 $\pm$ 0.57 <sup>a</sup>	5.23 $\pm$ 0.62 <sup>b</sup>	5.68 $\pm$ 0.61 <sup>b</sup>	6.23 $\pm$ 0.58 <sup>b</sup>	7.42 $\pm$ 0.22 <sup>b</sup>
T <sub>3</sub>	5.12 $\pm$ 0.48 <sup>a</sup>	5.06 $\pm$ 0.50 <sup>a</sup>	5.10 $\pm$ 0.50 <sup>b</sup>	5.63 $\pm$ 0.57 <sup>b</sup>	6.53 $\pm$ 0.56 <sup>b</sup>	7.87 $\pm$ 0.55 <sup>b</sup>
<b>CC</b>						
T <sub>1</sub>	4.39 $\pm$ 0.17 <sup>a</sup>	4.87 $\pm$ 0.49 <sup>a</sup>	5.52 $\pm$ 0.15 <sup>a</sup>	6.18 $\pm$ 0.53 <sup>a</sup>	7.08 $\pm$ 0.51 <sup>a</sup>	8.22 $\pm$ 0.59 <sup>a</sup>
T <sub>2</sub>	4.39 $\pm$ 0.17 <sup>a</sup>	4.40 $\pm$ 0.15 <sup>a</sup>	4.66 $\pm$ 0.49 <sup>b</sup>	5.09 $\pm$ 0.54 <sup>b</sup>	5.78 $\pm$ 0.58 <sup>b</sup>	6.78 $\pm$ 0.34 <sup>b</sup>
T <sub>3</sub>	4.39 $\pm$ 0.17 <sup>a</sup>	4.37 $\pm$ 0.20 <sup>a</sup>	4.53 $\pm$ 0.13 <sup>b</sup>	5.21 $\pm$ 0.19 <sup>b</sup>	6.06 $\pm$ 0.35 <sup>b</sup>	7.26 $\pm$ 0.56 <sup>ba</sup>

T<sub>1</sub> = Raw camel milk (without preservative), T<sub>2</sub> = Raw camel milk + *W. confusa* 22282 culture, T<sub>3</sub> = Raw camel milk + H<sub>2</sub>O<sub>2</sub>. Means bearing different superscript letters within the same column are significantly different ( $P < 0.05$ ).

**Figure 2.** Acidification curves for *W. confusa* 22282 and H<sub>2</sub>O<sub>2</sub> activated LPs and non-activated raw camel milk at room temperature.

effect due to the presence of higher levels of other indigenous antimicrobial components including the H<sub>2</sub>O<sub>2</sub>. It was reported that activation of LPs in camel milk decreased the multiplication of total bacteria for more than 12 hours of storage [40]. Other reports indicated that the activated LPs in milk had bacteriostatic effect against a mixed raw milk flora dominated by mesophilic bacteria [14].

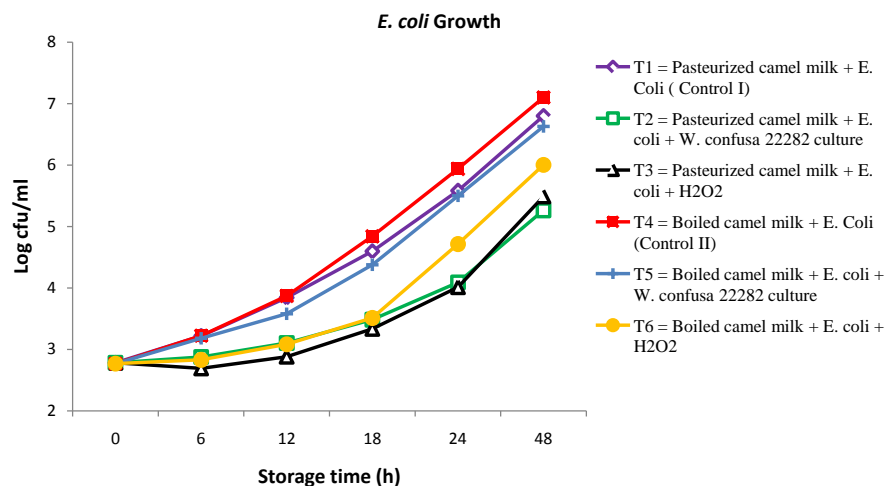
On the other hand, *W. confusa* 22282 activated LPs retarded level of CC by 1.09 log units as compared to the non-activated at 18 hours of storage showing

that the activated LPs exhibited antimicrobial effect on the coliform microorganisms in raw camel milk. This finding is in agreement with the result of other experiments [24] who reported reduction of CC by 1.14 log units in Saanen goats' milk activated with LPs compared to the non-activated milk. No significant difference ( $P > 0.05$ ) was observed in CC between the *W. confusa* 22282 and exogenous  $H_2O_2$  activated LPs samples throughout the storage period which indicated that the treatments have comparable effect on the growth of coliforms in camel milk.

### 3.4. Effect of Lactoperoxidase System Activation on Growth of *Escherichia coli*

The activation of LPs by *W. confusa* 22282 and exogenous  $H_2O_2$  in pasteurized camel milk remarkably reduced the growth rate of *E. coli* population compared their respective population in boiled milk throughout the storage period (Figure 3). This result indicated that the role of LP enzyme in boiled milk might be impaired in catalyzing the oxidation of thiocyanate by  $H_2O_2$ . The LPs improves keeping quality of milk pasteurized at  $72^\circ C/15$  s compared to milk heated to  $80^\circ C/15$  seconds [41]. Similar findings [42] was reported that activation of LPs extended the keeping quality of pasteurized milk ( $72^\circ C/15s$ ) inoculated with *Pseudomonas aeruginosa*, *S. aureus* and *S. thermophilus* while the milk heated at  $80^\circ C/5s$  and activated with the LPs had no effect on growth of these organisms. Moreover, this finding is supported by recommendation by FAO/WHO [8] that heating milk for 15 seconds at  $80^\circ C$ , completely inactivates enzymatic activity of the milk might be because of the destruction of indigenous inhibitory components by the higher temperature. Indigenous enzymes are heat-labile and can easily be destroyed under the boiling conditions [43].

Similarly, the rate of *E. coli* growth in the LAB and exogenous  $H_2O_2$  activated LPs in pasteurized camel milk was considerably lower than the rate in non-activated pasteurized milk (Figure 3). Reduction in microbial spoilage of LPs activated



**Figure 3.** Grow and survival of *E. coli* in the LPs activated pasteurized and boiled camel milk for 48 hours.

pasteurized cow milk inoculated with *E. Coli* compared to the non-activated one was also reported [44]. An inhibition of the growth of *E. coli* for 24 hours [45] and a nearly total inhibition of *E. coli* O157:H7 [21] was also reported in cow milk and in commercial fermented milk and traditional Madila activated with LPs, respectively.

No remarkable difference was observed in retarding *E. coli* population between the *W. confusa* 22282 activated and H<sub>2</sub>O<sub>2</sub> activated LPs in the pasteurized camel milk except a better retarding effect in the LAB activated samples toward the end of the storage period, which might be due to continuous production of H<sub>2</sub>O<sub>2</sub> in the milk (Figure 3). The *W. confusa* 22282 treated boiled milk had shown slight reduction compared to the untreated boiled milk samples might be because of the effects of residual LP in the milk [42] or due to production of metabolites by the LAB [46]. Metabolites produced by LAB were reported to affect the growth of *E. coli*, *S. aureus*, *Salmonella spp.* in food items [47]. On the other hand, the H<sub>2</sub>O<sub>2</sub> treated boiled milk shown remarkable reduction in *E. coli* counts compared to the untreated boiled samples. This clearly indicated that H<sub>2</sub>O<sub>2</sub> had antimicrobial effect by itself as supported by other finding [48].

#### 4. Conclusion

The current study showed that LAB can produce H<sub>2</sub>O<sub>2</sub> to activate the natural antimicrobial system in the milk. The optimization experiment indicated that *W. confusa* 22282 can produce sufficient amount of H<sub>2</sub>O<sub>2</sub> in MRS broth. The activation of LPs by *W. confusa* 22282 as a source of H<sub>2</sub>O<sub>2</sub> in raw camel milk significantly ( $P < 0.05$ ) reduced the rate of lactic acid development, TBC and CC compared to the non-activated samples at 12, 18, 24 and 48 hours of storage at room temperature. Similarly, the activation of LPs by *W. confusa* 22282 in pasteurized and boiled camel milk samples inoculated with *E. coli* as contaminant showed that the LPs activated pasteurized milk remarkably reduced the *E. coli* population compared to the *W. confusa* 22282 treated boiled milk throughout the storage period. The rate of growth of the *E. coli* population in the *W. confusa* 22282 activated LPs in pasteurized milk was also considerably lower than the rate in non-activated pasteurized milk. This indicated that pasteurization (63°C/30 minutes) cannot destroy the enzyme LP and the storage stability of the pasteurized milk samples was therefore due to the activation of the antimicrobial system in the milk. Generally, the present study showed that it is possible to activate the natural antimicrobial system in camel milk using H<sub>2</sub>O<sub>2</sub>-producing LAB (*W. confusa* 22282) as a source of H<sub>2</sub>O<sub>2</sub> in the presence of appropriate concentrations of natural thiocyanate in the milk. Therefore, it can be concluded that biological activation of LPs by *W. Confusa* 22282 in camel milk can substitute the chemical activation method provided that the milk has sufficient inherent thiocyanate.

#### Acknowledgements

The authors are grateful to Danish International Development Agency (DANIDA)

for financing this research project. Haramaya University is highly appreciated for providing facilities to conduct the research.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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