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Original article

Inhibition of *Escherichia coli* O157:H7 in commercial and traditional fermented goat milk by activated lactoperoxidase

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Abstract – The lactoperoxidase (LP) system has been reported to inhibit the production of acid by lactic starter cultures and can result in the survival and growth of acid-adapted enteropathogens in LP-activated fermented milk. The aim of this study was to investigate the effect of the LP system on growth and acid production by single strains and indigenous lactic acid bacteria (LAB) and the survival of *Escherichia coli* O157:H7 in fermented goat's milk. LP-activated raw and pasteurized goat's milks were inoculated with single strains of *Lactococcus* sp. and *Bifidobacterium longum* BB536 and incubated for 24 h at 30 °C to simulate commercial milk fermentation. Madila, a traditional fermented milk product, was fermented with indigenous LAB for 5 days at 30 °C. Goat's milk was also inoculated with *E. coli* O157:H7 to determine its survival during the fermentation of the LP-activated milk. The viability of LAB and *E. coli* O157:H7, pH and acid production were monitored during the fermentations. None of the LAB cultures tested displayed significant sensitivity to the LP system with respect to growth and acid production. However, *E. coli* O157:H7 was inhibited in the LP-activated milk in the commercial fermented milk and in the traditional Madila, where the counts were reduced by > 5.0 log cfu·mL^{-1}. Therefore, the LP system could be used during the fermentation of both traditional and commercial milk processing at ambient temperatures as an additional bacteriological control to improve the quality of commercial sour milk and traditional Madila-type products.

lactoperoxidase / goat milk / lactic acid / starter culture / *Escherichia coli* O157:H7

摘要 – 活性乳过氧化物酶对工业及传统发酵羊乳中大肠杆菌 O157:H7 的抑制作用摘要。 乳过氧化酶体系对乳酸菌发酵剂的产酸有抑制作用，进而导致肠道病原菌在高活力的乳过氧化酶发酵乳中存活和大量繁殖。 本文研究了发酵羊乳中的乳过氧化酶系统对单一菌株和内源性乳酸菌的生长以及对大肠杆菌 O157:H7 存活的影响。将含有活性乳过氧化酶的原料乳和巴氏杀菌的羊奶分别与 *Lactococcus* sp. 和 *Bifidobacterium longum* BB536 两株单一菌株在 30 °C 下培养 24 h 用以模拟发酵乳的工业生产过程。 Madila 是利用内源性乳酸菌在 30 °C 下发酵 5 d 生产的一种传统的发酵乳制品。 同样，在含有活性乳过氧化酶的羊乳中接种 *E. coli* O157:H7 以确定其存活能力。 检测了整个发酵过程中乳酸菌和 *E. coli* O157:H7 的存活能力以及 pH 和产酸能力。 根据乳酸菌的生长与产酸能力的对比，乳中活性乳过氧化酶对每一株乳酸菌的生长都没有显著的影响。 然而，无论是在工业发酵乳和传统 Madila

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发酵乳中，活性乳过氧化酶能够显著地抑制 *E. coli* O157:H7 的生长， *E. coli* O157:H7 的数目降到 > 5.0 log cfu·mL^{-1}。 因此，乳过氧化酶可以用来控制工业的和传统的发酵乳制品发酵过程中环境微生物的污染和影响以及改善发酵乳制品的质量。

乳过氧化物酶 / 羊乳 / 乳酸 / 发酵剂 / 大肠杆菌 O157:H7

Résumé – Inhibition d'*Escherichia coli* O157:H7 dans un lait de chèvre à lactoperoxydase activée fermenté par des cultures de souches pures ou indigènes de bactéries lactiques. Le système lactoperoxydase (LP) est considéré comme un inhibiteur de la production d'acide des cultures de levains lactiques qui peut entraîner la survie et la multiplication d'entéropathogènes adaptés à l'acide dans les laits fermentés à LP activée. Cette étude a été conduite pour rechercher l'effet du système LP sur la croissance et la production d'acide de souches pures ou indigènes de bactéries lactiques (LAB) et sur la survie d'*Escherichia coli* O157:H7 dans du lait de chèvre fermenté. Des laits de chèvre crus et pasteurisés à LP activée ont été inoculés avec des souches pures de *Lactococcus* sp. et de *Bifidobacterium longum* BB536 et incubés 24 h à 30 °C pour simuler la fermentation commerciale du lait, alors que le traditionnel Madila, un produit laitier fermenté, était fermenté avec des LAB indigènes pendant 5 jours à 30 °C. Le lait de chèvre était aussi inoculé avec *E. coli* O157:H7 pour en déterminer la survie pendant la fermentation du lait à lactoperoxydase activée. La viabilité des LAB et d'*E. coli* O157:H7, le pH et la production d'acide ont été suivis tout au long des périodes de fermentation. Aucune des cultures de LAB testées n'a montré de sensibilité significative au système LP en ce qui concerne la croissance et la production d'acide durant la fermentation du lait. *E. coli* était cependant inhibé par le lait à LP activée dans le lait fermenté commercial et le traditionnel Madila. Dans le Madila, les dénombrements d'*E. coli* O157:H7 étaient réduits de plus de 5,0 log cfu·mL^{-1}. Le système LP peut donc être utilisé en fermentation de lait traditionnel ou commercial fabriqué à température ambiante, comme contrôle bactériologique additionnel pour améliorer la qualité de produits laitiers fermentés commerciaux et traditionnels de type Madila.

lactoperoxydase / lait de chèvre / acide lactique / levain / *Escherichia coli* O157:H7

1. INTRODUCTION

Small-scale milk production in developing countries where ambient temperatures are well above 30 °C suffers high losses, particularly when the market chain lacks adequate infrastructure to preserve milk. For this reason, most small-scale agro-pastoralists process their leftover milk into artisanal fermented dairy products for home consumption, some of which enter the informal market for economic benefit. However, lack of process control results in inconsistent quality of traditional fermented dairy products. Recent implications of dairy and other acidic foods in *Escherichia coli* O157:H7 outbreaks [2, 15] have challenged the safety of goat milk products that were processed under uncontrolled conditions. Once acid adapted, *E. coli* O157:H7 can survive in high acid foods for extended periods of time and can survive lethal pH of the stomach to cause disease in the intestine [18, 26]. Since *E. coli* O157:H7 has common occurrence in raw milk, it can also contaminate milk post-pasteurization following poor milk handling. Therefore, proper handling of raw and pasteurized milk and the application of appropriate preservation methodologies are important to inhibit *E. coli* O157:H7 because low pH alone is no longer sufficient to eliminate its occurrence in fermented dairy products.

The lactoperoxidase (LP) system can be activated in raw milk and post-pasteurization of milk as an additional bacteriological control in dairy processing. LP is a naturally occurring enzyme in milk that catalyses the oxidation of thiocyanate into hypothiocyanite in the presence of hydrogen peroxide [19]. Hypothiocyanite has a bacteriostatic effect on *E. coli* in milk [25]. In spite of heat

sensitivity at temperatures above 70 °C [13], the LP enzyme is reported to maintain the activity at pasteurization temperatures of 63 °C for 30 min with ~ 30% loss of activity at 72 °C for 15 s [1].

Studies have shown that the LP system is not only antagonistic against undesirable microbes, but it also affects the growth and lactic acid production of some lactic acid bacteria (LAB) at both ambient and refrigeration temperatures [16, 24]. Acid production is critical in dairy fermentation because it is used to assess the quality of starter cultures [7]. Therefore, there are concerns that LP activation in milk will not only affect milk quality, but inhibition of acid production will also prolong the formation of casein gels and enable outgrowth of acid-adapted enteropathogens that occur in milk [9].

To our knowledge, there have been no studies on the effect of LP activation on indigenous lactic starter cultures that were used in artisanal fermented dairy products. Although there have been limited studies on the effect of LP activation on single strain lactic starter cultures [24], further studies are needed to enable the selection of LP-resistant lactic cultures that can be developed for the fermentation of specialized dairy products. In order to respond to the concerns of the application of LP system in milk intended for processing into fermented dairy products, this study was designed to first of all investigate the sensitivity of single *Lactococcus* sp. and *Bifidobacterium longum* to LP activation in pasteurized goat milk. Subsequently, selected sensitive and resistant LAB were used to ferment LP-activated goat milk that had been inoculated with *E. coli* O157:H7 as a model system to determine whether inhibition of acid production would affect the growth of *E. coli* O157:H7 in fermented milk. Finally, the application of LP system was tested in the fermentation of a traditional dairy product called Madila using indigenous lactic culture. Goat milk with inoculated *E. coli* O157:H7 was used to determine whether the effect of LP system on LAB and *E. coli* O157:H7 in the traditional product would differ from that of the commercial product.

2. MATERIALS AND METHODS

2.1. Milk source

Fresh Saanen goat milk was sourced from the University of Pretoria, experimental farm. The Saanen goats were milked following standard procedures with a milking machine. Milk from individual goats was pooled and delivered within 1 h of milking. One hundred millilitre portions of fresh goat milk were transferred into sterile 150 mL blue-capped Schott bottles and pasteurized at 63 °C for 30 min in a thermostatically controlled water bath before inoculation and activation of the LP system. Pasteurized milk was used for the processing of commercial fermented milk, and raw milk was used for traditional Madila fermentation.

2.2. Cultures

E. coli O157:H7 strains UP10 and 1062 were obtained from the Onderstepoort Veterinary Institute, Agricultural Research Council (Republic of South Africa (RSA)). Cultures were maintained on MacConkey agar (Oxoid, Hampshire, England) plates that were stored at 2 °C. Working cultures were prepared by transferring a single colony of each *E. coli* O157:H7 strain from MacConkey agar into sterile Tryptone Soy Broth (TSB; Biolab, Wadeville, RSA) and incubated for 24 h at 37 °C. The activating inoculum was prepared after two successive 24 h transfers of 0.5 mL of each of the *E. coli* O157:H7 strains UP10 and 1062 into 100 mL sterile TSB at 37 °C. This culture was used as inoculum for challenge tests.

The following lactic starter cultures were used in this study: single strain *Lactococcus lactis* subsp. *lactis* 345, *Lactococcus lactis*

subsp. *cremoris* 326, *Lc. lactis* subsp. *cremoris* 328, *Lactococcus lactis* subsp. *diacetylactis* 339 and *Lc. lactis* subsp. *diacetylactis* 340 in vacuum-sealed ampoules were obtained from the Department of Food Bioscience, University of Free State, RSA; *Lc. lactis* subsp. *lactis* AM1 isolated from traditional Amasi; and *B. longum* BB536 obtained from Morigana (South Korea). Activated cultures were prepared by growing cultures in 100 mg·L^{-1} sterile skim milk at 22 °C for 16 h.

2.3. Inoculation and fermentation

All the 100 mL volumes of pasteurized goat milk were inoculated with 1 mL LAB culture. Each LAB culture was inoculated into two separate bottles containing 100 mL pasteurized milk; the LP system was activated in one of the two bottles, and the second bottle served as the LP-untreated control. Before activation of the LP system, the thiocyanate content of goat milk was determined according to the International Dairy Federation [12]. The LP activity was determined by spectroscopic measurement using one-step ABTS (2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid, Sigma, St. Louis, USA) solution as substrate [25]. The LP system was activated by adding sodium thiocyanate (Saarchem, Krugersdorp, RSA) to a final concentration of 14 mg·L^{-1}. After thorough mixing, 30 mg·L^{-1} sodium percarbonate (Aldrich Chemical Company Inc., Milwaukee, USA) was added as a source of hydrogen peroxide [12]. The inoculated goat milk was then incubated at 30 °C for 6 h in a thermostatically controlled water bath.

To determine the effect of activated LP system on LAB in commercial fermented milk and its impact on the survival of *E. coli* O157:H7, 100 mL pasteurized goat milk samples inoculated with 1 mL selected single strain lactic cultures were also inoculated with 1 mL *E. coli* O157:H7 cocktail containing strains UP10 and 1062 prior to the activation of LP system. The initial concentration of LAB and *E. coli* O157:H7 was determined before incubation at 30 °C in a thermostatically controlled water bath for 24 h.

To prepare traditional Madila, fresh unpasteurized goat milk was transferred into two plastic buckets in 400 mL volumes. LP system was activated in one bucket containing 400 mL goat milk, and the second milk sample was used as the LP-untreated control. The LP-activated and control goat milk samples were each inoculated with 10% (v/v) traditional skim milk culture and 1% (v/v) *E. coli* O157:H7 strain UP10. Goat milk samples were allowed to ferment at 30 °C for 5 days. After 24 h and on each subsequent day for a total of 5 days, 1-day-old soured milk was added to fermenting Madila in a 4:1 (fermenting Madila:sour milk) ratio [17]. The 1-day-old soured milk was prepared by inoculating unpasteurized goat milk with 1% (w/v) freeze-dried traditional fermented milk and incubating at 25 °C for 24 h. On d 5, the whey from the fermented Madila was strained through a sterile jute bag. Madila was then mixed with cold unpasteurized goat milk in a ratio of 4 parts Madila:1 part goat milk.

2.4. Acid challenge

The surviving *E. coli* O157:H7 from LP-activated and control fermenting Madila samples were tested for acid adaptation after 24 h. Acid challenge test was conducted by transferring 1 mL of milk samples into 10 mL TSB acidified with 6 mol·L^{-1} lactic acid (Saarchem, Wadeville, RSA) to pH 4.0 for 4 h at 37 °C. Survival of *E. coli* O157:H7 from LP-activated and control Madila was compared to the survival of non-adapted *E. coli* O157:H7 challenged in acidified TSB (pH 4.0) for 4 h at 37 °C.

2.5. Chemical analyses

In order to determine the concentration of thiocyanate to add to milk, the thiocyanate

concentration of milk was determined according to the IDF method [12]. About 8 mL of raw milk were thoroughly mixed with 4 mL of 20% (w/v) trichloroacetic acid (Saarchem, Gauteng, RSA) and allowed to stand for 30 min. The mixture was then filtered through a Whatman No. 40 filter paper and 1.5 mL of the clear filtrate mixed with 1.5 mL of ferric nitrate reagent (16 g of Fe $(NO_3)_3 \cdot 9H_2O$ (Saarchem)) in 50 mL distilled water. The absorbance was measured at 460 nm wavelength with a Lamda EZ150 UV spectrophotometer (Perkin Elmer, USA), and the thiocyanate concentration was determined from a standard curve.

The titratable acidity (TA), used to measure lactic acid production, was determined by titrating 9 mL of milk with 0.1 mol·L^{-1} NaOH (Promark Chemicals, Robertsham, RSA). TA was expressed as percent lactic acid [3].

The pH readings were taken at the time of sampling of thoroughly mixed samples by inserting the pH electrode (Hanna Instruments, Italy) directly into the fermenting milk samples.

2.6. Microbiological analyses

Fermenting milk was sampled for viable *E. coli* O157:H7 and LAB counts after 0, 2, 4, 6 and 24 h for commercial Amasi/Maas-type fermented milk and 0, 1, 2, 3, 4 and 5 days for traditional Madila. Serial dilutions were prepared with 0.1% buffered peptone water (Oxoid, Hampshire, UK) and spread plated on M 17 agar (Oxoid) for *Lactococci* sp. counts, MRS agar (Oxoid) for *Lactobacillus* sp. and *Leuconostoc* sp. counts and Sorbitol MacConkey agar (SMAC, Oxoid) for *E. coli* O157:H7 counts. M 17 plates were incubated at 30 °C for 24–48 h, MRS plates were incubated at 37 °C for 48 h and SMAC plates were incubated at 37 °C for 24 h preceding enumeration of sorbitol negative *E. coli* O157:H7. Detection limit for microbial counts was 10 cfu·mL^{-1}.

2.7. Statistical analyses

Analysis of variance (ANOVA) was used to determine whether activated LP had a significant effect on lactic acid production, and viability of lactic starters and *E. coli* O157:H7 cultures throughout the processing of commercial fermented milk (24 h) and the Madila processing period (5 days). Each sample was analysed in duplicate, and the experiment was conducted three times. The significance level was set at $P \leq 0.05$. ANOVA was performed using Statistica (Tulsa, USA, 2008).

3. RESULTS

3.1. Quality of raw and pasteurized Saanen goat milk

The LP activity, TA and pH of raw and pasteurized Saanen goat milk are presented in Table I. The TA and pH of raw and pasteurized goat milk were within standard values. The standard deviation (SD) for pH of pasteurized goat milk (0.20) could be due to inconsistencies in time taken to reach pasteurization temperature, leading to an increase in pH. The average counts for *E. coli*, *Lactococcus* sp. and *Lactobacillus* sp. in fresh goat milk are presented in Table I. Zero counts were found in goat milk pasteurized at 63 °C for 30 min.

3.2. The effect of LP activation on single strain LAB in goat milk

All LAB cultures tested grew in pasteurized and LP-activated goat milk reaching populations of 9.1–9.4 log cfu·mL^{-1}, with the exception of *Lc. cremoris* 326 that reached a final concentration of 8.5 log cfu·mL^{-1} after 6 h. Although there was a significant strain ($P \leq 0.05$) effect on growth and acid production of the seven individual LAB strains tested (Tab. II), they did not show significant sensitivity to LP

Table I. Chemical and microbiological quality of raw and pasteurized Saanen goat milk ($N = 6$).

Analyses	Milk	Mean	SD
Lactoperoxidase activity	Raw	0.090 U·mL^{-1}	0.02
	Pasteurized	0.040 U·mL^{-1}	0.02
Titratable acidity	Raw	0.12%	0.01
	Pasteurized	0.14%	0.04
pH	Raw	6.52	0.04
	Pasteurized	6.47	0.20
Escherichia coli	Raw	3.07 log cfu·mL^{-1}	0.19
Lactococcus sp.	Raw	4.24 log cfu·mL^{-1}	0.26
Lactobacillus sp.	Raw	3.44 log cfu·mL^{-1}	0.04

system ($P > 0.05$). The highest acid production was observed in *Lc. lactis* AM1 that was isolated from traditional Amasi, while the lowest acid reduction was observed in *Lc. cremoris* 326 (Tab. II). The acid production correlated positively with the decrease in pH.

3.3. The effect of LP activation on single strain LAB in goat milk that has *E. coli* O157:H7 present

There was no significant difference between LP-activated and control populations of all LAB strains tested, although cell numbers differed significantly ($P \leq 0.05$) for individual cultures (Tab. III). Also, there was a significant ($P \leq 0.05$) overall LAB strain effect on acid production by single lactic cultures (Tab. III). All LAB tested in the presence of *E. coli* O157:H7 had a significantly higher ($P \leq 0.05$) acid production after 6 h compared to cultures that had no *E. coli* O157:H7. In the presence of *E. coli* O157:H7, acid production by *Lc. lactis* subsp. *cremoris* 326 culture was similar to those produced by *Lc. lactis* subsp. *diacetylactis* 340 and *Lc. lactis* subsp. *lactis* 345 after 6 h of fermentation. Also, all cultures tested with the exception of *Lc. lactis* AM1 showed a greater increase in acid production in the LP-activated milk after 6 h of fermentation compared to the LP-untreated controls. *Lc. cremoris* 326 showed the greatest difference in acid production.

Like the single strain LAB cultured in the absence of *E. coli* O157:H7, LP system did not have a significant effect on acid production in the presence of *E. coli* O157:H7 throughout the fermentation period. Nonetheless, a marginal reduction in acid production was observed in the LP-activated 24 h culture of *B. longum* BB536 and *Lc. lactis* AM1. *Lc. diacetylactis* 340 on the other hand showed resistance to activated LP system with 4.18% increase in acid production compared to control cells after 24 h (Tab. III).

The *E. coli* O157:H7 numbers generally increased in goat milk during the first 6 h of fermentation. Inhibition of *E. coli* O157:H7 numbers was subsequently observed after 24 h in all single strain LAB cultured goat milk (Tab. III). Inhibition of *E. coli* O157:H7 was however not uniform for all the LAB strains tested. Here, LP system had a significant effect ($P \leq 0.05$) on *E. coli* O157:H7 over time. Although LP inhibition of E. *coli* O157:H7 was not apparent in the *Lc. diacetylactis* 340 culture (19% in LP-activated culture compared to 18% in the control), significant reductions were observed in *Lc. lactis* 345 (18% in LP-activated culture compared to 13%

Table II. Changes in the mean values (†SD) of pH, acid production and LAB counts in pasteurized and lactoperoxidase-activated Saanen goat milk fermented at 30 °C by single strains of LAB.

LAB strains	Time (h)	pH		Titratable acidity (%)		LAB counts (log cfu·mL^{-1})	
		No LP	LP	No LP	LP	No LP	LP
Lc. diacetylactis 339	2	5.92 (†0.09)	5.93 (0.09)	0.21 (0.005)	0.20 (0.003)	8.97 (0.04)	8.95 (0.03)
	6	4.37 (0.03)	4.37 (0.01)	0.55 (0.017)	0.54 (0.019)	9.41 (0.06)	9.38 (0.04)
Lc. diacetylactis 340	2	6.02 (0.05)	6.03 (0.04)	0.19 (0.003)	0.17 (0.007)	8.72 (0.07)	8.73 (0.10)
	6	4.46 (0.02)	4.46 (0.02)	0.53 (0.012)	0.53 (0.012)	9.10 (0.11)	9.05 (0.07)
Lc. lactis 345	2	6.01 (0.05)	6.06 (0.06)	0.19 (0.006)	0.17 (0.003)	8.73 (0.12)	8.66 (0.06)
	6	4.46 (0.02)	4.47 (0.03)	0.56 (0.014)	0.53 (0.012)	9.13 (0.12)	9.09 (0.16)
Lc. cremoris 326	2	6.28 (0.01)	6.28 (0.01)	0.16 (0.009)	0.15 (0.007)	8.01 (0.19)	8.03 (0.11)
	6	5.30 (0.25)	5.28 (0.27)	0.34 (0.080)	0.34 (0.077)	8.49 (0.25)	8.41 (0.31)
Lc. cremoris 328	2	5.90 (0.06)	5.94 (0.06)	0.21 (0.007)	0.20 (0.009)	8.65 (0.37)	8.59 (0.43)
	6	4.37 (0.01)	4.39 (0.01)	0.58 (0.019)	0.56 (0.012)	9.32 (0.07)	9.40 (0.05)
B. longum BB536	2	6.06 (0.24)	6.14 (0.22)	0.16 (0.023)	0.16 (0.023)	8.16 (0.36)	8.06 (0.23)
	6	4.70 (0.21)	4.75 (0.22)	0.62 (0.078)	0.59 (0.073)	9.25 (0.01)	9.19 (0.10)
Lc. lactis AM1	2	5.94 (0.03)	5.97 (0.04)	0.16 (0.023)	0.16 (0.023)	8.52 (0.11)	8.60 (0.09)
	6	4.96 (0.02)	4.56 (0.02)	0.66 (0.015)	0.66 (0.021)	9.30 (0.05)	9.25 (0.02)
P value (time effect)		0.000		0.000		0.000	
P value (LP effect)		0.658		0.257		0.724	
P value (LAB strain effect)		0.000		0.000		0.000	
P value (LP vs. time)		0.986		0.911		0.982	
N		3		3		3	

Table III. Changes in the mean values (†SD) of acid production and *E. coli* O157:H7 counts in pasteurized and LP-activated Saanen goat milk fermented by single strain LAB at 30 °C.

LAB strains	Time (h)	Titratable acidity (%)		LAB (log cfu·mL^{-1})		*E. coli* O157:H7 (log cfu·mL^{-1})	
		No LP	LP	No LP	LP	No LP	LP
Lc. diacetylactis 340	2	0.24 (†0.009)	0.25 (0.032)	8.41 (0.017)	8.43 (0.134)	6.56 (0.031)	6.69 (0.076)
	6	0.66 (0.012)	0.66 (0.009)	9.27 (0.004)	9.21 (0.126)	7.31 (0.25)	7.34 (0.063)
	24	0.70 (0.015)	0.82 (0.028)	8.95 (0.123)	9.01 (0.068)	5.97 (0.229)	5.94 (0.197)
Lc. lactis 345	2	0.22 (0.015)	0.26 (0.022)	8.71 (0.035)	8.47 (0.047)	6.68 (0.015)	6.54 (0.095)
	6	0.66 (0.032)	0.66 (0.017)	9.09 (0.082)	9.21 (0.029)	6.93 (0.135)	7.12 (0.052)
	24	0.82 (0.015)	0.81 (0.009)	8.91 (0.166)	8.83 (0.016)	6.02 (0.170)	5.84 (0.216)
Lc. cremoris 326	2	0.25 (0.009)	0.22 (0.009)	8.50 (0.070)	8.43 (0.016)	6.72 (0.195)	6.89 (0.020)
	6	0.64 (0.025)	0.66 (0.019)	9.11 (0.016)	9.13 (0.013)	6.85 (0.090)	7.07 (0.073)
	24	0.81 (0.015)	0.81 (0.009)	9.22 (0.310)	8.92 (0.134)	6.17 (0.371)	5.42 (0.074)
B. longum BB536	2	0.23 (0.009)	0.21 (0.009)	8.83 (0.097)	8.77 (0.054)	6.88 (0.054)	7.05 (0.121)
	6	0.70 (0.012)	0.67 (0.006)	9.31 (0.026)	9.24 (0.023)	7.07 (0.197)	6.98 (0.041)
	24	0.84 (0.10)	0.81 (0.012)	9.25 (0.111)	9.13 (0.087)	5.41 (0.117)	5.19 (0.147)
Lc. lactis AM1	2	0.29 (0.032)	0.28 (0.032)	8.93 (0.028)	8.86 (0.027)	6.70 (0.103)	6.76 (0.107)
	6	0.70 (0.013)	0.69 (0.015)	9.28 (0.018)	9.26 (0.020)	6.96 (0.204)	7.03 (0.066)
	24	0.83 (0.015)	0.80 (0.009)	9.00 (0.153)	9.21 (0.050)	5.65 (0.164)	5.32 (0.283)
P (LAB strain effect)		0.000		0.000		0.151	
P (LP effect)		0.745		0.822		0.635	
P (time)		0.000		0.000		0.000	
P (LP vs. time)		0.959		0.735		0.012	
P (LAB vs. *E. coli* O157:H7)		0.000		0.000		NA	
P (LP vs. LAB vs. *E. coli* O157:H7)		0.954		0.960		NA	
N				3			

Table IV. Changes in the mean values (†SD) of pH, titratable acidity and counts of *E. coli* O157:H7 and indigenous LAB during processing of traditional Madila at 30 °C.

LP treatment	Time (days)	*E. coli* O157:H7 (log cfu·mL^{-1})	*Lactococcus* (log cfu·mL^{-1})	*Lactobacillus* and *Leuconostoc* (log cfu·mL^{-1})	pH	Titratable acidity (%)
No LP	0	6.95 (†0.08)	6.68 (0.19)	7.30 (0.19)	6.35 (0.08)	0.17 (0.01)
	1	7.23 (0.73)	9.13 (0.16)	9.28 (0.04)	4.19 (0.08)	0.75 (0.00)
	2	4.39 (0.79)	8.55 (0.16)	8.77 (0.40)	4.19 (0.12)	0.77 (0.03)
	3	3.43 (0.33)	8.51 (0.16)	8.90 (0.22)	4.36 (0.04)	0.73 (0.01)
	4	3.89 (0.15)	8.44 (0.10)	8.84 (0.23)	4.09 (0.04)	0.96 (0.04)
	5	4.25 (1.15)	7.35 (0.59)	7.30 (0.52)	3.92 (0.05)	1.17 (0.05)
LP	0	6.95 (0.07)	6.67 (0.11)	7.21 (0.12)	6.44 (0.02)	0.16 (0.01)
	1	7.27 (0.75)	9.03 (0.13)	9.13 (0.15)	4.22 (0.08)	0.77 (0.05)
	2	4.85 (0.38)	8.49 (0.18)	8.45 (0.17)	4.11 (0.04)	0.73 (0.03)
	3	3.75 (0.19)	8.28 (0.17)	8.61 (0.19)	4.33 (0.12)	0.71 (0.05)
	4	2.76 (0.22)	8.33 (0.17)	8.40 (0.24)	3.97 (0.10)	1.09 (0.08)
	5	0.52 (0.52)	7.44 (0.42)	7.48 (0.37)	3.79 (0.08)	1.35 (0.03)
P (LP)		0.052	0.636	0.243	0.382	0.062
P (LP vs. time)		0.010	0.993	0.884	0.625	0.043
N				3		

in the control); *B. longum* BB536 (26% in LP-activated culture compared to 24% in the control culture) and *Lc. lactis* AM1 (24% in LP-activated culture compared to 19% in the control). Overall, *Lc. cremoris* 326 showed the greatest difference in *E. coli* O157:H7 inhibition between the LP-activated culture (23%) and the control culture (10%).

3.4. The effect of LP system on the processing of a traditional fermented product that has *E. coli* O157:H7 present

The LP system did not significantly affect the growth and acid production of indigenous LAB in Madila fermentation. The numbers of LAB increased reaching an optimum of 9.28 log cfu·mL^{-1} after 24 h of fermentation (Tab. IV). The LAB concentration subsequently declined marginally maintaining a level of ~ 8 log cfu·mL^{-1} throughout the fermentation period until d 5 when LAB numbers declined further. Similarly, pH of fermenting Madila was unaffected by activated LP throughout the fermentation period ($P > 0.05$) (Tab. IV). The pH of the LP-activated Madila declined to 4.22 and 4.19 in LP-untreated Madila after 24 h. The pH did not change significantly during the subsequent fermentation period. The TA of both LP-activated and control Madila increased after 24 h followed by constant acid production until d 3 (Tab. IV). On d 4 and d 5, both LP-activated and control Madila showed a progressive increase in TA; however, the LP-activated Madila had a higher TA compared to the control. This LP effect on TA was significant ($P \leq 0.05$) over the 5-day period.

The *E. coli* O157:H7 numbers in both LP-activated and control Madila increased marginally after 24 h of fermentation. Subsequently, *E. coli* O157:H7 counts in

LP-activated Madila declined progressively until they reached < 1.0 log cfu·mL^{-1} at the end of the fermentation period (Tab. IV). The *E. coli* O157:H7 numbers in the control Madila also declined until d 3 after which the cell numbers levelled reaching 4.25 log cfu·mL^{-1} at the end of the fermentation period. The LP effect on *E. coli* O157:H7 survival during fermentation of Madila was statistically significant ($P \leq 0.05$).

After 24 h of fermentation, *E. coli* O157:H7 cells in LP-activated and control Madila were challenged to lethal acid treatment at pH 4.0 for 4 h to determine whether *E. coli* O157:H7 in the fermenting medium had become acid adapted. Acid challenge caused 1.81 log cfu·mL^{-1} and 1.65 log cfu·mL^{-1} reductions in *E. coli* O157:H7 counts in LP-activated and control Madila, respectively. The non-adapted *E. coli* O157:H7 cells were reduced beyond detection after 4 h acid challenge at pH 4.0 (data not shown).

4. DISCUSSION

While lactic acid produces fresh flavour to fermented milk products [11], it is also important for the coagulation of milk. Therefore, rapid production of lactic acid is the most important attribute of lactic starter cultures [7]. In this study, all the single strain LAB tested with the exception of *Lc. cremoris* 326 were fast acid producers that reduced the pH of pasteurized goat milk to an average of pH 4.5 in 6 h. The relatively low acid production of *Lc. cremoris* 326 correlated positively with the cell concentration in pasteurized goat milk. Since all conditions were the same, the difference in the rate of growth and lactic acid production was characteristic of the strain.

The lack of activated LP inhibition of all the LAB strains tested is supported by other authors. For example, Nakada et al. [16] observed no significant difference in viability for single strain cultures *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* in yoghurt with or without subjection to the LP system, although acid production was inhibited in LP-activated cultures at 41 °C. In this study, acid production by single strain *Lactococcus* sp. and *B. longum* BB56 was not significantly suppressed in LP-activated goat milk. This apparent resistance could be due to the low LP activity of Saanen goat milk used in this study. LP activity of milk has been found to be highly variable depending on the type of milk and the period of lactation [6]. However, the level of LP activity in Saanen goat milk recorded in this study falls within the range of 0.04–0.16 U·mL^{-1} reported by Fonteh et al. [10] for raw goat milk during the lactation period. Regardless of the low LP activity of milk, the marginal reduction of acid production by *B. longum* BB536, *Lc. cremoris* 328 and *Lc. lactis* 345, compared to acid production in control milk, suggests that these LAB could potentially be sensitive to the LP system at a higher LP activity.

The presence of *E. coli* O157:H7 did not affect the growth of LAB in goat milk. The increased acid production of LAB in *E. coli* O157:H7 inoculated milk compared to the milk that had no *E. coli* O157:H7 was due to the additional lactic acid production by *E. coli* O157:H7 metabolism of lactose. The LP effect on acid production in the presence of *E. coli* O157:H7 was variable for the individual LAB tested. This difference lies in the strain-to-strain variation of lactic cultures [21], and the interaction between the lactic cultures, *E. coli* O157:H7 and the stresses encountered in the fermenting medium. Although the nature of this interaction was not investigated, the lactic cultures were clearly influenced by the presence of *E. coli* O157:H7 because lactic acid production in LP-activated milk differed from the cultures that had no *E. coli* O157:H7 present. The greater increase of acid production in LP-activated milk was unexpected. Given that *E. coli* O157:H7

cells were significantly inhibited by the LP system, the difference in acid production could not be attributed to acid production by *E. coli* O157:H7 alone. It appears that increased acid production was stimulated by lactic starter cultures in the presence of an antagonistic pathogen.

Apart from lactic acid inhibition of *E. coli* O157:H7 in fermented milk, other factors such as the production of bacteriocins and ethanol could have contributed to *E. coli* O157:H7 inhibition. Some species of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are known to produce nisin and lactococcin, respectively, that have broad antimicrobial spectra [15, 20]. Although studies have indicated that these antimicrobial peptides inhibit Gram positives, when coupled with activated LP, these bacteriocins could have an additional inhibitory effect on *E. coli* O157:H7 as observed in the LP-activated *Lc. lactis* subsp. *cremoris* 326 24 h fermented milk culture compared to the control culture. The increased inhibition of *E. coli* O157:H7 observed in *Lc. lactis* AM1 and *B. longum* BB536 cultures in both LP-activated and control milk could be due to their characteristic antimicrobial properties. Both *Lc. lactis* subsp. *lactis* and *B. longum*, particularly strain *B. longum* BB536, have been classified as probiotics that are antagonistic against pathogenic microbes [14, 22].

In the traditional Madila product, the indigenous LAB were resistant to LP system. The resistance of indigenous LAB from traditional fermented milk to activated LP system has not been reported. Previous studies examining the sensitivity of mixed and single strain lactic starter cultures to activated LP system found that acid production and survival of lactic starter cultures in activated LP milk vary from one investigation to another. Seifu et al. [24] reported that activated LP inhibits acid production of commercially mixed lactic starter cultures. In this study, the indigenous LAB were not only insensitive to activated LP, but lactic acid production was not inhibited in LP-activated milk. The lack of LP inhibition of lactic cultures could be due to the reversal of antimicrobial hypothiocyanite by the enzyme NADH-OSCN oxidoreductase into thiocyanate [5]. This reversal factor exhibited by NADH oxidoreductase together with NADH oxidase and peroxidase enzymes is stimulated during oxidative stress [23]. Investigation of the molecular basis for resistance of these indigenously mixed lactic starters could shed more light on the mechanism of resistance against LP activation. These indigenous LAB cultures could be developed for upscale Madila processing from LP-activated milk.

Although inhibition of acid production was not observed in LP-activated fermented milk in this study, acid challenge of the 24 h culture during Madila fermentation indicated that the inoculated *E. coli* O157:H7 had become acid adapted. This finding is consistent with those of other authors who have reported acid resistance of *E. coli* during fermentation of dairy products [29]. Though acid adapted, the *E. coli* O157:H7 cells were inhibited in LP-activated Madila. Previous studies have indicated a limited period of LP efficacy in milk [8]. It was stated in the guideline for raw milk preservation [4] that the activated LP system can extend the keeping quality of raw milk stored at 30 °C for 7–8 h. In this study, activated LP inhibition of acid-adapted *E. coli* O157:H7 was evident after d 4 of Madila fermentation. This observation suggests that when activated LP system was coupled with low pH, the combined inhibitory effect was extended for at least 5 days at 30 °C. The delayed LP inhibition of *E. coli* O157:H7 in LP-activated Madila suggests that low pH sensitized acid-adapted *E. coli* O157:H7 to activated LP. The increased enzymatic production of HOSCN/$OSCN^-$ and the easy passage of uncharged hypothiocyanite into the cell at low pH [27, 28] could have contributed to the inhibition of acid-adapted *E. coli* O157:H7 in LP-activated Madila.

Since the combination of LP activation and low pH caused > 5.0 log cfu·mL^{-1} reduction in *E. coli* O157:H7, it can be applied in traditional milk processing and storage at ambient temperature to improve the microbiological safety of fermented milk with respect to *E. coli* O157:H7.

5. CONCLUSION

This study has shown evidence that the application of activated LP did not inhibit lactic acid production by single strain and indigenous LAB during the first 6 h of fermentation, which is a crucial period for growth and acid production of LAB in the processing of fermented dairy. These cultures can therefore be developed for the processing of specialized dairy products from activated LP milk. Though *E. coli* O157:H7 cells were inhibited in LP-activated milk, the high numbers in fermented milk after 24 h indicate that the application of LP system in the industrial processing of milk may not be sufficient to reduce *E. coli* O157:H7 that occur in milk. However, in the traditional processing of milk products, like Madila, where milk is slowly fermented at ambient temperatures over long periods, LP system can be applied to improve the safety of the product.

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