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Effect of the lactoperoxidase system on the activity of mesophilic cheese starter cultures in goat milk

E. Seifu[a,*], E.M. Buys[a], E.F. Donkin[b]

[a] Department of Food Science, University of Pretoria, Lynnwood Road, Pretoria 0002, South Africa
[b] Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

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Abstract

The effect of goats' milk lactoperoxidase (LP) system on the activity of commercially available mesophilic cheese starter cultures was investigated. The growth and acid production of the starter cultures were measured at 2 h intervals for 8 h in goats' milk kept at 30 °C. Most of the starter cultures examined were found to be sensitive to the LP system, but varied in their susceptibility to inhibition. The activity of the mixed starter cultures CHN11, CHN22, CHN19, DCC240 and Flora Danica Normal was strongly inhibited by the LP system. However, the mixed starter culture LL 50C showed resistance to the LP system. The single strain culture *Lactococcus lactis* subsp. *lactis* NCDO 605 was inhibited by the LP system. However, the cultures *Lactococcus lactis* subsp. *diacetylactis* NCDO 176 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313 were insensitive to the LP system. The results of this study indicate the need for routine screening of starter cultures for resistance to the LP system before using them for cheesemaking from goats' milk preserved by the LP system.

Keywords: Mesophilic cheese starter cultures; Goat milk; Lactoperoxidase system

1. Introduction

The use of starters in cheese manufacture is as old as cheesemaking. Lactic acid starters are mainly prepared cultures of mesophilic and/or thermophilic lactic acid bacteria intended to be used in the manufacture of cheese, cultured butter, fermented milks or fermented milk products that are used to initiate desirable changes (IDF, 1991a).

The primary function of starter cultures in cheesemaking is to ferment the lactose of the milk to lactic acid, which aids in separation of curd from whey during cheese manufacture, modifies texture of cheeses and enhances preservation (IDF, 1980). Starters also have other important functions in cheese manufacture such as flavour and aroma development and controlled proteolysis of cheese curd during the ripening process. Some starter cultures also contribute to the formation of gas, which is desirable in some varieties of cheese (IDF, 1980).

Different factors can inhibit the activity of starter cultures such as bacteriophages, the presence of agglutinins in raw milk, the lactoperoxidase (LP) system, lysozyme, lactoferrin, antibiotics, bacteriocins and chemical sanitizers (Lewis, 1987; Frank & Hassan, 1998).

The LP system is the most significant microbial inhibitor in raw milk (Frank & Hassan, 1998). LP is an enzyme that is naturally present in raw milk and together with thiocyanate and hydrogen peroxide constitutes the LP system. LP is usually present in sufficient amount in milk; however, the level of thiocyanate is more variable in milk and depends on the feeding of the animal (FAO, 1999). The third component, hydrogen peroxide, is not normally detected in raw milk (Pruitt & Kamau, 1994; FAO, 1999). The enzyme LP catalyzes the oxidation of thiocyanate by hydrogen peroxide and generates hypothiocyanite ($OSCN^-$) ion, which has proven antibacterial activity (Reiter, 1985).

The LP system has been recommended for preservation of raw milk in areas where it is not possible to use mechanical refrigeration for technical and/or economic reasons (IDF, 1988; FAO, 1999). Due to the growing

*Corresponding author. Tel.: +27-12-420-3413; fax: +27-12-420-2839.
E-mail address: eyassu_seifu@yahoo.com (E. Seifu).

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interest in the use of the LP system for preservation of raw milk, there is a possibility that LP-activated milk could be used for cheese manufacture. However, one of the concerns regarding the use of the LP system for manufacture of fermented dairy products, such as cheese, is its inhibitory effect on starter cultures. Earlier reports (Sarkar & Misra, 1992) indicated that activation of the LP system resulted in a reduced rate of acid production in fermented milk, which may lead to an abnormal product or extend the production time significantly. Therefore it is necessary to identify starter cultures, which are resistant to the inhibitory effect of the LP system.

Goats are important milk producers in several parts of the tropics and significantly contribute to human nutrition in many developing countries (Devendra, 1999). Milk production from goats in South Africa has increased over the last two decades (Donkin, 1998) and the majority of goats' milk producers in South Africa are small-scale farmers and they process the milk into various types of cheese. The potential of the LP system for preservation of raw goats' milk has been reported (Haddadin, Ibrahim, & Robinson, 1996).

Activation of the LP system in cows' milk was found to inhibit the activity of thermophilic lactic starter cultures (de Valdez, Bibi, & Bachmann, 1988; Basaga & Dik, 1994). The activity of individual starter cultures varies to a considerable extent with the type of milk used (Dutta, Kuila, Ranganathan, & Laxminarayana, 1971). Rysstad and Abrahamsen (1983) found variations in the biochemical performance of two mixed strain starters, Christian Hansen 01 (CH01) and Flora Danica Normal, grown in goats' and cows' milk. They attributed this difference to the variation in the composition of goats' and cows' milk and suggested the use of different starters for fermented dairy products made from goats' milk. Similarly, the acidifying and proteolytic activities of a given strain of lactic starter culture were found to be different in cows' and goats' milk (Lodi, Brasca, Carcano, & Sangalli, 1996). Thus, it can be expected that the effect of goats' milk LP system on cheese starter cultures may be different from that of cows' milk. The objective of this experiment was therefore to study the effect of the LP system on the activity of commercial mesophilic cheese starter cultures and to screen for LP-resistant cultures which could be used for cheesemaking from goats' milk preserved by activation of the LP system.

2. Materials and methods

2.1. Starter cultures

The cultures used in this study were CHN11, CHN22, DCC240, CHN19, Flora Danica Normal (FDN), LL 50C, *Lactococcus lactis* subsp. *diacetylactis* NCDO 176, *Lactococcus lactis* subsp. *lactis* NCDO 605 and *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 33313. The first five were mixed strain cultures which were obtained from the supplier (Chr. Hansen, Denmark) in a freeze-dried form. All these five cultures were composed of *L. lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *diacetylactis* and *L. mesenteroides* subsp. *cremoris*. The mixed strain culture LL 50C was obtained from Anchor Biotechnologies (Johannesburg, South Africa) in a freeze-dried form and it was composed of *L. lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. The single strain cultures *L. lactis* subsp. *lactis* and *L. lactis* subsp. *diacetylactis* were obtained from Irene Animal Nutrition and Animal Products Institute, of the Agricultural Research Council (Irene, South Africa). *L. mesenteroides* subsp. *cremoris* was obtained from the South African Institute of Medical Research. The cultures were activated by growing in 10% (w/v) sterile reconstituted skim milk powder at 22°C for 16 h before use.

2.2. Source of milk samples

Saanen goat milk samples were obtained from the Onderstepoort Teaching Animal Unit of the Faculty of Veterinary Science, University of Pretoria. The goats were fed on a commercial ration composed of 23.8% w/w yellow maize meal, 19.84% w/w malt dust, 4.96% w/w sunflower oil cake, 7.93% w/w yeast, 29.76% w/w eragrostis, 11. 9% w/w molasses, 0.5% w/w mono calcium phosphate, 0.5% w/w salt, 0.5% w/w limestone powder, 0.3% w/w premix, and 0.02% w/w Romensin. The milk samples were chilled and delivered to the laboratory for analysis within 1 h of milking. The average composition of the milk samples was: 3.1% w/w fat, 3.1% w/w protein and 11.4% w/w total solids. The milk samples had a thiocyanate content ranging from 1.27 to 4.67 mg L^{-1} with an average value of 2.78 mg L^{-1}. The thiocyanate content of each milk sample was determined and then the LP system was activated by addition of sodium thiocyanate (see Section 2.4).

2.3. Treatment of the milk sample

One percent (v/v) of each starter culture was inoculated into 400 mL of Saanen goats' milk that had been pasteurized at 63°C for 30 min in a thermostatically controlled water bath. The initial concentration of the cultures in the milk was determined immediately after addition of the starter cultures. The inoculated milk was then divided into two equal portions: one was used for the activation of the LP system whereas the other was used as untreated control. Both the treated

and control milk samples were incubated at 30°C for 8 h.

2.4. Growth and activity tests of the starter cultures

The inhibitory effect of the LP system on the starter cultures was measured by determining the number of colony forming units (cfu mL^{-1}) and lactic acid production at 2 h intervals over the 8 h incubation period. Prior to activation of the LP system, the thiocyanate content of each milk sample was determined and then the LP system was activated by addition of sodium thiocyanate (Saarchem, Midrand, South Africa) as a source of thiocyanate to a final concentration of 14 mg L^{-1}, as recommended by the International Dairy Federation (IDF, 1988). After 1 min thorough mixing, 30 mg L^{-1} sodium percarbonate (Sigma Aldrich Chemical Co., Johannesburg, South Africa) was added as a source of hydrogen peroxide (IDF, 1988). Lactic acid production was determined by titration with 0.1 N NaOH and expressed as percent (w/w) lactic acid (Bradley et al., 1993). For viable counts, suitable dilutions, made in quarter strength Ringer's solution, were plated in duplicate by the pour plate method using MRS agar (de Man, Rogosa, & Sharpe, 1960). Plates were incubated at 30°C for 48 h. In the case of the culture LL 50C, only the activity test (titratable acidity) was conducted, and in the case of the culture *L. mesenteroides* subsp. *cremoris*, only the growth of the culture was measured.

2.5. Determination of chemical composition

Thiocyanate concentration in raw milk was determined spectrophotometrically as described by IDF (1988). In all, 4 mL of raw milk was mixed with 2 mL of 20% (w/v) trichloroacetic acid solution. The mixture was blended well and then allowed to stand for 30 min. It was then filtered through Whatman No. 40 filter paper. The clear filtrate (1.5 mL) was then mixed with 1.5 mL of ferric nitrate reagent and the absorbance measured at 460 nm. The thiocyanate concentrations were calculated from a standard curve. The fat content of the milk samples was determined by the Gerber method as described by Bradley et al. (1993). The protein content of the milk samples was determined after measuring the total nitrogen content of the milk by the Dumas method (IDF, 2000) using a Leco FP-528 Protein/Nitrogen Analyser (Leco Corporation, Michigan, USA) and multiplying the total nitrogen by the factor 6.38. The total solids content of the milk samples was determined after measuring the water content of the milk samples using the forced draft oven method (Bradley et al., 1993) and subtracting the values from 100.

2.6. Statistical analysis of the data

The differences in the growth and activity of the starter cultures in the treated and control milk samples at each incubation period were analyzed by the Wilcoxon Mann Whitney test of the Statistical Analysis System (SAS, 1999). The mean, maximum and minimum values were calculated and the significance of these differences was calculated at the 5% significance level.

3. Results and discussion

3.1. Mixed strain starter cultures

Fig. 1 indicates the growth and acid production of various mixed starter cultures in LP-activated Saanen goats' milk. The growth ($\log_{10}$ cfu mL^{-1}) of most of the mixed starter cultures in LP-activated milk samples was less than their growth in the untreated control milk samples, throughout the incubation period. However, significant differences ($p < 0.05$) in growth were observed between LP-activated and control milk samples for the cultures CHN22 (Fig. 1b) and DCC240 (Fig. 1c) only, at 6 h and 8 h of incubation.

Similarly, the lactic acid production of the mixed starter cultures used in this experiment was also affected by activation of the LP system. A significant difference ($p < 0.05$) in the level of acid production between LP-activated and control milk samples was observed for the cultures CHN11 (Fig. 1a), CHN22 (Fig. 1b), DCC240 (Fig. 1c), CHN19 (Fig. 1d) and FDN (Fig. 1e) at 6 and 8 h of incubation. However, the level of acid production in LP-activated and control milk samples throughout the incubation period was not significantly different for the starter culture LL 50C (Fig. 1f).

For all cultures no significant increase in acid production was observed in the control milk samples until 4 h of incubation. Scott (1986) reported that the increase in titratable acidity by a starter culture in cows' milk at 30°C is expected to reach an average value of 0.45% (w/w) after 4 h of incubation although it depends on the type of starter culture used. However, the activity of individual starter cultures varies to a considerable extent with the type of milk used (Dutta et al., 1971; Cárcoba, Delgado, & Rodríguez, 2000), an effect which may be associated with the difference in the composition of milk from different species. Freshly drawn cows' milk has bacteriostatic property which lasts for 1–2 h (FAO, 1999). The delay in acid production in the control milk samples observed in this study suggests that goats' milk might have a strong bacteriostatic property which lasts longer after milking.

The difference in acid production and growth in LP-activated milk samples between the cultures CHN11, CHN22, DCC240, CHN19, and FDN at 8 h of

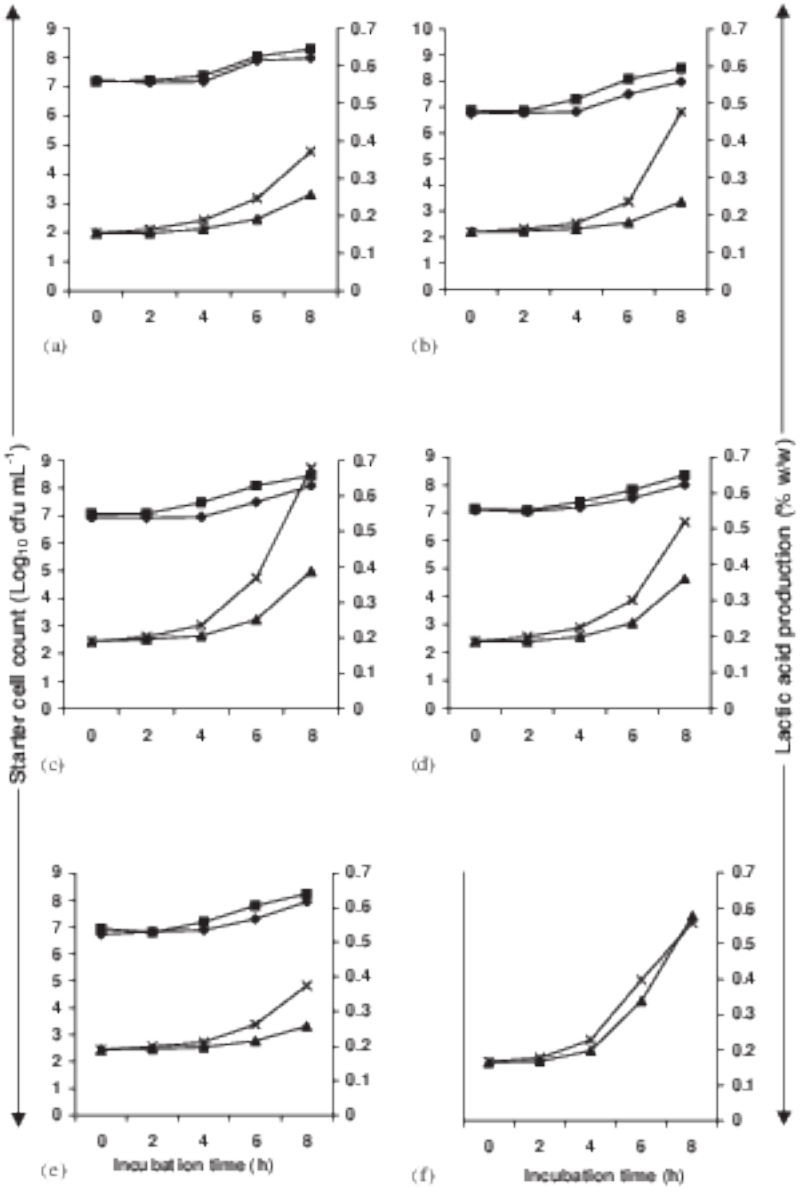

Fig. 1. Starter cell growth (◆, ■) and acid production (▲, ×) of mixed strain mesophilic cheese starter cultures CHN11 (a), CHN22 (b), DCC240 (c), CHN19 (d), FDN (e) and LL 50C (f) in LP-activated goats' milk (◆, ▲) and control milk (■, ×) during 8 h of incubation at 30°C. ◆, growth in LP-activated milk; ■, growth in control milk; ▲, acid production in LP-activated milk; and ×, acid production in control milk ($n = 5$ for the starter cultures CHN11, CHN22, DCC240 and FDN; $n = 4$ for the starter cultures CHN19 and LL 50C).

incubation is indicated in Table 1. For all, apart from one culture, the level of acid production was lower in the LP-activated milk than in the control. However, the decrease in acid production in LP-activated milk at 8 h among the cultures was different (Table 1). The level of acid production by the LL 50C culture in the LP-activated milk at 8 h was higher than that of the control (Table 1). This indicates that the starter cultures examined in this experiment showed varying degrees of sensitivity to the LP system. This is in agreement with an earlier report by Guirguis and Hickey (1987). They reported that thermophilic starter strains were found to be sensitive to the LP system inhibition in cows' milk, but varied in their susceptibility to inhibition by the system.

In mixed strain cheese starters, inhibition, insensitivity or stimulation of activity by the LP system depends on the ratio of sensitive, insensitive and stimulated strains or variants within a starter (IDF, 1991b). The experiment on single strain cultures showed varying degree of sensitivity to the LP system among the single strain cultures used. The cultures *L. lactis* subsp. *diacetylactis* (Fig. 2b) and *L. mesenteroides* subsp. *cremoris* (Fig. 2c) were insensitive to the LP system whereas the culture *L. lactis* subsp. *lactis* (Fig. 2a) was inhibited by the LP system. Thus, the difference in

Table 1
Difference in the level of acid production and growth between mixed and single strain mesophilic cheese starter cultures in lactoperoxidase activated Saanen goats' milk at 8 h of incubation at 30°C

Starter culture	Acid production (% w/w lactic acid)		Growth (Log_{10} cfu mL^{-1})	
	% reduction[a]	% increase[a]	% reduction	% increase
CHN-11[b]	30.7		3.7	
CHN-22[b]	50.8		6.0	
DCC 240[b]	42.9		4.4	
CHN-19[c]	30.4		4.0	
FDN[b]	31.2		3.8	
LL 50C[c]		9.7	nd[d]	
L. lactis[e]	23.9			0.8
L. diacetylactis[e]	11.5			1.6
Leuc. mesenteroides[e]	nd			1.1

[a] Percent reduction or increase in acid production was calculated as follows: 100 × (acid production in the control milk at 8 h − acid production in the lactoperoxidase milk at 8 h)/(acid production in the control milk at 8 h).
[b] Mean of five experiments.
[c] Mean of four experiments.
[d] nd = not determined.
[e] Mean of three experiments.

sensitivity of the various mixed starter cultures examined in this study to the LP system might have been attributed to the differences in the proportions of the various strains between the mixed cultures used. The balance of species and/or strains within a mixed culture may alter and reduce the capacity of the culture to produce acid (Scott, 1986). The strains can be slow or fast acid producers; hence, a shift in the proportion of the different strains can affect the activity of the cultures.

The resistance of some starter cultures to the LP system could be associated with the presence of a 'reversal factor' as reported by Oram and Reiter (1966) and Reiter (1985). This 'reversal factor' is an enzyme, NADH:OSCN oxidoreductase, that catalyzes the reduction of the inhibitor $OSCN^-$, by NADH, to the inert thiocyanate. The starter strains in the LL 50C culture might be naturally resistant to the inhibitory effect of the LP system and may possess mechanisms that counteract the oxidation product produced by the LP system.

The inhibitory effect of the LP system on the acid production by mixed starter cultures was greater than that on growth, as measured by colony count. This might have been attributed to the difference in composition of the single strain starter cultures used in the mixed cultures. The single strain culture *L. mesenteroides* subsp. *cremoris* (Fig. 2c) was found to be insensitive to the LP system; however, it produces insufficient acid in milk. In contrast *L. lactis* subsp. *lactis* which is the major lactic acid producer among the lactic starter cultures used for cheesemaking (Scott, 1986) was sensitive to the LP system (Fig. 2a). Thus, if a mixed starter culture has a high proportion of the resistant culture *L. mesenteroides* subsp. *cremoris* and low proportions of the sensitive species *L. lactis* subsp. *lactis*, it can be expected that the acid production by the mixed culture will be inhibited more than their growth by the LP system.

3.2. Single strain starter cultures

Acid production by *L. lactis* subsp. *lactis* (Fig. 2a) in LP-activated milk was significantly ($p < 0.05$) lower than that in the control milk at 6 and 8 h of incubation. However, no significant differences in growth were observed between LP-activated and control milk samples for *L. lactis* subsp. *lactis* throughout the incubation period. This finding is consistent with that reported previously by Nakada, Dosako, Hirano, Oooka, and Nakajima (1996). They found that the LP system suppressed the rate of acid production by, but not the rate of growth of, yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). The greater inhibition of acid production than growth of *L. lactis* subsp. *lactis* by the LP system might be attributed to inhibition of key metabolic enzymes by the LP system that are responsible for the production of lactic acid by the starter culture. It has been reported that the hypothiocyanite ion produced during activation of the LP system causes inhibition of specific glycolytic enzymes such as aldolase, hexokinase and glyceraldehydes-3-phosphate dehydrogenase (Condon, 1987).

Both the growth of, and acid production by, the single strain culture *L. lactis* subsp. *diacetylactis* (Fig. 2b) in LP-activated milk and in control milk did not differ significantly ($p > 0.05$) throughout the 8 h incubation period. The growth of *L. mesenteroides* subsp. *cremoris* (Fig. 2c) was not affected by the LP system. An attempt

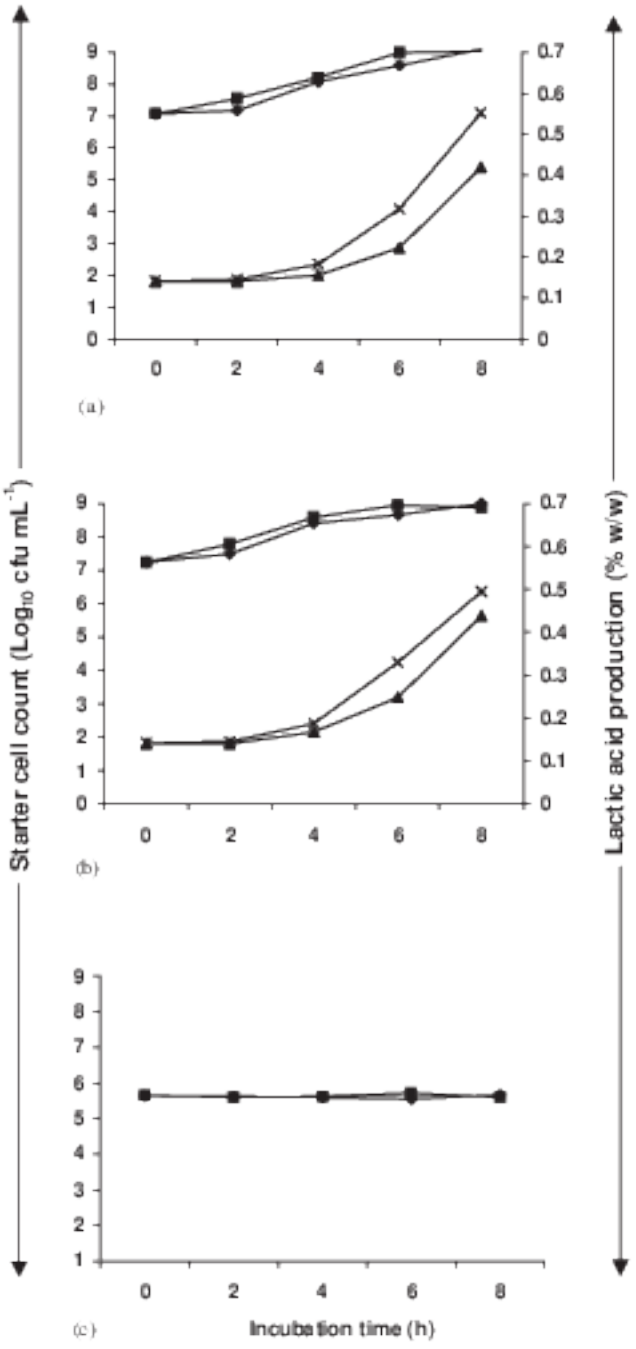

Fig. 2. Starter cell growth (◆, ■) and acid production (▲, ×) of single strain mesophilic cheese starter cultures *L. lactis* subsp. *lactis* (a), *L. lactis* subsp. *diacetylactis* (b) and *L. mesenteroides* subsp. *cremoris* (c) in LP-activated goats' milk (◆, ▲) and control milk (■, ×) during 8 h of incubation at 30 °C. ◆, growth in LP-activated milk; ■, growth in control milk; ▲, acid production in LP-activated milk; and ×, acid production in control milk ($n = 3$).

was made to measure the activity of *L. mesenteroides* subsp. *cremoris*; however, it did not produce any measurable acidity in the milk samples during the 8 h incubation period. This trend is consistent with that of Frank and Hassan (1998) who reported that *L. mesenteroides* does not produce sufficient acidity in milk to coagulate it.

The difference in acid production between the single strain starter cultures *L. lactis* subsp *lactis* and *L. lactis* subsp. *diacetylactis* at 8 h in LP-activated milk is indicated in Table 1. The percentage reduction in acid production by both cultures in LP-activated milk was lower than the values for mixed starter cultures (Table 1). In contrast to the trend for mixed starter cultures, the growth of the single strain starter cultures *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis* and *L. mesenteroides* subsp. *cremoris*, in LP-activated milk at 8 h of incubation was higher than that in the control milk (Table 1).

Although the single strain starter cultures exhibited less sensitivity to the LP system compared to mixed strain cultures, differences in sensitivity to the LP system were observed among the single strain starter cultures. The variation in sensitivity between the single strain cultures to the LP system may be associated with differences in their genetic makeup. Roginski, Broome, Hungerford, and Hickey (1984) reported that inhibition of starter cultures by the LP system is strain dependent. They showed that the growth of, and acid production by, *Lactococcus lactis* subsp. *cremoris* C1 were stimulated by the LP system in cows' milk whereas the growth of *Lactococcus lactis* subsp. *cremoris* BK5 and *L. lactis* subsp. *lactis* C10 were inhibited by the LP system.

4. Conclusions

Most of the starter cultures examined were found to be sensitive to the LP system but varied in their susceptibility to inhibition by the system. Activation of the LP system resulted in greater inhibition of acid production than growth of the starter cultures. Of the mixed starter cultures evaluated in this study, only one culture (LL 50C) was found to be insensitive to the LP system and thus could be used for cheesemaking from goats' milk preserved by the LP system. The results of this study indicate the need for routine testing of starter cultures for their sensitivity to the LP system before using them for cheesemaking from goats' milk preserved by the LP system.

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