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Full Length Research Paper

Interactions between *Lactobacillus acidophilus* strains and the starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* during fermentation of goats' milk

Su Li, Helen Walsh, Sumangala Gokavi and Mingruo Guo*

Department of Nutrition and Food Sciences, University of Vermont, Burlington, VT 05405, United States.

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It has been observed that *Lactobacillus acidophilus* has poor survivability in fermented goats' milk. In this study, interactions between *L. acidophilus* and starter cultures during goats' milk fermentation were investigated using three strains of *L. acidophilus*: ATCC-11975, LA-5 and NCFM, and the starter cultures (*Lactobacillus delbrueckii* subsp*. bulgaricus* (LB350) and *Streptococcus thermophilus (ST350*), isolated from a commercial yogurt starter. Selective enumeration methods were validated; de Mann, Rogosa and Sharpe (MRS) agar with 0.2% bile and anaerobic incubation at 37°C (72 h) was found to be suitable for all *L. acidophilus* strains; MRS agar with pH adjusted to 5.2 and anaerobic incubation at 37°C (24 h) was used for strain LB350 and M17 agar with 0.5% lactose and aerobic incubation at 37°C (24 h) for strain ST350. Addition of LB350 and/or ST350 into the goats' milk inoculated with *L. acidophilus* strains accelerated pH decrease compared to *L. acidophilus* strains used alone. Antagonism between each of the *L. acidophilus* strains and LB350 occurred, most noticeably with the LA-5 culture. However, it varied widely between the *acidophilus* strains indicating that antagonism is likely to be strain specific.

Key words: *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp*. bulgaricus*, *Streptococcus thermophilus*, interactions, goats' milk.

INTRODUCTION

The consumption of foodstuffs containing probiotics is widely considered advantageous with many different positive qualities being attributed to these bacteria. According to Mortazavian et al. (2006) there are more than 90 *Acidophilus/Bifidobacterium* product types produced worldwide and Japan, for example, has at least 53 dairy probiotic products. Yogurt, buttermilk, sauerkraut and fermented soy all have live lactic acid bacteria and are suitable for the growth of probiotics, kefir is a fermented milk drink from the Balkans, while ProViva (Skane Dairy, Malmö, Sweden), was the first probiotic food that does not contain milk or milk constituents. Because of its therapeutic benefits, including immuno-modulation, antagonism against pathogens, lowering of cholesterol level and reduction of occurrence of certain diseases, *Lactobacillus acidophilus* (LA) is becoming one of the most commonly used probiotics incorporated into dairy products (Kailasapathy and Rybka, 1997; Kailasapathy and Chin, 2000; Saarela et al., 2000). To receive these benefits, a minimum number (10^6 CFU/ml in food products) with regular consumption (400 to 500 g) of products per week are recommended (Kailasapathy and Rybka, 1997; Guo, 2009). Required minimum levels vary so, for example, the Canadian food inspection agency requires that a serving of stated size of a product

*Corresponding author. E-mail address: mguo@uvm.edu. Tel: 802 656 8168. Fax: 802 656 0001.

should contain a minimum level of 1.0×10^9 cfu of one or more of the eligible microorganism(s) (CFIA, 2009) while the European food safety authority recommends 10^8 CFU/serving for the alleviation of lactose intolerance (EFSA, 2010).

However, unsatisfactory viability of *L. acidophilus* has been observed, both in commercial products during refrigerated storage (Shah et al., 1995; Rybka and Fleet, 1997) and in laboratory scale studies (Nighswonger et al., 1996; Vinderola et al., 2000; Sodini et al., 2002; Li et al., 2006). Therefore, to meet the requirement the initial viable *L. acidophilus* count immediately after product processing is very important. In practice, *L. acidophilus* is, together with starter cultures, incorporated into milk before fermentation. This allows propagation of *L. acidophilus* to some extent in milk, which improves the initial number after processing and assists its adaptation to the product environment, which will help the survivability during storage (Tamime, 2005).

The probiotics generally do not cause rapid fermentation, which is a limitation in their usage in processing. It is always preferable to add the probiotic in conjunction with a specialized starter culture (such as the *Streptococcus thermophilus*/*L. delbruekii* subsp. *bulgaricus* (ST/LB) combination required for standard yogurt production). The storage conditions of each bacterial type before addition (freeze dried, frozen, microencapsulated, etc.) will have an influence on the relative viability of the organisms being added (Mattila-Sandholm et al., 2002). The symbiotic relationship between the yogurt starter cultures *S.thermophilus* and *L. delbrueckii* subsp*. bulgaricus* has been well established.

L. delbrueckii subsp*. bulgaricus* possesses proteolytic enzymes, oligopeptides and free amino acids which can be used as a nitrogen source for *S. thermophilus* during fermentation (Shihata, 2000). Conversely *S. thermophilus* produces substances which stimulate the growth of *L. delbrueckii* subsp*. bulgaricus*, including formic acid, pyruvate and CO_2 (Tamime and Robinson, 1999). In addition, yogurt produced by these two bacteria has good texture quality and flavor development and the food and drug administration (FDA) and other regulatory organizations require them to be present during fermentation to fulfill the definition of 'yogurt' (WHO/FAO, 2003; FDA, 2005).

However, the combined starter cultures and probiotics further interact and the resulting effect may be positive or negative. The effects tend to be strain-specific and therefore strain-specific data are required when determining which micro-organisms to use in a given product. Much research work has been carried out into the interaction of these bacteria in cows' milk products (Heller, 2001; Mattila-Sandholm et al., 2002; Grosso and Fávaro-Trindade, 2004; Mortazavian et al., 2006), however there is much less data on the effect of the use of goats' milk. The aim of this study was to investigate the interactions between different *L. acidophilus* strains and commonly used starter cultures during fermentation of goats' milk.

MATERIALS AND METHODS

Bacterial strains and propagation

L. delbrueckii subsp*. bulgaricus* strain LB350 and *S. thermophilus* strain ST350 were isolated from a commercial yogurt starter culture YC350. Three *L. acidophilus* strains were assessed: ATCC-11975 was purchased from ATCC (American Type Culture Collection, Manassas, Va. U.S.A.), LA-5 from Chr. Hansen Inc (Milwaukee, Wis., U.S.A.) and NCFM from Danisco US Inc. (Rochester, N.Y., U.S.A.). All the strains were tested for purity using Gram stain. Each culture was propagated weekly in sterile MRS broth with 1% inoculum at 37°C for 24 h, and maintained in the same medium at 4°C. Before experimentation, the cultures were transferred successively three times for activation, then either used for validation of selective enumeration methods directly, or transferred to 9% reconstituted skim milk (RSM) at 37°C for 24 h for fermented milk preparation.

Validation of selective enumeration methods

Activated bacterial strains were enumerated on deMann, Rogosa and Sharpe (MRS) agar as control, aerobically and anaerobically at 37°C for 48 h. Bacteria from the same dilution bottles were also enumerated on MRS to 0.2% Bile, MRS to pH 5.20 and M17 to 0.5% Lactose agar, to check the selectivity of each medium (incubation conditions are detailed under microbiological analysis in this section). In addition, to further check the performances of selective enumeration methods, mixed cultures were tested (Table 1).

Fermented milk preparation

A total of 3.6 L of commercial pasteurized (73°C for 20 sec) whole goats' milk from Oak Knoll Dairy Company (Windsor, Vt., U.S.A.) was heated to 43°C in a hot water bath. Reconstituted skim milk (RSM) containing one *Lactobacillus acidophilus* strain was inoculated into goats' milk (designated as LA milk) at a rate of: 3% for ATCC-11975, 2% for LA-5 and 1% for NCFM, and then each was divided into 4 batches. One of the batches was inoculated with 1% in RSM LB350 (designated as LA/LB), one with 0.4% in RSM ST350 (LA/ST), and one with both 1% in RSM LB350 and 0.4% in RSM ST350 (LA/LB/ST). The milk from each of the 4 batches was divided into 5 plastic cups, and then fermented by incubation at 43°C for 24 h. At each 6 h time interval (including 0 h), one cup of each batch was taken out to determine pH and viable bacterial count. Each trial was repeated three times and averages calculated.

Microbiological analyses

Fermented milk samples were diluted in commercial sterile phosphate buffer (Butterfield (Remel Products), Lenexa, Kans., U.S.A.), and appropriate dilutions were subsequently plated in duplicate. *L. acidophilus* strains were enumerated on MRS agar with 0.2% bile under anaerobic incubation at 37°C for 72 h. LB350 was enumerated on MRS agar with pH adjusted to 5.20 anaerobically at 37°C for 24 h; since *L. acidophilus* produced pinpoint colonies on this medium they were excluded from this analysis and only large colonies were counted (Table 1). ST350 was enumerated on M17 agar with 0.5% lactose under aerobic incubation at 37°C for 24 h. The pour plate technique was used, as

Table 1. Validation of selective enumeration methods for probiotic and starter culture strains. All samples were incubated at 37°C.

Parameter	Sample	MRS Ae48 h	MRS An48 h	MRS-0.2% BileAn, 72 h (LA)	MRS-pH 5.20An, 24 h (LB)	M17-0.5% Lactose Ae, 24 h (ST)
L. acidophilus strains	11975	7.70(0.07)	7.89(0.02)	7.89(0.04)	Pinpoint	No growth
	LA-5	8.18(0.01)	8.20(0.01)	8.19(0.02)	Pinpoint	No growth
	NCFM	8.30(0.03)	8.28(0.03)	8.30(0.02)	Pinpoint	No growth
	LB350	8.31(0.01)	8.40(0.03)	No growth	8.61(0.02)	No growth
	ST350	9.05(0.02)	8.99(0.03)	No growth	No growth	9.06(0.02)
Starter cultures	LB350/ST350	N/A	N/A	No growth	8.64(0.03)	9.04(0.02)
	11975/LB/ST	N/A	N/A	7.86(0.02)	8.63(0.04)	9.04(0.02)
	LA-5/LB/ST	N/A	N/A	8.14(0.02)	8.70(0.02)	9.06(0.01)
	NCFM/LB/ST	N/A	N/A	8.26(0.03)	8.66(0.02)	9.08(0.01)

Standard errors are shown in parentheses. (Ae = aerobic, An = anaerobic).

described by Shah (2000).

Statistical analysis

Data were analyzed using SPSS Software. In the analyses, $P < 0.05$ was used as the critical value for significance.

RESULTS AND DISCUSSION

Validation of selective enumeration methods

Although there are several existing selective enumeration methods for *L. acidophilus* and starter cultures, these methods showed strain specificity and are not valid for all strains (Dave and Shah, 1996; Shah, 2000; Tharmaraj and Shah, 2003). The data for validation of selective enumeration methods used in this study are shown in Table 1. MRS medium is commonly used to give a control viable bacterial number in selective enumeration studies, and here both aerobic and anaerobic incubation were conducted. For both ATCC-11975 and LB350, anaerobic conditions gave significantly ($p<0.05$) higher counts than those given by aerobic conditions for the other bacterial strains. There was no statistical significant difference in counts between aerobic and anaerobic conditions. There was no growth of either LB350 or ST350 on MRS to 0.2% Bile agar (selective for LA strains), nor growth of LA strains or LB350 on M17 to 0.5% Lactose agar (selective for ST strains).

On MRS-pH 5.20 agar (selective for LB strains), ST350 did not show any growth, and LA strains gave pinpoint colonies, which can be differentiated from the much bigger round white LB350 colonies. All these demonstrated that each selective enumeration method gives the required selectivity. They also gave bacterial numbers no less than the control number, indicating good recovery ability for relevant strains. For each strain, pure culture and mixed cultures gave similar viable bacterial count on the corresponding medium. For LB350, the counts obtained using MRS to pH 5.20 agar was significantly ($P<0.05$) higher than the control count given by MRS agar under anaerobic condition. For all the other strains there was not a statistically significant difference. The data demonstrated the validity of these selective enumeration methods, for use with these strains in the studies of interactions between *L. acidophilus* strains and starter cultures LB350 and ST350. However, validation of other strains is recommended when carrying out a similar experiment.

Changes in pH during fermentation

During the fermentation of goats' milk the drop in pH causes coagulation of the milk proteins initiating at around pH 5.2 and producing a firm texture as the pH drops below 4.6, the IP of casein. A typical yogurt has a pH of between 4.2 and 4.4. As can be seen in Figure 1, of the pure cultures (black line to the left of each group), 11975 and NCFM fell to these levels but only after considerable time had passed (18 to 24 h), while LA-5 (center group) was well above even the outside pH limit for coagulation after 24 h. Sodini et al. (2002) has shown that it takes around 12 h

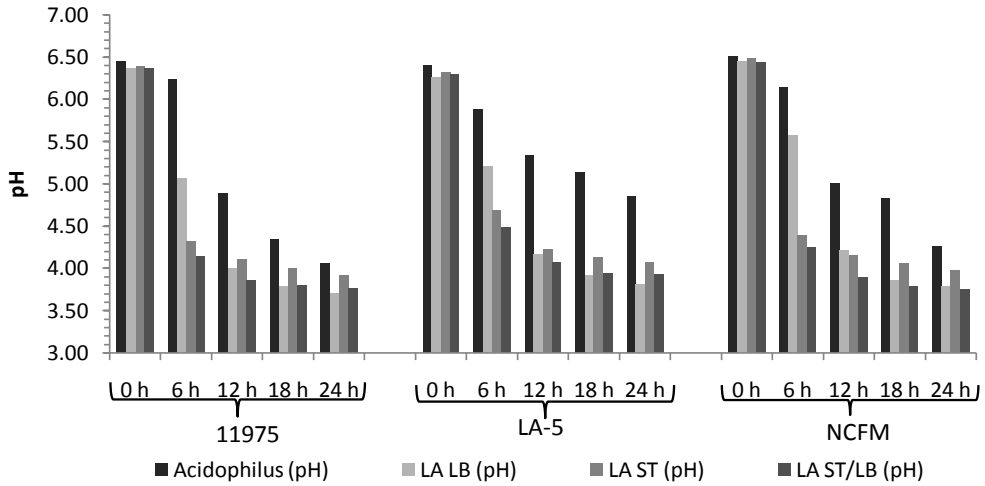

Figure 1. Changes in pH of goats' milk fermented by acidophilus strains ATCC-11975, LA-5 and NCFM. From left, each set: LA strain alone; LA strain with LB starter culture; LA strain with ST starter culture; LA strain with combination of LB and ST.

for LA-5 to reduce cow's milk pH to 5.0; this is more rapid than in this study, however, the discrepancy may be caused by different milk composition and/or the inoculation rate. Overall these results were consistent with observations by Saxelin et al. (1999) who reported that it is difficult to use probiotic organisms on their own for milk fermentation, the time for pH reduction being unrealistic for fermented milk manufacture.

In addition, in this work, separation, off-odors and an unpleasant foamy layer on the surface were observed with LA milk samples after 12 h. Alcohol dehydrogenase is produced by *L. acidophilus* and this converts acetaldehyde to ethanol which may be responsible for this defect (Marshall and Cole, 1983). For each of the probiotics, when combined with one or more starter cultures, pH was adequately reduced after 12 h and for 11975 and NCFM, this reduction was seen after 6 h for both LA/ST and LA/ST/LB combinations similar to that for commercial yogurt manufacture (Lankaputhra and Shah, 1997; Sodini et al., 2002). Considering that both *L. delbrueckii* subsp*. bulgaricus* and *S. thermophilus* are lactic acid bacteria, and their lactic acid producing ability is stronger than that of probiotics (Tamime and Robinson, 1999), it is reasonable to observe much faster pH decrease when they are present during milk fermentation, as in this study. Bacterial interaction may change their growth pattern or metabolism, further affecting the lactic acid production and changes in pH.

Interactions between *L. acidophilus* strains and starter cultures LB350/ST350

Survivability of LA strains

Poor survivability of *L. acidophilus* during refrigerated storage has been observed in surveys of commercial yogurt products (Shah et al., 1995; Rybka and Fleet, 1997). Our research of goats' milk yogurt beverage products also showed a similar trend, in which *L. acidophilus* died out within 3 to 4 weeks of storage, regardless of initial pH (data not shown). Antagonism from starter cultures has been speculated to be one responsible factor, Joseph et al. (1998) and Vinderola et al. (2002) observed antagonism between *L. acidophilus* and starter cultures, using modified spot-on-lawn and agar well diffusion assays, and noted that it was strain specific. In addition, inhibition of *L. acidophilus* by *L. delbrueckii* subsp*. bulgaricus* was observed in fermented milk (Gilliland and Speck, 1977), and the presence of *L. delbrueckii* subsp*. bulgaricus* was postulated to be responsible for the poor survivability of *L. acidophilus* (Dave and Shah, 1997; Rybka and Fleet, 1997).

L. acidophilus used alone

In this study, as shown in Figures 2 to 4, the growth pattern of *L. acidophilus* depended on the strain used and combination with starter cultures. Of the three probiotic strains used, starting from similar initial levels of bacteria, the strain 11975 (Figure 2) had the greatest increase over 24 h, at which stage its population was almost 100 times the initial number. NCFM (Figure 4) showed a 10X increase over the incubation time. NCFM, as pure culture, also had a slight increase over initial counts for all combinations for the first 6 h and, as with the LA-5, pure cultures remained stable.

LA strains combined with LB

When the LA strains were combined with LB350 alone this

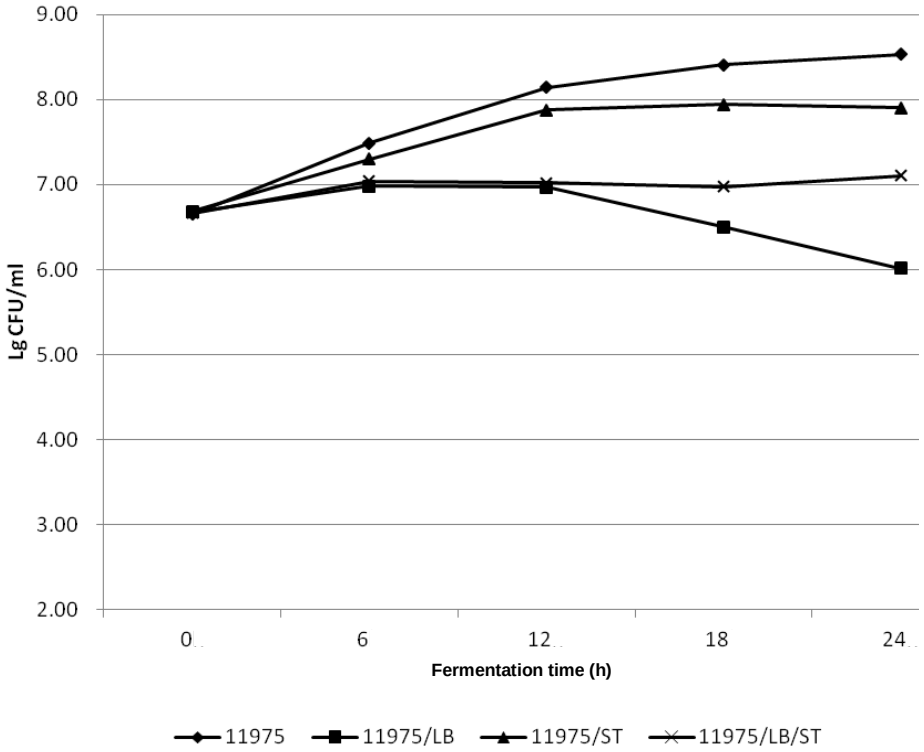

Figure 2. Changes in population of probiotic ATCC-11975 during fermentation of goats' milk when different combinations of starter cultures are used.

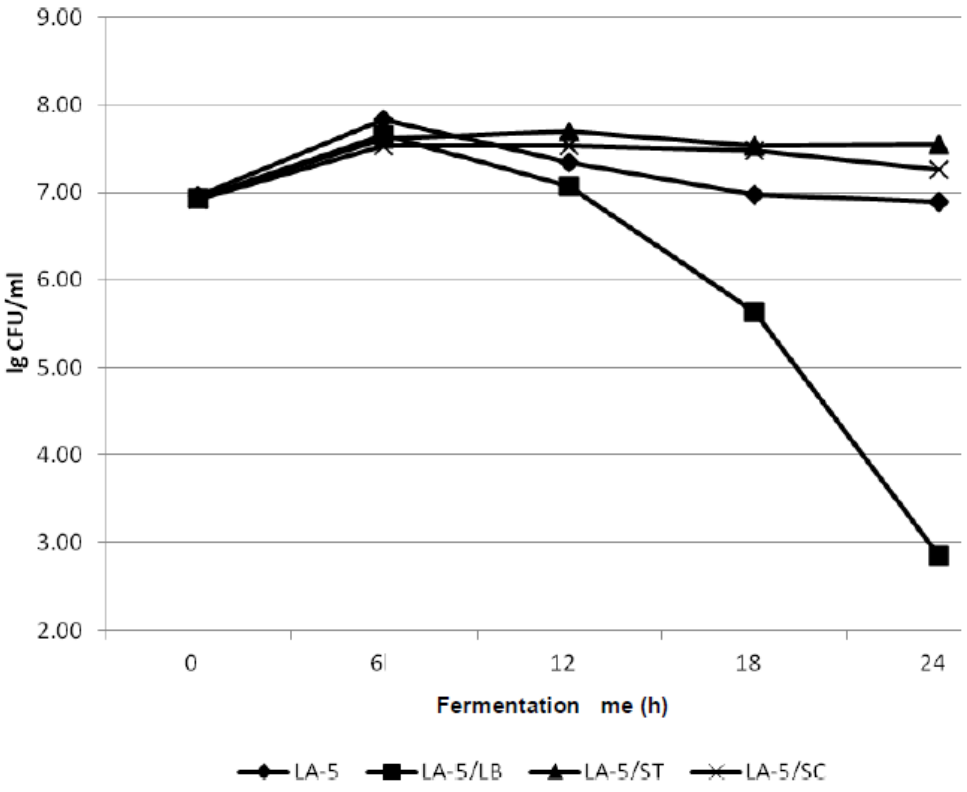

Figure 3. Changes in population of probiotic LA-5 during fermentation of goats' milk when different combinations of starter cultures are used.

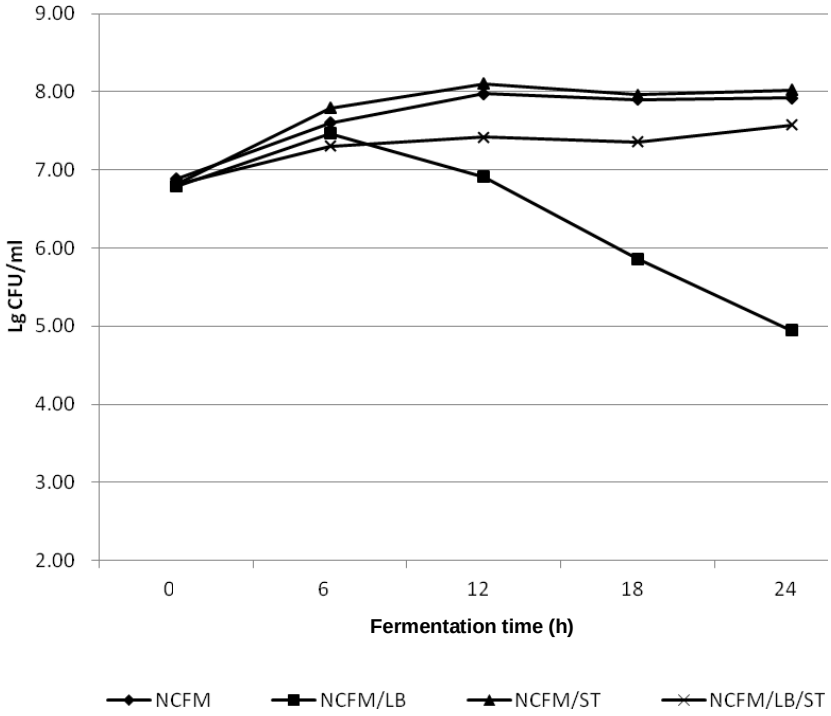

Figure 4. Changes in population of probiotic NCFM during fermentation of goats' milk when different combinations of starter cultures are used.

had a negative survival effect on the probiotic though 11975 was more resistant to decline than the other types. In each case LA numbers increased during the first 6 h, and then dropped at a much faster rate than all the other combinations. LA-5 was severely affected when combined with LB alone (Figure 3). Interestingly this falloff was not seen where LB/ST combination was used. Again NCFM had better survivability of LA in combination with ST and LB/ST than with LB alone where a significant falloff in counts resulted.

LA combined with ST or both LB and ST

LA/ST and LA/LB/ST milk samples yielded significantly better LA numbers than those of LA/LB milk, close to or exceeding pure culture counts for LA-5 and NCFM after 12 h though in ATCC-11975 experimental samples they were still not as high as those seen in ATCC-11975 milk alone. From 6 h onwards, the pH of LA/ST and LA/LB/ST samples was lower than that of LA and LA/LB samples (Figure 1). Taking this into account, together with the observation that viable LA counts did not decrease as dramatically as when LA/LB were combined (Figures 2 to 4), a lower pH does not seem to be the relevant factor in loss of LA population. Conversely, the addition of ST350 alone does not show an effect on the growth/survival of *L. acidophilus* strains different to pure culture. Therefore, it seems that the difference in the LA counts can be attributed to the presence of LB and that when both *L. acidophilus* strains and LB are present, the addition of ST is the factor reducing the inhibition of *L. acidophilus* strains by LB.

Survivability of starter cultures

Research on the symbiotic nature of starter cultures shows that *S. thermophilus* produces lactic acid, pyruvic acid, formic acid and CO_2, with lactic and formic acid in particular stimulating the growth of *Lb. bulgaricus*. Also *S. thermophilus* assimilates oxygen in milk, thereby creating favorable conditions for *L. bulgaricus* growth. *L. bulgaricus* in turn produces peptides and amino acids that stimulate the growth of *S. thermophilus* (Angelov et al., 2009). A good quality yogurt, without probiotics, with initial counts of log 6 bacteria should increase to around log 8 over the first 6 h of processing at 43°C (Bylund, 2003).

Effect of *L. acidophilus* strains

Addition of ST350 decreased LB350 counts during fermentation in LA-5 experimental samples (Figure 6) compared to LB alone. For the other two groups, 11975 (Figure 5) and NCFM (Figure 7), though there was a decline in LB counts, it was much more gradual and did

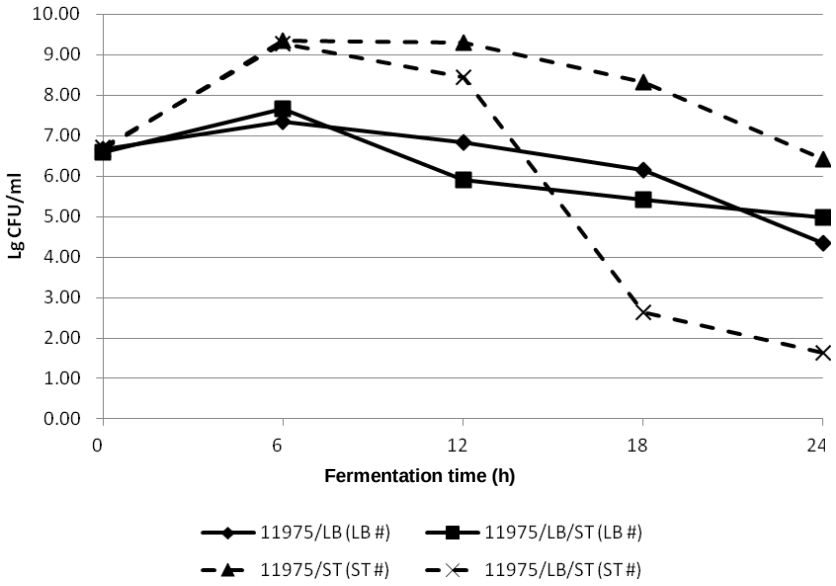

Figure 5. Changes in population of starter cultures LB350 and ST350 during fermentation of goats' milk (ATCC-11975 group). # indicates the enumerated strain.

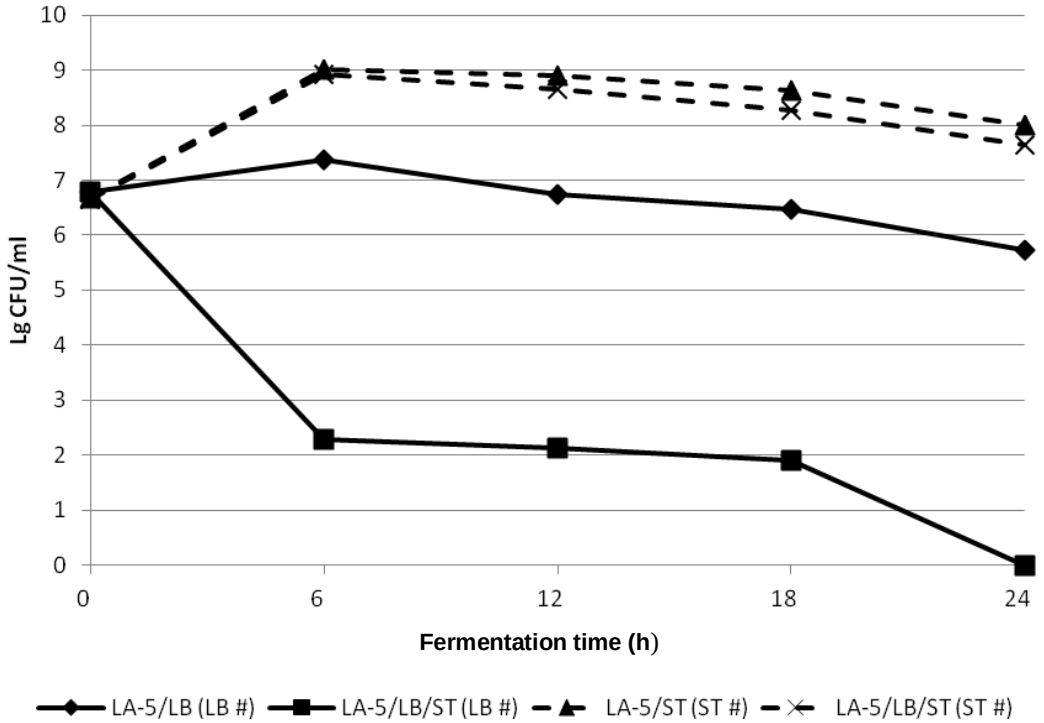

Figure 6. Changes in population of starter cultures LB350 and ST350 during fermentation of goats' milk (LA-5 group). # indicates the enumerated strain.

not seem to be influenced by the presence of ST. For 11975 and NCFM group, both types showed a significant ($P<0.05$) decline in ST when it was combined with LB compared to use as the only starter. However, no effect of LB on ST counts in LA-5 added samples was shown. This appears to show both an unexpected, non-symbiotic interaction between the LB and ST when grown with LA probiotic cultures and one which has very different effects

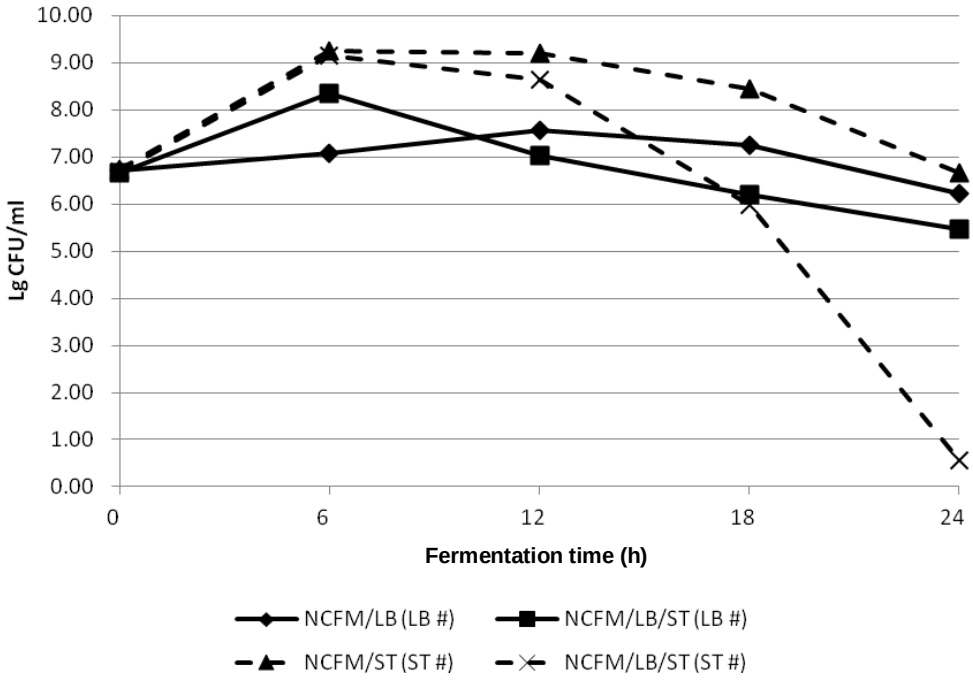

Figure 7. Changes in population of starter cultures LB350 and ST350 during fermentation of goats' milk (NCFM group). #, the enumerated strain.

depending on the strain. In the case of ATCC-11975 and NCFM one possible theory is that these probiotic bacteria are preferentially using the byproducts of LB metabolism and preventing their use by ST thereby stabilizing the probiotic counts while causing the decline of the ST. However a contrasting theory is then required for the opposite effect seen with the LA-5 samples. Further investigation of the cause of these contrasting results may be required.

This effect does not become noticeable until after the first 6 h so it would not necessarily have an impact in a commercial product, where the survival rates of the starters up to 6 h are of primary importance. It is possible, however, that this variation in interaction might produce different flavors and textures of yogurts if they were made over a longer period. When we consider the viable *L. acidophilus* number in all LA/LB/ST samples at 6 h fermentation (the most relevant for commercial yogurt manufacture) LA-5 gives the highest bacterial count (7.54 lg CFU/ml), followed by NCFM (7.31 lg CFU/ml) and ATCC-11975 (7.04 lg CFU/ml), as shown in Figures 2 to 4. Therefore, under these conditions, LA-5 would appear to be the best choice among these three.

However, further studies, such as survivability of each *L. acidophilus* strain during refrigerated storage, should be conducted, using the study's selective enumeration methods. Selection of probiotic strains and their combination with *L. delbrueckii* subsp*. bulgaricus* and *S. thermophilus* are essential for probiotic yogurt manufacture and these can be used in conjunction with other effective methods such as addition of oxygen scavengers (catalase, ascorbic acid, L-cysteine), packaging material (glass bottles instead of plastic containers), and micro encapsulation. According to previous studies, all of these showed their efficacy in improving probiotic count, thus the combination of these approaches may be more effective and promising for application in probiotic yogurt manufacture.

Conclusion

Procedures for clearly differentiating between *L. acidophilus* probiotic strains and starter cultures *S. thermophilus* and *L. bulgaricus* have been developed. Results obtained under these conditions show that addition of LB350 and/or ST350 into the goats' milk inoculated with *L. acidophilus* strains accelerated the acid production in goat milk yogurt. Antagonism between *L. acidophilus* strains and LB350 was observed. There is a stronger symbiotic relationship between *L. acidophilus* strains and ST350 than that between LB350 and ST350, indicating the promise of using starter culture devoid of *L. delbrueckii* subsp*. bulgaricus* for fermented goats' milk products.

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