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Research Article

Production of kefir like product from mixed cultures of *Saccharomyces cerevisiae*, *Streptococcus cremoris* and *Streptococcus lactis*

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Abstract

Various ratios of *Streptococcus cremoris* to *Streptococcus lactis*, 0.5 : 1.5, 1 : 1 and 1.5 : 0.5% were added to milk supplemented with 5% (w/v) sucrose. Fermentation temperature was kept constant at 30°C. For the first 15 h of fermentation, the milk was fermented by 5% (v/v) *Saccharomyces cerevisiae*, followed by lactic acid bacteria. The total fermentation time was 51 h. Chemical compositions and microbial counts were analysed. It was found that 30 h of fermentation seemed to be the optimal fermentation time. After 30 h of fermentation and bacterial ratio of 0.5 : 1.5%, pH, total soluble solids, yeast cell and bacterial cell counts were 4.97, 8.2°Brix, 5.8×10^7 and 3.2×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 16.47, 3.03 and 1.2 g/L, respectively. For bacterial ratio of 1 : 1%, pH, total soluble solids, yeast cell and bacterial cell counts were 4.93, 8.2°Brix, 8.2×10^7 and 4×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 16.57, 2.54 and 1.3 g/L, respectively. For bacterial ratio of 1.5 : 0.5%, pH, total soluble solid, yeast cell and bacterial cell counts were 4.95, 8.4°Brix, 7.8×10^7 and 3.65×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 15.35, 2.95 and 1.1 g/L, respectively.

Keywords: kefir, lactic acid bacteria, *Saccharomyces cerevisiae*, *Streptococcus cremoris*, *Streptococcus lactis*

Introduction

Kefir, as the origin of fermented milk beverages is very old and dates back to the domestication of certain mammals such as the cow, goat and sheep. It is safe to assume that the first types of product were made accidentally by the fermentation of milk, which was stored at ambient temperature (~20-30°C depending on the region). Due to variations in the climatic and environmental conditions in different parts of the world, specific strains of microorganisms became dominant in these products and, as a consequence, specific types of fermentations evolved and became distinct to a given region.

The production of fermented milk was known to the ancient Greeks and Romans. The Greek historian Herodotus reported that a refreshing drink produced from mare's milk was popular among the Getae tribe. According to Caucasian legend, Mahomet (ca. 570 – 632) gave Mahomet's grains (also known as mushrooms) together with the secret kefir recipe to the inhabitants of the region [1]. Thus, kefir is one of oldest fermented milk beverages, and the technology of production and the use of specific starter cultures have developed through the ages. Its origin can be traced back to the Caucasus region where it has been produced by a traditional method in bags made from animal hides, or in oak barrels or earthenware pots. As these containers were used continuously (i.e. after some kefir being consumed, a new batch of milk was added), the microorganisms tended to form a thin layer and later clusters on the surface of the containers. This process of microbial film formation was helped by the warm conditions of the Caucasus [2].

The kefir grains can be produced by using the same traditional method of handling the milk. A goatskin bag (4 L capacity) was used, which was washed several times with sterile water and filled with pasteurized milk and the intestinal flora from a sheep. It was kept at 24 – 16°C for 48 h and shaken every hour. When the milk coagulated, 75% of the fermentate was replaced with fresh milk and this procedure was repeated for 12 weeks. Gradually, a polysaccharide layer appeared on the surface of the hide. The layer was removed aseptically from the hide and propagated in pasteurized cow's milk where grains were formed (0.5 – 3.2 cm in diameter) and added several times to fresh cow's milk [3].

The objective of this research was to investigate a kefir-like product by using pure *Saccharomyces cerevisiae* and various ratios of *Streptococcus cremoris* to *Streptococcus lactis*.

Materials and Methods

Microorganisms

Saccharomyces cerevisiae from the Persian Type Culture Collection, Iranian Research Organization for Science and Technology is maintained at the Department of Biotechnology, Faculty of Agro-Industry, Chiang Mai University. *Streptococcus cremoris* TISTR No. 058 and *Streptococcus lactis* TISTR No. 457 were obtained from the Thailand Institute of Scientific and Technological Research.

Media

The suitable agar media for *Saccharomyces cerevisiae* is PDA, *Streptococcus cremoris* is MRS agar and *Streptococcus lactis* is NA.

Potato Dextrose Agar (PDA) [4], medium with the following composition, per litre: Potato 200.0 g, D-Glucose 20.0 g, Agar 15.0 g, Deionized water 1.0 L. Preparation of potato infusion ; Peel and dice potato (200 g), add 500 ml of deionized water, gently heat and bring to boil, continue boiling for 30 min. Filter through cheese cloth. Bring volume of filtrate to 1 L. Preparation of medium; to 1 L of potato infusion, add glucose and agar. Mix thoroughly, gently heat and bring to boil. Distribute into tubes or flasks. Sterilize for 15 min at 15 psi and 121°C.

deMan Rogosa Sharpe (MRS) [4], medium with the following composition, per litre: Peptone 10.0 g; Beef extract 10.0 g; Yeast extract 5.0 g; Glucose 20.0 g; Tween 80 1.0 g; K₂HPO₄ 2.0 g; Sodium acetate 5.0 g; Tri-ammonium citrate 2.0 g; MgSO₄.7H₂O 0.2 g; MnSO₄.4H₂O 0.2 g; Deionized water 1.0 L.

Nutrient agar (NA) [4], medium with the following composition, per litre: Beef extract 3.0 g; Peptone 5.0 g; Agar 15.0 g; Deionized water 1.0 L.

Methods

Preparation of milk

Cow's milk supplemented with 5% (w/v) sucrose was boiled to 90°C, then it was placed in a water bath at 90°C for 30 min.

Stock cultures

Streptococcus cremoris was maintained in MRS agar, *Streptococcus lactis* was maintained in NA. In addition, *Saccharomyces cerevisiae* was maintained in PDA. The microorganisms were stored at 4°C and periodically sub-cultured every month.

Starter preparation

Lactic acid bacteria

One loop of cultures from MRS or NA slant was inoculated into 30 ml of pasteurized milk and incubated at 37°C for 17 -19 h .

Saccharomyces cerevisiae

For *Saccharomyces cerevisiae*, 1 loop of culture from PDA slant was inoculated into 60 mL of PD broth and incubated in shaker incubator at 35°C for 15 h.

Investigation of fermentation

For yeast, 5% of starter culture was inoculated into 1000 mL of 5% (w/v) of sucrose supplemented milk. Incubation temperature was 30°C. For the first 15 h of fermentation, it was only fermented by yeast.

For LAB ratios, 0.5 : 1.5, 1: 1, 1.5 : 0.5% of *Streptococcus cremoris* to *Streptococcus lactis* were carried out. The volume of sucrose supplemented milk was 1000 mL. The incubation temperature was 30°C. After yeast fermentation for the first 15 h, it was

further fermented with mixed cultured of lactic acid bacteria. The total fermentation time was 51 h.

Biochemical analysis

pH determination

The pH of the sample was determined by using pH meter according to AOAC standard method [5]. It was calibrated with standard buffer solutions pH 7.0 and 4.0 before measuring the samples.

Total soluble solids determination

Total soluble solids of the sample were determined by using hand refractometer 0 – 32°Brix according to AOAC standard method [5]. It was calibrated with distilled water before use.

Microbiological analysis

Enumeration of Streptococcus cremoris and Streptococcus lactis

Fermented milk samples were diluted using 10-fold dilution with sterile saline with 0.85% NaCl. deMan Rogosa Sharpe (MRS) agar with bromocresol purple 0.05% was used for enumeration of *Streptococcus cremoris* and *Streptococcus lactis*. After the agar solidified, the plate of *Streptococcus cremoris* and *Streptococcus lactis* was incubated at 37°C for 48 h.

Enumeration of Saccharomyces cerevisiae

Fermented milk samples were diluted using 10-fold dilution with sterile saline with 0.85% NaCl. Potato Dextrose agar (PDA) was used for enumeration of *Saccharomyces cerevisiae*. After the agar solidified, the plate of *Saccharomyces cerevisiae* was incubated at 37°C for 48 h.

HPLC Analysis

A combination of sucrose and lactose, lactic acid and ethanol concentrations were measured by HPLC using a ODS column (C-18) (SHIMADZU, Kyoto) and a refraction index detector (RID). The mobile phase is 5 mM H₂SO₄ at a flow rate of 0.75 mL/min. The column is maintained at 37°C. Twenty µl of samples by sequential treatments with centrifugation (12,000 rpm, 15 min) and filtering (0.45 µm; Whatman, Kent, UK) are applied.

Results and Discussion

Various ratios of lactics

Various ratios of lactics were added into the milk supplemented with 5% (w/v) sucrose by using pure culture of *Saccharomyces cerevisiae* 5% (v/v). The ratios of *Streptococcus cremoris* to *Streptococcus lactis* were 0.5 : 1.5, 1 : 1 and 1.5 : 0.5%. Fermentation temperature was 30°C. For the first 15 h of fermentation, the milk was fermented by yeast and then further fermented with lactic acid bacteria. The total fermentation time was 51 h. Then, chemical compositions and microbial counts, pH, lactic acid, total soluble solids (°Brix), combination of sucrose and lactose, ethanol and cell numbers were analysed.

Figure 1 shows the pH change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). All of the experiments were carried out during 51 h. LAB were added after 15 h of yeast-fermented milk. It was found that pH of all experiments decreased until the end of fermentation, because LAB produced acid during fermentation. All strains of lactic acid bacteria reduced pH of MRS medium from an initial value of 6.35 – 6.41 to lower than 4.97 of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%), 4.93 of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) and 4.95 of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) during 51 h of fermentation. Katsiari *et al.* [6], reported that the pH decreased with storage time in other fermented milks like yoghurt because of lactose breakdown by the bacteria in the culture. Rao *et al.* [7], reported that *Lactobacillus plantarum* could produce lactic acid and reduce the pH to values lower than 4.00. Yoon *et al.* [8], reported that *Lactobacillus acidophilus* and *Lactobacillus plantarum* reduced the pH of beet juice from an initial value of 6.30 to lower than 4.50 after 48 h of fermentation. Garcia *et al.* [9], reported that *Lactococcus spp.* and *Lactobacillus spp.* could produce lactic acid during the first 24 h of fermentation in kefir made from cows' milk the pH decreased to 4.24.

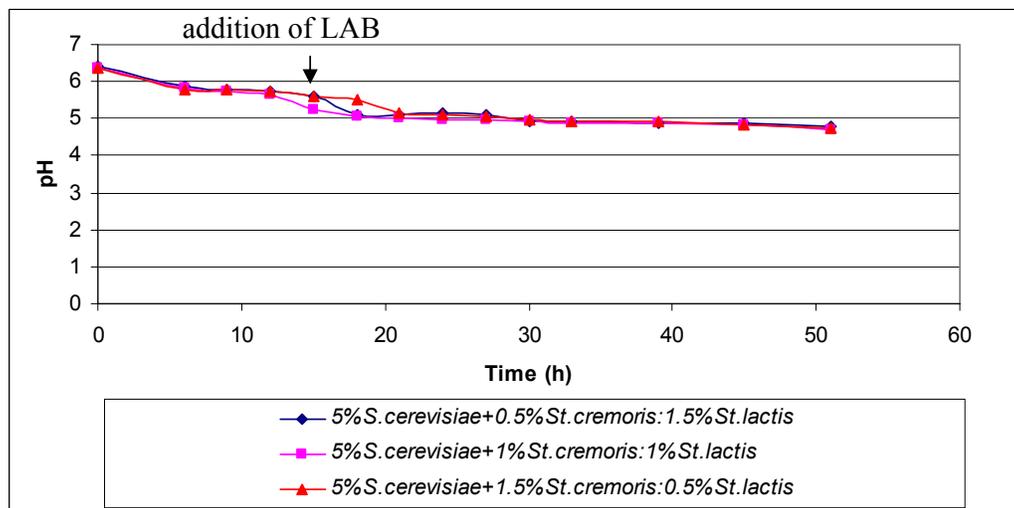


Figure 1. pH Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5%).

Figure 2 shows the lactic acid change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis*

(1.5:0.5%). All of experiments were carried out during 51 h. LAB were added after 15 h of yeast-fermented milk. It was found that lactic acid in all of experiments increased continually from 15 to 39 h of fermentation and thereafter slightly decreased until the end of fermentation.

The optimum yield of lactic acid of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) were 3.03, 2.54 and 2.95 g/L, respectively. It was found that after about 27 - 30 h of fermentation, lactic acid production flattened out until the end of fermentation.

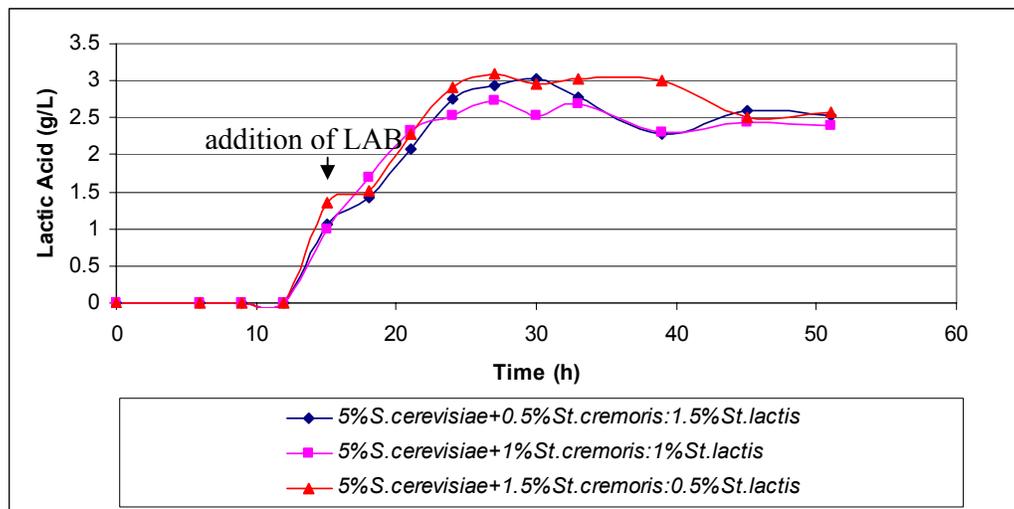


Figure 2. Lactic Acid Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5%).

Figure 3 shows the total soluble solids change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). All of the experiments were carried out during 51 h. Lactic acid bacteria were added after 15 h of yeast-fermented milk. It was found that total soluble solids of all experiments decreased progressively during fermentation until the end of fermentation. It was found that the lowest total soluble solids were 8.2, 8.2 and 8.4°Brix, respectively, after 30 h of fermentation with mixed lactic acid bacteria.

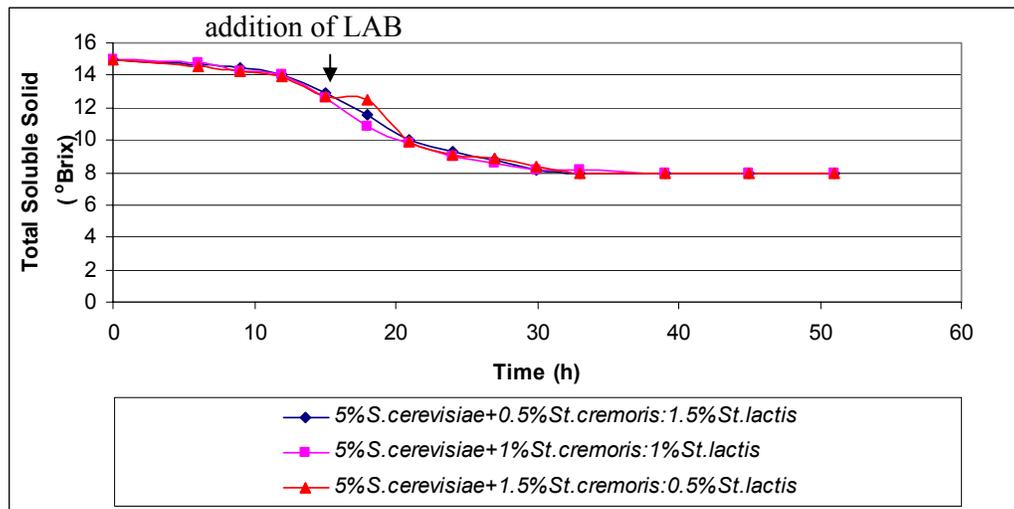


Figure 3. Total Soluble Solids Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5 %).

Figure 4 shows the combination of sucrose and lactose change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). All of the experiments were carried out during 51 h. LAB were added after 15 h of yeast-fermented milk. It was found that sucrose and lactose in all of experiments rapidly decreased during the first 15 h of fermentation and then sucrose and lactose slowly decreased until the end of fermentation.

The optimum yields of the combination of sucrose and lactose of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) after 30 h of fermentation were 16.47, 16.57 and 15.35 g/L, respectively.

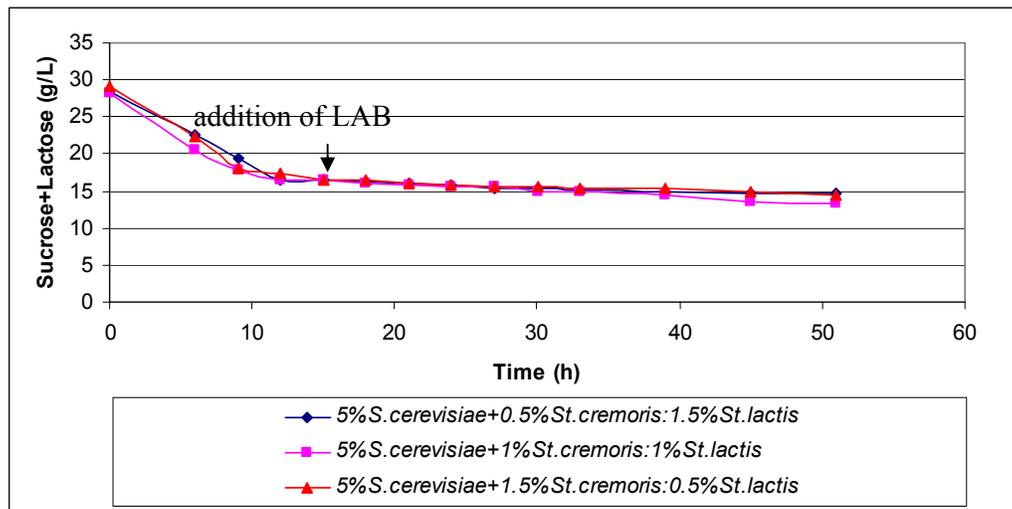


Figure 4. Sucrose and Lactose Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5 %).

Figure 5 shows the ethanol change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). All of the experiments were carried out during 51 h. LAB were added after 15 h of yeast-fermented milk. It was found that ethanol in all of the experiments increased continually from 15 to 30 h of fermentation and thereafter ethanol decreased slightly until the end of fermentation.

The optimum yields of ethanol from *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) were 1.20, 1.30 and 1.10 g/L, respectively. It was found that at 30 h of fermentation, maximum yield of ethanol was obtained. Motaghi *et al.* [3], reported that *Saccharomyces cerevisiae* produced 2.0 g/L of alcohol in fermented milk in 72 h.

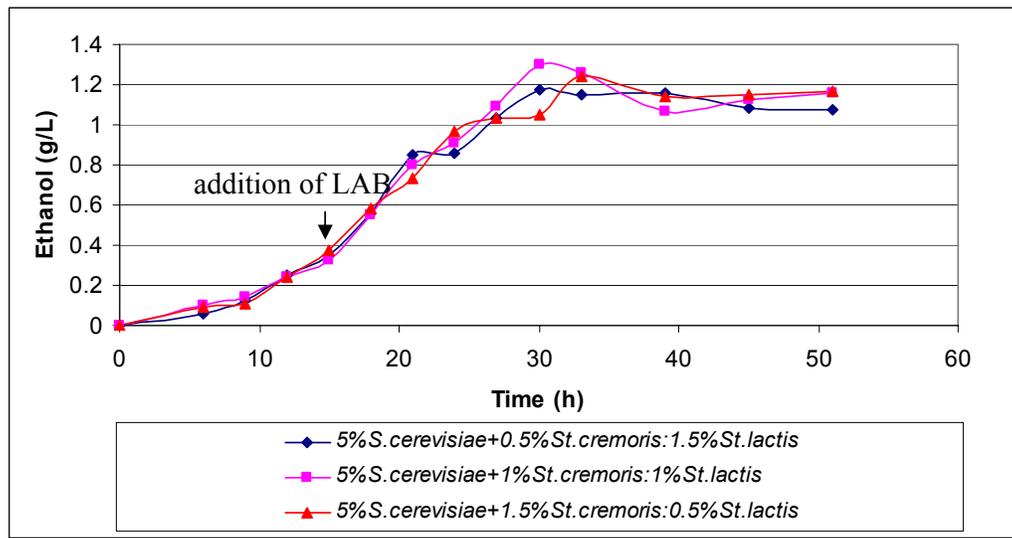


Figure 5. Ethanol Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5 %).

Figure 6 shows the cell number of yeast change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). All of the experiments were carried out during 51 h. LAB were added after 15 h of yeast-fermented milk. It was found that cell numbers of yeast in all of the experiments increased continually during the first 30 h of fermentation and thereafter decreased until the end of fermentation. The highest viable cell count of yeast was found in the additional condition of *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) were 5.80×10^7 , 8.20×10^7 and 7.80×10^7 cfu/mL, respectively. The yeast counts in these experiments were in the range found in fermented milk reported elsewhere [10]. It was reported that *Saccharomyces cerevisiae* counts ranged from 6.0 to 8.0 log cfu/ml and 4.3 to 7.4 log cfu/mL in Tanzanian and Kenyan traditional fermented milks, respectively [10].

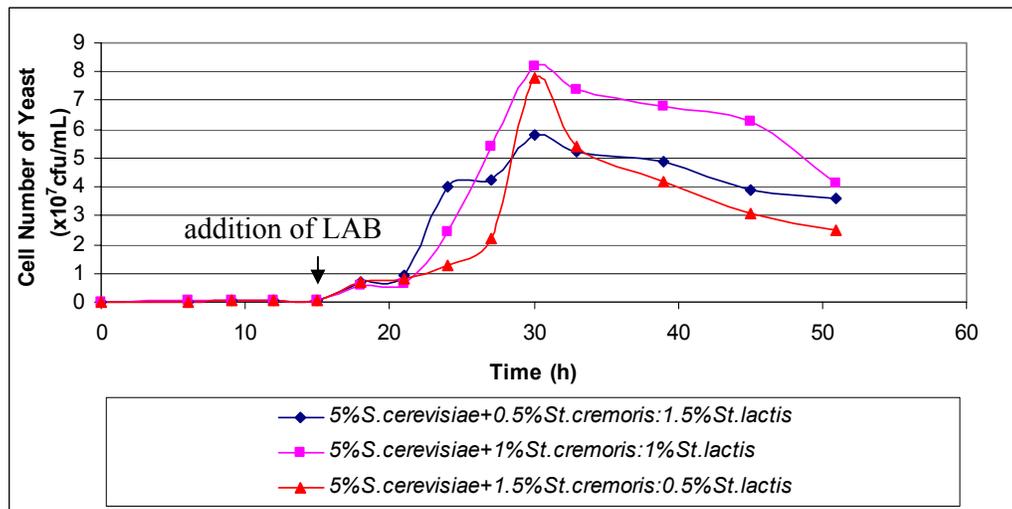


Figure 6. Cell Number of Yeast Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5%).

Figure 7 shows the cell number of LAB change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%). The count in all of the experiments increased continually in the period from 15 to 39 h of fermentation and thereafter decreased until the end of fermentation. The highest viable cell counts of LAB found in *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris* : *Streptococcus lactis* (1.5:0.5%) at 30 h of fermentation from the initial fermentation time were 3.20×10^9 , 4.00×10^9 and 3.65×10^9 cfu/mL, respectively. This was the highest cell numbers. It was reported elsewhere that lactic acid bacteria reached log 9 cfu/mL after 48 h of fermentation at 30°C. Garcia *et al.* [9], reported that kefir made from cows' milk using a commercial starter culture, *Lactococcus spp.* predominated during the first 48 h of fermentation ($\sim 8 \log_{10}$ cfu g⁻¹) and *Lactobacillus spp.* became the predominant species after 48 h ($\sim 8.5 \log_{10}$ cfu g⁻¹).

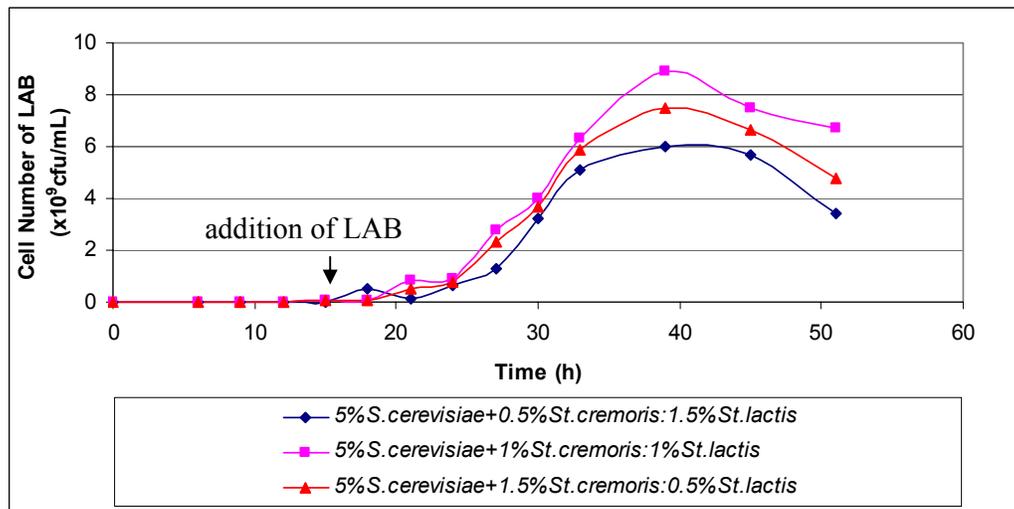


Figure 7. Cell Number of LAB Change due to *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (0.5:1.5%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.0:1.0%) or *Saccharomyces cerevisiae* (5%) and *Streptococcus cremoris*:*Streptococcus lactis* (1.5:0.5%).

Conclusions

The ratios of *Streptococcus cremoris* to *Streptococcus lactis* are 0.5 : 1.5, 1 : 1 and 1.5 : 0.5%. Fermentation temperature was 30°C. For the first 15 h of fermentation, it was fermented by yeast and followed up with lactic acid bacteria. The total fermentation time was 51 h. Then chemical compositions and microbial counts were analysed. It was found that 30 h of fermentation seemed to be the optimal fermentation time.

At this fermentation time and bacterial ratio *Streptococcus cremoris* to *Streptococcus lactis* was 0.5 : 1.5%, pH, the total soluble solids, yeast cells and bacterial cell counts were 4.97, 8.2 °Brix, 5.8×10^7 and 3.2×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 16.47, 3.03 and 1.2 g/L, respectively.

For bacterial ratio *Streptococcus cremoris* to *Streptococcus lactis* was 1 : 1%, pH, the total soluble solids, yeast cells and bacterial cell counts were 4.93, 8.2°Brix, 8.2×10^7 and 4×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 16.57, 2.54 and 1.3 g/L, respectively.

For bacterial ratio *Streptococcus cremoris* to *Streptococcus lactis* was 1.5 : 0.5%, pH, the total soluble solids, yeast cells and bacterial cell counts were 4.95, 8.4°Brix, 7.8×10^7 and 3.65×10^9 cfu/mL, respectively. Combination of sucrose and lactose, lactic acid and ethanol were 15.35, 2.95 and 1.1 g/L, respectively.

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