ISSN 1330-9862
(FTB-1558)

original scientific paper

The Effect of Incubation Temperature on the Survival and Growth of Yeasts in Sethemi, South African Naturally Fermented Milk

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Received: September 21, 2005
Accepted: July 5, 2006

Summary

The effect of temperature on the growth of yeasts during the production of Sethemi, South African naturally fermented milk (NFM), was studied by incubating raw milk and milk inoculated with selected yeast strains at 7, 15, 25 and 37 °C. The different temperatures were selected to represent the average ambient temperatures around Bloemfontein, South Africa, during winter, spring, summer, and in the human body, respectively. The yeast strains used had previously been isolated from Sethemi and identified as *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Candida albicans* and *Debaryomyces hansenii*. The yeast strains were inoculated into raw milk separately and also as a mixture of the four strains. The yeast counts, lactic acid bacteria counts and pH were monitored over a period of 20 days. It was observed that although all the yeast strains grew in the milk at all temperatures, the fastest growth was at 37 °C but there was a prolonged lag phase at 7 and 15 °C. The highest yeast counts of 8.30 log (CFU/mL) were obtained at 25 °C in the milk inoculated with *K. marxianus*. At all temperatures, the initial yeast count in the control was significantly ($p<0.05$) lower than the counts in the inoculated milk. Lactic acid bacteria also grew to high numbers both with added yeast and in the control. The highest LAB counts of about 11.59 log (CFU/mL) were obtained in the presence of *S. cerevisiae* after about 4 days of incubation at 25 °C. The addition of different yeast strains did not affect significantly the growth of LAB at all temperatures. After 3 days, the LAB counts decreased rapidly at 37 °C, while from day 2 to day 5 the LAB numbers remained stable at 25 °C. There was a rapid decrease in pH at higher temperatures than at 7 or 15 °C, corresponding to the LAB growth. A temperature of 25 °C was found to be ideal for producing fermented milk with high LAB counts, low pH and a visually acceptable coagulum.

Key words: yeasts, naturally fermented milk, Sethemi, lactic acid bacteria

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Introduction

Yeasts are widely distributed in nature and are therefore often found as contaminants in both commercial and traditional fermented milk (*1,2*). Several researchers have reported yeast counts ranging between 10^3–10^7 log (CFU/mL) in fermented milk products (*1–5*). Depending on the type of the fermented milk produced, their contribution may either be positive or negative. In yoghurt, their occurrence is mainly a consequence of the contamination and hence they are a major cause of yoghurt spoilage (*6*). On the other hand, during the commercial production of kefir and koumiss they are deliberately introduced into milk to bring about the desired aroma and flavour of the final product (*7*). In naturally fermented milk, yeasts are part of the indigenous microflora, coming into the product with the raw milk or from the environment and containers (*2,8*). In a previous study, yeast counts of up to 6 log (CFU/mL) were reported in Sethemi, South African naturally fermented milk (*9*).

Sethemi is produced by allowing raw milk to spontaneously ferment in gourds or clay pots with a final pH of about 4.1–4.3. As with other naturally fermented types of milk, the characteristics of Sethemi are influenced by the type of the predominant fermenting microorganisms including lactic acid bacteria, yeasts and possibly coliforms. However, the final microbial ecology will be determined by the fermentation conditions.

In a previous study, *Debaryomyces hansenii*, *Candida albicans*, *Clavispora lusitaniae*, *Cryptococcus curvatus*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* were found to be the predominant yeasts in traditionally fermented Sethemi (*9*). *K. marxianus* is a lactose fermenting yeast with good proteolytic and lipolytic properties (*6,10*). *D. hansenii* assimilates lactate, while *S. cerevisiae* is only able to utilize glucose and galactose (*1,6*). However, *S. cerevisiae* has often been isolated from naturally fermented milk (*2,11,12*). *Candida albicans* is an opportunistic pathogen that can cause superficial, localized, and/or systemic infection (*13,14*). The growth of these different types of yeasts during fermentation of milk under different conditions will help in understanding the characteristics of the naturally fermented milk (NFM). The current study, therefore, aims at monitoring the growth of *K. marxianus*, *S. cerevisiae*, *D. hansenii* and *C. albicans* during the production of NFM at different temperatures that depict the ambient conditions in Bloemfontein, South Africa at different times of the year and the temperature in the human body. The associated growth of the LAB and changes in pH were also monitored.

Materials and Methods

Milk samples

Raw milk (4 L) was collected in sterile 5-litre bottles from a dairy farm near Bloemfontein, South Africa, and transported to the Food Biotechnology Laboratory, University of the Free State, in a cooler box. The samples were used within 1 h of sampling.

Preparation of yeast cultures

Pure cultures of *K. marxianus*, *S. cerevisiae*, *D. hansenii* and *C. albicans* strains, previously isolated and identified from Sethemi and kept on yeast extract-malt extract (YM) agar slants (Biolab Diagnostics, Midrand, South Africa), were obtained from the Food Biotechnology Laboratory, University of the Free State. Actively growing cultures were prepared by separately inoculating pure colonies of each yeast strain from the slants into 50 mL of YM broth (Biolab) in 200-mL Erlenmeyer flasks and incubating at 25 °C for 24 h with shaking. Portions of the broth culture (1 mL) were further transferred to other flasks containing 50 mL of YM broth (Biolab) and incubated at 25 °C for 48 h. The microbial load in each flask was estimated by making appropriate serial dilutions of portions of the broth culture and spread plating on YM agar (Biolab).

Preparation of fermented milk

Based on the microbial load in the broth culture, a volume was calculated to give approximately 3 log (CFU/mL) of yeast after inoculation into raw milk (200 mL). A mixed yeast culture was also obtained by mixing appropriate volumes of broth cultures of the yeast strains to obtain a volume that would give approximately 3 log (CFU/mL) of total yeast in the inoculated milk. Four sets of each of the following cultures were inoculated: *K. marxianus*, *S. cerevisiae*, *C. albicans* and *D. hansenii*, as well as a mixed yeast culture of all four strains, and control (uninoculated raw milk).

A set of each was incubated at 7, 15, 25 and 37 °C, representing the mean temperatures for winter, spring/autumn and summer seasons in the Bloemfontein area, and that of the human body, respectively.

A portion (1 mL) of the fermenting milk was withdrawn for analysis from each flask at the following intervals: 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18 and 20 days. At each sampling stage, yeast and lactic acid bacteria counts as well as pH were determined. The experiment was repeated three times.

Microbiological analysis

Yeast counts were determined by spread plating appropriate dilutions (0.1 mL) of the fermenting milk at the different intervals onto Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Basingstoke, UK), and incubating at 25 °C for 72 h. LAB counts were determined by spread plating appropriate dilutions (0.1 mL) onto MRS agar (Merck, Darmstadt, Germany), and incubating at 30 °C for 48 h.

Determination of pH

The pH of the samples was measured at 25 °C with an HI 9321 Microprocessor pH meter (Hanna Instruments, Germany), calibrated using standard buffers to pH=4 and 7.

Statistical analysis

The differences in counts between the different treatments were compared using one way analysis of vari-

ance with the NCSS 2000 statistical software (*15*) at 95 % level of significance.

Results and Discussion

The results represent the mean of 3 independent trials. This study simulated typical fermentation conditions of the different seasons in Bloemfontein (7, 15, 25 °C) and in the human body (37 °C). The changes in yeast and lactic acid bacteria (LAB) counts at the four different temperatures are shown in Figs. 1 A, B, C and D. The control samples had significantly ($p<0.05$) lower initial yeast counts, and the counts in these cultures increased at a lower rate than the inoculated samples. The yeast counts in the inoculated samples, however, represented the total yeast count in the sample. The rate of yeast growth in the fermented milk samples increased with temperature. At 7 and 15 °C, microbial growth was monitored for 20 days because both the yeast and the LAB counts continued to increase during this period (Figs. 1 A and B). The yeasts grew at these two temperatures but at a much slower rate than at either 25 or 37 °C (Figs. 1 C and D). At 7 °C, the highest yeast counts were obtained with *K. marxianus* after 14 days, while at 15 °C the highest yeast counts of 7.54 log (CFU/mL) were also obtained with *K. marxianus* after 5 days. After the maximum numbers were attained at both temperatures, the yeast counts remained stable up to day 20, except for *C. albicans*, whose counts at 15 °C slightly decreased from the high 7.33 to 6.40 log (CFU/mL) at day 20. At 7 °C, *C. albicans* counts increased up to day 20.

At 25 °C, yeast counts in all cultures increased rapidly and the *K. marxianus* culture again had the highest yeast count of 8.30 log (CFU/mL) after 4 days (Fig. 1 C). Thereafter, the yeast numbers remained stable and there was no significant ($p>0.05$) difference between the highest counts and the counts at day 10 for all cultures. These yeast counts are similar to those recorded in ultra-high temperature (UHT) treated milk (*1,8*).

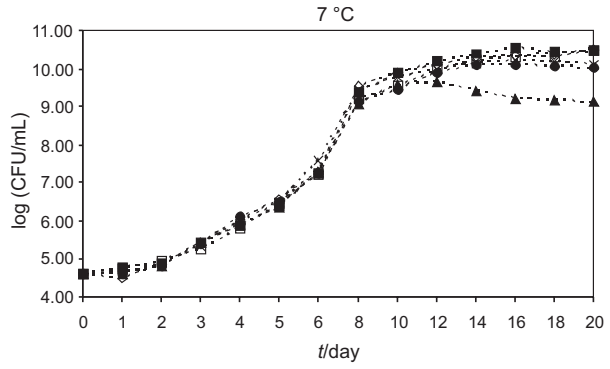
At 37 °C the yeast had a very short lag phase and maximal yeast numbers were obtained after 2 days, with *K. marxianus* having the highest counts (Fig. 1 D). However, the yeast numbers slightly decreased after day 4 in the inoculated samples, while in the control the decrease was noticeable after 3 days. After 10 days, yeast counts in the control were about 5.7 log (CFU/mL). This could indicate that the yeasts quickly exhausted their nutrient sources at this temperature and died. In addition, the milk showed obvious signs of excessive gas formation and was evidently spoiled after 10 days.

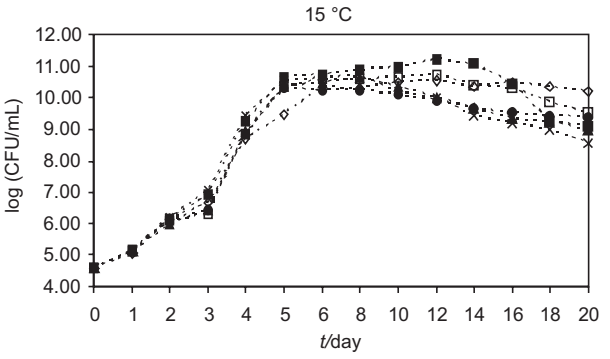
The mixed yeast culture had no significant advantage over the individual yeast cultures and its growth had similar trend as the single cultures at the four different temperatures. For example, at 15 and 25 °C, the mixed culture had similar counts as *K. marxianus* and *D. hansenii* throughout the fermentation period. All the yeast cultures, therefore, showed significant growth at different temperatures. This suggests that all the strains used in the study have a potential to proliferate in the milk during fermentation. *K. marxianus* is well known for its lactose fermenting abilities and therefore had an obvious advantage over the other cultures in the milk (*1,16,17*). The mechanism for growth of *S. cerevisiae* and *C. albicans* in milk is not clear. However, these yeasts could use the glucose and galactose produced by the LAB from lactose. Previous reports have also suggested that *S. cerevisiae* has some weak proteolytic and lipolytic abilities (*16,18,19*). Enzymes associated with the yeast cell wall or intracellular enzymes released into the milk through autolysis are thought to be involved (*16*). This activity and the trace levels of free fatty acids and free amino acids that may already be in the milk could be used for growth. *D. hansenii* is capable of utilizing lactic and citric acids and galactose that accumulate in the milk due to lactose breakdown by LAB (*20*).

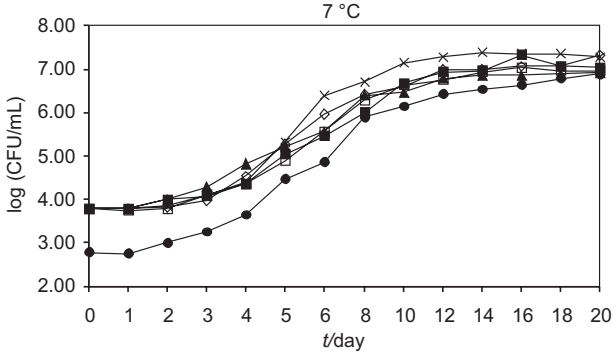
C. albicans, although rarely isolated from milk, grew well in the milk at all temperatures. This is of concern since this organism is known to be an opportunistic pathogen in humans. There was no evidence that *C. albicans* could be inhibited by the LAB and lactoferrin in the milk, in contrast to earlier reports (*21–24*). This means that the conditions in the milk allow the growth and repair of this yeast. Hence, further study on the growth kinetics of *C. albicans* in milk is needed. Torija *et al*. (*25*) observed similar temperature effects on the growth of strains of *S. cerevisiae* in grape must and noted significant changes in the metabolic products produced at different temperatures. In the current study, the lower temperatures only managed to slow down the metabolic rate of the yeasts but the high final counts suggest that yeast metabolism will play an important role in the characteristics of the milk with prolonged fermentation.

In all trials, the LAB counts were significantly higher than yeast counts during the initial stages of fermentation, which may be attributed to better adaptation of the LAB to the environmental conditions, giving them a competitive advantage. Most yeasts, on the other hand, rely on the growth of LAB to support their growth based on the breakdown of lactose. In general, yeasts were overgrown by LAB, only at elevated temperatures (37 °C) the yeasts competed well against them and in fact showed higher loads than the LAB counts (Fig. 1 D). There were no significant ($p<0.05$) differences between LAB counts in the yeast inoculated cultures and the control at all temperatures during the fermentation (Figs. 1 A, B, C and D). At 7 and 15 °C, the lactic acid bacteria had prolonged lag phases both in the yeast-inoculated samples and in the control. However, the LAB increased in numbers up to day 20. At 7 °C the highest LAB counts in the control sample of about 10.0 log (CFU/mL) were obtained after 20 days, while in the mixed culture, LAB counts were about 10.5 log (CFU/mL) at day 20. At the end of the fermentation, there were no significant ($p>0.05$) differences in the LAB counts between the cultures although there was a slight decrease in LAB in the *C. albicans*-inoculated milk from 9.56 log (CFU/mL) at day 10 to 9.11 log (CFU/mL) at day 20 (Fig. 1 A). These observations therefore suggest that even if a fermented milk product can be prepared at low temperatures, it would take several days for the milk to coagulate. This is detrimental to the production of a safe product as psychrotrophic pathogenic bacteria may also multiply to infectious levels.

However, the highest LAB counts of about 11.59 log (CFU/mL) were obtained in the presence of *S. cerevisiae* after about 4 days of incubation at 25 °C (Fig. 1 C), but







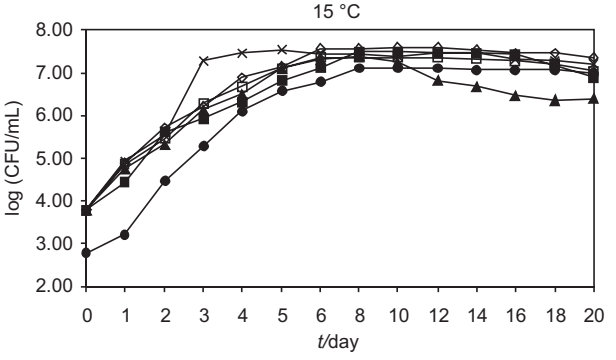
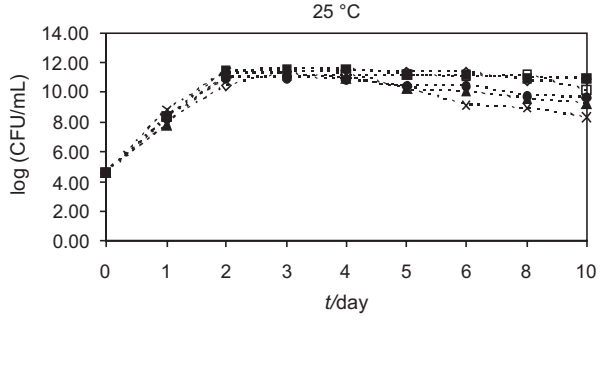
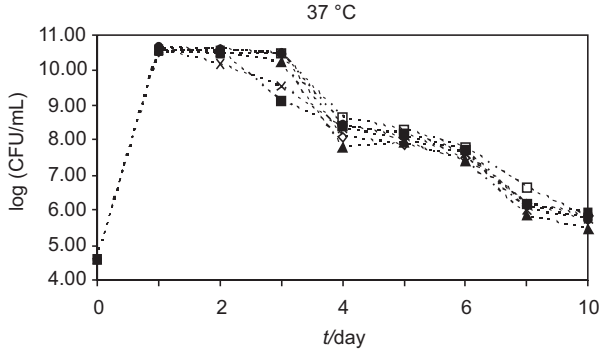
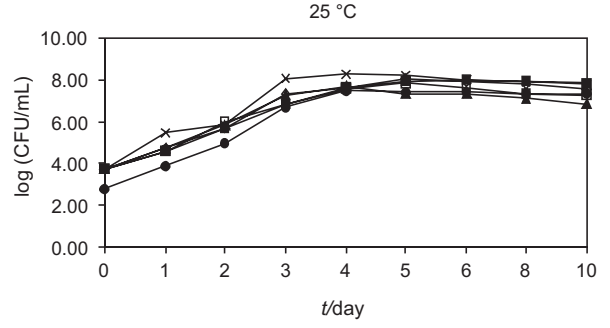


Fig. 1A **Fig. 1B**







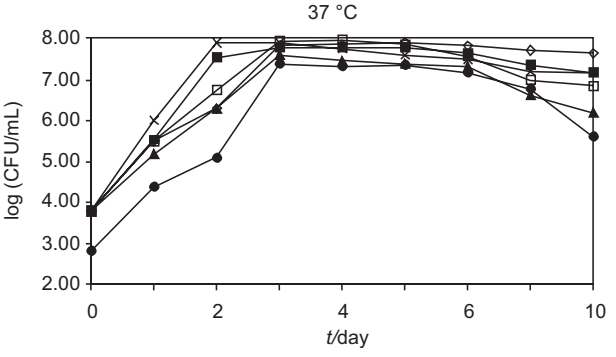


Fig. 1C **Fig. 1D**

Fig. 1. The changes in yeast (——) and lactic acid bacteria (----) counts during the fermentation of raw milk at 7 (1A), 15 (1B), 25 (1C) and 37 °C (1D) after inoculation with *Kluyveromyces marxianus* (×), *Saccharomyces cerevisiae* (■), *Candida albicans* (▲), *Debaryomyces hansenii* (◊) and a mixture of these four yeasts (□). The control (●) had no added yeast

this was not significantly ($p>0.05$) different from the other cultures. High counts of up to 11 log (CFU/mL) were recorded after 2 days in all cultures at 25 °C. In comparison, at 37 °C, maximum LAB counts of 11 log (CFU/mL) were obtained after 12 h (Fig. 1 D). At this higher temperature LAB counts decreased rapidly after 3 days, while at 25 °C the LAB numbers remained stable from day 2 to day 5. The decrease in LAB counts at both 25 and 37 °C was most significant in the milk inoculated with *K. marxianus*. The final LAB counts at 25 °C in the presence of *K. marxianus* were about 8.2 log (CFU/mL). These lower counts suggest possible inhibition of LAB in the presence of *K. marxianus* due to high levels of gas and alcohol produced as it grows in milk. Green and Ibe (*4*) had previously reported that high levels of alcohol in naturally fermented milk retarded the growth of LAB. Lactose is not limiting in milk and therefore competition for nutrients was unlikely to cause the sharp decline in LAB numbers.

The pH of the milk also decreased significantly faster at the higher incubation temperatures (results not shown). For example, at 37 °C the pH dropped sharply from an average of 6.69 to about 4.3 in about 12 h, while at 15 °C the pH was still about 5.37 after 24 h. The pH of the different cultures was not significantly different at any given temperature, suggesting that the yeast did not affect the acidification process of the LAB. The final pH was therefore determined mainly by the incubation temperature and not by the type of yeast culture.

A smooth coagulum with high numbers of LAB could, therefore, be produced in a reasonably short time at 25 °C. This coagulum was stable until the yeast counts increased to high levels of about 6–8 log (CFU/mL), resulting in gas production and disruption of the coagulum. This suggests that during summer when the ambient temperature is about 25 °C, an organoleptically acceptable Sethemi could be produced but should be consumed within 2 to 4 days of fermentation. At 37 °C, on the other hand, the fermented milk was evidently spoiled after 2–3 days.

Conclusion

It can be concluded from the above that 25 °C is the ideal temperature for preparing Sethemi. Fermented milk with a smooth coagulum, high numbers of LAB and low pH can be obtained in a reasonably short time. Again at this temperature, the yeasts grow to high numbers, especially *K. marxianus*. Possible yeast-LAB interactions in the presence of *K. marxianus* are manifested through the reduction in LAB counts during prolonged incubation. However, the significant growth of the opportunistic pathogen *C. albicans* at all temperatures is a safety concern for the fermented milk.

Acknowledgements

The study was supported through grants from the South African National Research Fund (NRF).

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