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The effects of different concentrations of natamycin and the point of addition on some physicochemical and microbial properties of vanilla-flavoured yoghurt under refrigerated condition

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Abstract

Yogurt is among the most common dairy products consumed around the world. Although it has many desirable properties, the product is still susceptible to deterioration during refrigeration. The study was carried out to determine the effects of different concentrations of natamycin and the point of its addition on some physicochemical properties of vanilla-flavoured yoghurt stored under refrigeration at 5 ± 1°C. Yoghurt samples were preserved by addition of Natamycin at concentrations of 5, 6, 7, 8, 9 and 10 ppm. Total soluble sugars and pH decreased while titratable acidity increased during the 28 days of storage. The changes in quality parameters were not as pronounced in samples containing 8, 9 and 10 ppm compared to those with lower concentrations of the preservative. The preservative effectively and significantly reduced yeast counts at these higher concentrations, while yeast levels increased in yoghurts with zero, 5 and 6 ppm. The concentration of 8 ppm resulted in the highest percentage decrease in yeast counts of yoghurt samples and was consequently used as the appropriate concentration of natamycin for preservation. Generally the addition of 8 ppm resulted in no significant differences ($p > 0.05$) in the changes in four quality parameters of yoghurt analyzed.

Introduction

Milk is a highly unstable food as it is a very good medium for the growth of many microorganisms. Preservation of milk by fermentation aims at converting it into more stable nutritious and desirable products such as yoghurt, cheese and butter milk (Sivansankar, 2010). Fermented foods are popularly accepted for their flavour, better keeping quality, and the fact that fermentation creates variety among foods. Yoghurt is produced through the fermentation of pasteurized milk by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Tamime and Robinson, 2007).

Research work has been done on the effect of several preservation techniques on yoghurt (Davies, 1970; Viljoen *et al.*, 2003). The efficacy of several chemical preservatives (Akpan *et al.*, 2007; Al. Otaibi and El. Demerdash, 2008) and bacteriocins (Gupta and Prasad, 1989; Var *et al.*, 2004) have also been published. One such bacteriocin is the natural polyene macrolide antibiotic known as natamycin or pimaricin which is obtained from the bacterium *Streptomyces natalensis*. It inhibits microbial activity by binding to and altering fungal cell membrane sterols so that vital structures inside the cell pass through the cell membrane and out of the cell (Hamilton-Miller, 1974; Deacon, 1997). In view of the fact that bacteria do not possess membrane sterols, they are insensitive to this antimicrobial agent. Natamycin has been successfully used in the preservation of food products such as cheese, meats, juices and wines without any adverse effect on the organoleptic characteristics of the product. The concentration of natamycin for yoghurt preservation has been suggested to be in the range of 5-10 ppm (Thomas and Delves-Broughton, 2001).

Despite its many health benefits, yoghurt generally has a short shelf life of about 30 days when stored at low temperatures of about 5°C (Davies, 1970, Lourens-Hattingh and Viljoen, 2002). Although yeast cells are not involved in the fermentation process during yoghurt production, they are the major cause of spoilage of the products. Due to the inherent low pH of yoghurt and the ability of yeasts to assimilate sugars, lactose and organic acids, the product acts as a selective environment for the growth of yeasts. Yeast appear as contaminants from the processing equipment and to a lesser extent, from the fruit, honey and sugar used as additives in the product (Suriyarachchi and Fleet, 1981; Fleet and Mian, 1987; Davies, 1970). In some instances, its resistance to preservatives might be an added cause of its prevalence in the product (Green and Ibe, 1986).

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The use of natamycin as a natural preservative in dairy products and other foods has been approved in over sixty countries (Delves-Broughton *et al.*, 2005). The preservative is used widely in foods and many researchers have found it to be effective against microbial activities of yeasts and moulds (Jay *et al.*, 2005). The objectives of this study was to determine the effects of different concentrations of natamycin and its point of addition (during production) on some physicochemical and microbial properties of vanilla-flavoured yoghurt stored under refrigeration conditions

Materials and Methods

Source of raw materials

Whole milk powder (TMC Dairies (N.I) ltd., Northern Ireland, UK) was obtained from a local market and Natamycin (CAPE Natamycin) was bought from South Africa.

Preparation of yoghurt

Stirred yoghurt was prepared based on described procedures for yoghurt preparation and the selected pasteurization conditions for temperature and time were 90°C for 15 minutes (*http://necfe.foodscience.cornell.edu/ publication/pdf/FS_YoghurtProduction.pdf*). An amount of 1050 g of whole milk powder was mixed with 7 liters of tap water and the mixture was gently and thoroughly stirred to dissolve the milk powder and obtain a homogenous solution. The milk was pasteurized at a temperature of 90°C for 15 minutes with continuous stirring. The pasteurized milk was cooled to a temperature of 45°C in a container of cold water which was at a temperature of 5°C and then inoculated with 2% yoghurt starter culture. The bulk preparation was poured into seven plastic containers with one liter capacity and they were incubated at 45°C (Gallenkamp Plus II Incubator, England) for four hours. After incubation the containers were stirred to break the coagulum and obtain a consistent product after which vanilla flavour and 50 g of sugar were added.

Addition of different concentrations of natamycin to yoghurt

Natamycin was added to each one liter container of yoghurt at concentrations of zero (as control) 5, 6, 7 8, 9 and 10 ppm. Each of the seven containers of yoghurt was distributed into 200 ml labeled bottles (N0, N5, N6, N7, N8, N9 and N10) to represent natamycin at zero, 5, 6, 7, 8, 9, and 10 ppm respectively. The bottles of yoghurt were stored under refrigeration conditions at 5 ±1ºC. The procedures of preparation of yoghurt and addition of natamycin to yoghurt were repeated under exact conditions to obtain duplicate samples of yoghurt. Based on results obtained after adding the different concentrations of natamycin, 8 ppm of natamycin was added to yoghurt samples at two stages of preparation: (a) before incubation and (b) after incubation.

Physicochemical analyses of samples during refrigeration

Analyses were done on weekly basis starting from the day of preparation up to a total storage period of 28 days.

Determination of total soluble solids

The total soluble sugar (TSS) was determined by placing a few drops of yoghurt on the prism surface of a hand-held Refractometer (ATAGO Manual Refractometer) and reading the results on a percentage scale. The results were adjusted based on tabulated values for the temperature correction from the temperature of the sample (25ºC) to the reference temperature (20ºC) of the refractometer.

Determination of pH and titratable acidity

About 30 ml of yoghurt was poured into three 50 ml beakers and the pH was measured with an electric digital pH meter (BECKMAN Φ 340 pH/ Temp. Meter). The titratable acidities (TAs) of the yoghurt samples were determined by titrating a diluted amount of sample (20 ml of yoghurt diluted with 20 ml of distilled water) with 0.1N NaOH using phenolphthalein indicator, according to AOAC methods (AOAC, 2005). Calculation was based on percentage lactic acid.

Determination of Yeast counts

The total yeast loads of samples were determined by the pour plate method using yeast extract agar (CM0019 –OXOID Ltd.) as the culture medium. Serial dilutions (10^{-1} to 10^{-6}) of the samples were prepared, plated and incubated for 48 hours at 37°C after which yeast colonies were counted with a Colony counter (Stuart Scientific). Yeast colonies of plates inoculated with dilutions of 10^{-4} were recorded. The microbial loads in Colony Forming Units (CFU) were converted to log_{10} and the results reported as the average from duplicate samples.

Statistical analyses

Statgraphics Centurion XV.I statistical program was used for the data analyses. Means and standard deviations were determined. The results were subjected to analysis of variance (ANOVA) using a two factor complete block design to determine differences in means of quality parameters of yoghurts. Significance differences between means were determined at 95% confidence interval using the least significant difference (LSD) test.

Results and Discussion

Changes in the physicochemical properties of yoghurt preserved with different concentrations of natamycin

Changes in pH

Table 1 shows changes in pH of yoghurts during the four week period of storage at 5 ± 1°C. Generally as the number of days of storage increased, pH of samples decreased. The pH reduced from 4.56 to 4.18 in yoghurts preserved with natamycin at 10 ppm, and 4.56 to 4.12 in the control sample. The pH of yoghurts containing natamycin at concentrations of 8, 9 and 10 ppm did not reduce as much from the 14th day onwards compared to yoghurt samples with lower concentrations of the preservative which had pH up to 4.11 at the end of the storage period while those with more preservative had pH reduction to a range between 4.15 and 4.19. There were no significant differences ($p > 0.05$) in the pH of yoghurts of all concentrations on the day of preparation, on the 7th and 14th days of storage. However on the 21st and final days of storage, significant differences ($p < 0.05$) existed between the samples. Both the concentration of natamycin and days of storage had a significant effect ($p < 0.05$) on the pH. As the number of days increased, the pH values decreased. The extent of decrease in pH varies with the rate of growth of bacteria, interactions between yoghurt culture bacteria and other probiotic or spoilage microorganisms present in the product, additives present (e.g. fruits), storage temperatures and physicochemical properties such as total solids (TS) and total soluble solids (TSS) which provide fermentable substrate for lactic acid bacteria (LAB) and other spoilage bacteria such as coliforms. Several researchers have reported different degrees of decrease in pH under different storage conditions, as affected by the factors mentioned above (Yeganehzad *et al.*, 2007; Akpan *et al.*, 2007; Viljeon *et al.*, 2003). In the use of natamycin as a preservative in yoghurt, El-Diasty *et al.* (2009) also reported a reduction of pH from 4.56 to 4.50 when 10 and 20 mg/kg of the preservative were used, while the pH of the control reduced from 4.56 to 4.14 in 28 days. Similarly, Akpan *et al.* (2007) reported a decrease in pH from 4.4 to 3.79 at the end of 21 days of storage under refrigeration of yoghurt preserved with 10 mg/ml sodium benzoate, while the drop in pH was not all that low (4.07) when higher

Table 1. The pH of yoghurts with different concentrations of natamycin during storage at 5°C

Concentration of natamycin	Day zero	7th Day	14th day	21st day	28th day
N0	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.42 ± 0.00^{a}	4.22 ± 0.01^{a}	4.12± 0.01^{a}
N5	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.41 ± 0.00^{b}	4.23 ± 0.01^{a}	4.11± 0.01^{a}
N6	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.41 ± 0.00^{b}	4.22 ± 0.00^{a}	4.11± 0.00^{a}
N7	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.41 ± 0.00^{b}	4.24 ± 0.01^{ab}	4.15± 0.00^{b}
N8	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.42 ± 0.01^{ab}	4.26 ± 0.01^{c}	4.19± 0.01^{c}
N9	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.41 ± 0.00^{b}	4.25 ± 0.01^{cb}	4.19± 0.01^{c}
N10	4.56 ± 0.01^{a}	4.55 ± 0.00^{a}	4.41 ± 0.00^{b}	4.26 ± 0.01^{c}	4.18± 0.00^{c}

N0, N5, N6, N7, N8, N9 and N10 represent the control sample and samples containing natamycin at 5, 6, 7, 8, 9 and 10 ppm respectively. Mean values with different superscripts in the same column indicate significant differences ($p < 0.05$).

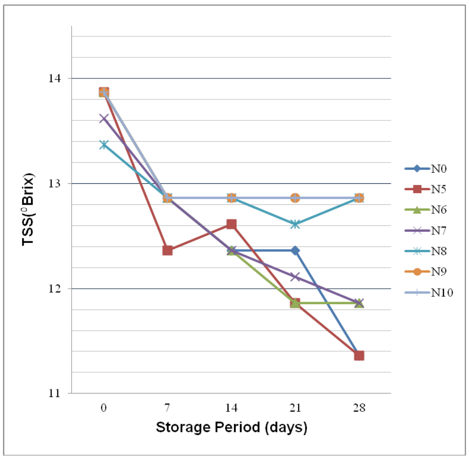

Figure 1. Effect of concentrations of natamycin on TSS of yoghurts under refrigeration

concentration (20 mg/ml) of the same preservative was used. Probiotic bacteria are slow acid producers (Marshall and Tamime, 1997). The yoghurt starter cultures including *L. delbrueckii* subsp *bulgaricus* and *S. thermophilus* are active even at refrigerated temperature and still can produce small amounts of lactic acid by fermentation of lactose which results is noticeable pH decrease (Shah *et al*., 1995).

Changes in total soluble sugars (TSS) of yoghurt samples

Figure 1 shows the progressive decrease of TSS of the yoghurts with different concentrations of natamycin from the day of preparation to the end of the storage period. The TSS reduced throughout the 28 days of storage. Both the concentration of natamycin and the number of days of storage had significant effects ($p < 0.05$) on the TSS of yoghurt samples. As the number of days of storage increased, the TSS of yoghurts containing higher concentrations (8, 9 and 10 ppm) of natamycin decreased to a lesser extent from 13.87 to 12.86°Brix compared to those of lower concentrations (5, 6, 7 ppm) where a much lower reduction up to 11.36 and 11.86°Brix was established The decrease in TSS may be attributed to yeast utilization of sugars in metabolic processes for energy production (*http://biochemie.web.med.*

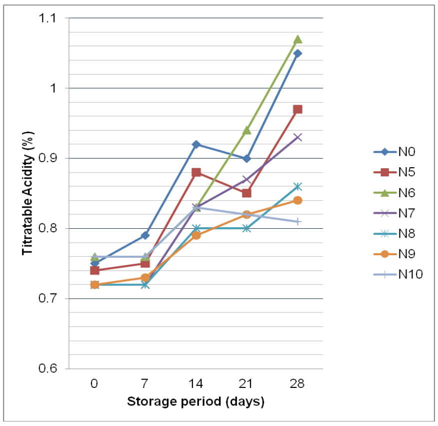

Figure 2. Effect of concentrations of natamycin on TA of yoghurts under refrigeration

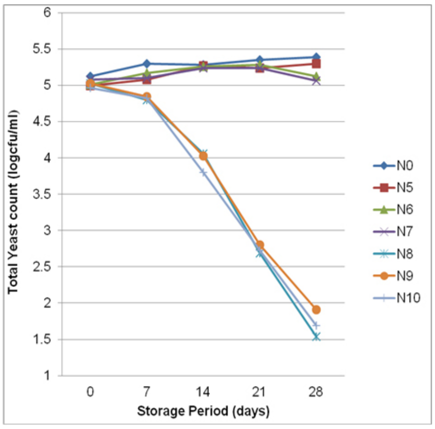

Figure 3. Effect of concentrations of natamycin on yeast counts in yoghurts under refrigeration

unimuenchen.de/Yeast_Biol/03%20Yeast%20 Metabolism.pdf). Decreases in total soluble solids in yoghurts from 7.33% to 6.83% and 15.33% to 14.93%, for corn milk and cow milk yoghurts respectively, have also been reported and these reductions have also been attributed to the utilization of sugar by the starter cultures (Vasiljevic and Jelen , 2002; Wang *et al.*, 2002).

Changes in titratable acidity (TA)

The higher lactic acid contents of samples N0 (1.05%), N5 (0.98%), N6 (1.07%) and N7 (0.93%) at the end of storage period indicate that these samples comparatively supported the proliferation of more Lactic Acid Bacteria (LAB) than samples N8, N9 and N10 in which there were lower acid levels of 0.87, 0.84 and 0.80% respectively (Figure 2). As the number of days of storage increased, TA also increased. The lower acid level observed in samples with higher concentrations of natamycin at the end of the storage period correlated with the less pronounced decrease in TSS and pH levels in these samples. Titratable acidity of yoghurts tends to increase during cold storage. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are responsible for the post-acidification of yoghurt (Donkor, 2006). This post-acidification, during storage, is due to beta-galactosidase which is still active at 0–5°C (Kailasapathy, 2006).

The final acidities of all yoghurts at the end of the storage period were comparable to values recorded by El-Diasty *et al.* (2009). They reported titratable acidities ranging from 0.78 to 0.89% for yoghurt treated with 10 and 20 ppm natamycin and from 0.78 to 0.92% for control samples. This is a common observation during the storage of yoghurt when some other preservatives like sodium benzoate, potassium metabisulphite and essential oils are used to improve the shelf life of yoghurt (Al. Otaibi and El. Demerdash, 2008; Akpan *et al.*, 2007).

Changes in Total Yeast counts of Yoghurts

Figure 3 shows that yeast counts reduced drastically from 5.03, 5.03 and 4.90 log cfu/ml to 1.54, 1.91 and 1.69 log cfu/ml in samples containing 8, 9 and 10 ppm of natamycin respectively, whereas in samples containing no natamycin and 5 ppm, yeast counts continued to increase throughout the period. In these samples yeast cells increased from 5.13 and 5.0 log cfu/ml on the day of preparation to 5.39 and 5.30 log cfu/ml respectively on the final day of storage. However, samples with natmycin concentration of 6 and 7 ppm increased slowly from the 7th day up to the 21st day and then on the 28th day decreased slightly. Similar to the case of pH, TSS and TA, both the concentration of natamycin and number of days of storage had significant effects ($p < 0.05$) on the total yeast counts in the yoghurt samples. There was no significant difference in yeast counts in all samples on the day of preparation ($p > 0.05$). However on the 7th to 28th day of storage, there were significant differences ($p < 0.05$) in yeast counts of the yoghurts samples.

Yeasts are a major cause of spoilage of yoghurt and fermented milks in which the low pH provides a selective environment for their growth (Fleet, 1990; Rohm *et al.*, 1992). The nutritional requirement of yeast, the ability to grow at low temperature, low pH, low moisture content, and high salt or sugar concentrations together with their enzymatic activity make yeast a natural part of the microbiota in dairy products (Hansen and Jakobsen, 2004). Studies of yeast in retail yoghurt have been reported from different countries. It is not unusual to detect yeast in yoghurt in numbers of 10^3 cells/g and yeast count up to 10^8 cells/g has been reported. The contamination rate of yeast in yoghurt was in the order of 10^3 for 20% of the examined samples in United Kingdom

Table 2. Changes in pH, TA and TSS of yoghurts with natamycin added before and after incubation of milk base

	Day zero	7th Day	14th Day	21st Day	28th Day
pH					
N0	4.54 ± 0.03^{a}	4.33 ± 0.02^{a}	4.24 ± 0.02^{a}	4.20 ± 0.01^{a}	4.00 ± 0.04^{a}
NB	4.53 ± 0.02^{a}	4.44 ± 0.01^{b}	4.31 ± 0.01^{b}	4.25 ± 0.01^{b}	3.97 ± 0.01^{a}
NA	4.55 ± 0.01^{a}	4.46 ± 0.01^{b}	4.29 ± 0.02^{ab}	4.29 ± 0.00^{c}	4.01 ± 0.01^{a}
Titratable Acidity					
N0	0.71 ± 0.00^{a}	0.73 ± 0.00^{a}	0.98 ± 0.01^{a}	0.99 ± 0.01^{a}	1.01 ± 0.02^{a}
NB	0.72 ± 0.00^{a}	0.72 ± 0.01^{a}	0.86 ± 0.01^{b}	0.92 ± 0.04^{a}	0.95 ± 0.02^{a}
NA	0.72 ± 0.01^{a}	0.72 ± 0.01^{a}	0.90 ± 0.01^{c}	0.93 ± 0.00^{a}	0.95 ± 0.04^{a}
TSS					
N0	13.37 ± 0.00^{a}	13.11 ± 0.35^{a}	12.61 ± 0.35^{a}	12.36 ± 0.00^{a}	11.36 ± 0.00^{a}
NB	13.37 ± 0.00^{a}	12.86 ± 0.00^{a}	12.36 ± 0.00^{a}	12.60 ± 0.33^{a}	11.86 ± 0.00^{ab}
NA	13.62 ± 0.35^{a}	12.61 ± 0.35^{a}	12.36 ± 0.00^{a}	12.36 ± 0.00^{a}	12.11 ± 0.35^{b}

N0, NB and NA respectively represent yoghurt samples without natamycin, with natamycin added before fermentation and those added after fermentation. Mean values of a particular property with different superscript in the same column indicate significant differences ($p < 0.05$).

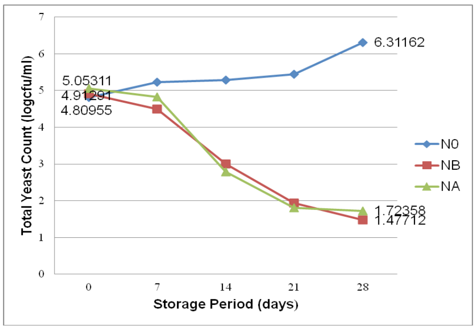

Figure 4. Effect of point of addition of natamycin during yoghurt preparation on the yeast counts in yoghurts under refrigerated condition

(Davis, 1975) and Canada (Arnott *et al.*, 1974). The occurrence and growth of yeast in yoghurt are closely related to poor hygiene and sanitation (Fleet and Mian, 1987). It has been emphasized that spoilage can be prevented through implementation of general principles of good manufacturing practice (Fleet, 1990; Jakobsen and Narvhus, 1996). In the present study high concentrations of the preservative reduced greatly yeast counts with increase in days of storage. Natamycin acts by disrupting cell membranes of yeasts and moulds, causing leakage and eventual lysis (Food Standards, 2004).

The effects of addition of 8 ppm of natamycin at two stages of yoghurt preparation

In the study of the effect of different concentrations of natamycin on yoghurt during storage, addition of 8 ppm of natamycin resulted in the highest percentage decrease in yeast counts of yoghurt samples. The percentage decrease in yeast counts were 69.36%, 61.99% and 65.99% in yoghurts with natamycin at 8, 9 and 10 ppm respectively. A concentration of 8 ppm of natamycin was, therefore, added to yoghurts during preparation before incubation of the milk base (NB) and after incubation (NA).

On the day of preparation (day zero), there were no significant differences ($p > 0.05$) in pH, TA and TSS of the yoghurts with natamycin added before fermentation and those with the preservative added after fermentation (Table 2). The pH were 4.54, 4.53 and 4.55 for samples N0, NB and NA respectively immediately after preparation of yoghurts. Significant differences ($p < 0.05$) between NB and NA in terms of TA and pH occurred on only on the 14th day and 21st day respectively. There were no significance differences in TSS in the two points of addition throughout the storage period of 28 days. Generally the addition of natamycin before or after fermentation did not have significant effects on the pH, TA and TSS.

Figure 4 shows that the yeast loads increased from 4.81 log cfu/ml to 6.31 log cfu/ml in the control (N0), and decreased drastically from 4.91 log cfu/ml and 5.05 log cfu/ml to 1.48 log cfu/ml and 1.72 log cfu/ml in NB and NA respectively. Yeast counts decreased in the treated samples, while the controls supported the proliferation of yeast cells. This reflects in the lower TSS in the control at the end of the storage period and consequently in their higher levels of lactic acid compared to the samples treated with natamycin. There were no significant differences ($p > 0.05$) in total yeast counts between NB and NA.

Conclusion

Natamycin at concentrations of 8-10 ppm in vanilla-flavoured yoghurt effectively and drastically reduced yeast growth in yoghurt compared to lower concentrations of 5-7 ppm. These higher concentrations of natamycin also resulted in relatively minimal changes in important physicochemical properties (pH, TA and TSS) than the lower concentrations. Among the range of concentrations used in the preservation, the natamycin concentration of 8 ppm resulted in the highest percentage decrease in yeast counts of yoghurt samples. The percentage decrease in yeast counts were 69.36%, 61.99% and 65.99% in yoghurts with natamycin at 8, 9 and 10 ppm respectively. A concentration of 8 ppm of natamycin was consequently added to yoghurts during preparation before incubation of the milk base (NB) and after incubation (NA). Generally the addition of natamycin to the product before or after fermentation of the milk base did not have any significant effect ($p > 0.05$) on quality parameters measured in this study.

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