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Manufacture and Quality of Fermented Milks Prepared Using Pure Strains of Lactic Acid Bacteria (LAB) and Yeast

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Abstract: In the present study lactic acid bacterial strains previously identified as the main fermenting microorganisms of the Sudanese *garris* and yeast strains previously identified as the dominating yeast strains of the Sudanese *robe*, were used to prepare fermented milk products in High Temperature Short Time (HTST) pasteurized cows milk. The lactic acid bacteria included *Lactobacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus fermentum* while the yeast strains included *Pichia membranefaciens* and *Candida famata*. The strains were inoculated into HTST milk and incubated at 25°C. The changes in proximate chemical composition, organic acids and viable microbial counts were estimated. The LAB reached maximum counts after 18 h, while the yeast attained maximum populations after 24 h. The study showed that a stable fermented milk product with high nutritive value could be produced using a mixed culture of lactic acid bacteria and yeast and that a fermentation period of 24 h at 25°C would be sufficient.

Key words: Quality, lactic acid bacterial, yeast, organic acid

INTRODUCTION

In fermentation, the raw materials are converted by microorganisms (bacteria, yeasts and moulds) to products that have acceptable qualities of food (Ray and Daeschel, 1992). In natural fermentation, the conditions are set so that the desirable microorganisms grow preferentially and produce metabolic byproducts which give the unique characteristics of the product. When the yield is unstable and where the desired microorganisms might not grow, a controlled fermentation is used. In a controlled fermentation the fermentative microorganisms are isolated and characterized then maintained for use as starter culture. Starter cultures are added to the raw materials in large numbers and incubated under optimal conditions.

Combinations of different bacterial strains belonging to the genera *Lactobacillus*, *Streptococcus* and *Bifidobacterium*, have been used traditionally in fermented dairy products to promote human health (Dunnel *et al*., 1999).

Fermented milks are products prepared from milks, whole, partially or fully skimmed, concentrated or milk substituted from partially or fully skimmed dried milk, homogenized or un-homogenized, pasteurized or sterilized and fermented by means of specific microorganisms. Milk from eight species of domesticated mammals (cow, buffalo, sheep, goat, horse, camel, yak and zebu) has been used to make traditional fermented milk products throughout the world (Kroger *et al*., 1989). The

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characterization of the microorganisms responsible for the fermentation led to the isolation of starter cultures, which could be produced, on a large scale to supply factories involved in the manufacture of these products (Cogan and Accolas, 1996).

Lactic Acid Bacteria (LAB) and yeasts have been reported to be the predominant microorganisms in most of the African indigenous fermented food products (Steinkraus, 1996; Halm and OLson, 1996; Olasupo and Azeez, 1992; El-Hadi, 2001).

Sudan has a very long tradition in producing a great variety of fermented dairy products. Possibly more than 50% of annual milk production is converted into fermented dairy products (Dirar, 1993). These fermented milks are widely popular and consumed by a larger section of the human population. Of the many fermented milks of Sudan, a few of them are important like *robe*, *garris*, *mish*, *zabady* and others. In general, these milk products are resultant of natural fermentation. The quality and safety of final product is dependent upon various influencing factors, especially the hygienic status of raw milk used and the presence of lactic acid bacteria and yeasts in good numbers and their ability to grow and causing in desirable fermentation. The present study aims at the preparation of quality consistent and safe traditional fermented milk products using different culture combinations of lactic acid bacteria and yeasts, investigation of the possible interactions between the yeasts and LAB through studying final populations in the fermented milks as well as determination of the nutritive value of these products.

MATERIALS AND METHODS

Preparation of Starter Cultures and Inoculation into HTST Milk

The cultures of lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus fermentum*) which had been previously isolated and identified as the main fermenting LAB in *garris* (El-Hadi *et al*., 2004) and yeast strains *Pichia membranefaciens* and *Candida famata* which had been previously identified as the main yeast strain in *robe* product (El-Hadi, 2001) were used. The LAB cultures had been stored at -80°C in sterile tubes containing MRS broth. LAB was cultivated by streaking on MRS agar (Merck) and incubated anaerobically using Gas Pack at 30°C for 24 h. A colony was picked from each pure culture plate, grown successively in MRS broth and centrifuged. The pellet was washed in peptone physiological salt solution centrifuged again and redistributed in peptone physiological salt solution.

Pure cultures of yeasts *Pichia membranefaciens* and *Candida famata* were cultivated by streaking on Potato Dextrose Agar (PDA), incubated at 30°C for 48 h then centrifuged and washed. Yeast cultures had been stored on potato dextrose agar slants at 4°C until required. Cell suspensions of the individual cultures of LAB and yeast were prepared from 36 h culture broths of Lactobacillus MRS and potato dextrose media, respectively. Appropriate dilutions of the cell suspension were prepared in sterile 0.85% saline, to obtain desirable levels in the product preparation. The inoculated milk samples were aseptically transferred into screw-capped glass bottles.

Preparation of the Fermented Milk Products

Normal, previously pasteurized (HTST) and cooled cow's milk was used. The milk was standardized to 3.0% (v/v) fat, homogenized and poured into sterile jars. Each jar was inoculated 0.1% (v/v) of the individual culture combinations as follows: (I) *Lactobacillus plantarum, Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus fermentum* (product-A); (ii) *Lacotbacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus fermentum* and *Pichia membranefaciens* yeast (product -B); and (iii) *Lacotbacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus fermentum* (LAB) and *Candida famata* yeast (product-C). The culture combinations were then thoroughly mixed. Inoculated milk samples were incubated at 25°C for 18-20 h. The fermented product was then dispensed in sterile 200 mL capacity glass bottles.

Viable Microbial Counts

The Lactic acid bacterial strains were enumerated (pour plate) on MRS agar (Merck, Darmstadt, Germany) and incubated at 30°C for 48 h. Serial dilutions in both cases were done in quarter strength Ringers' solution (Oxoid, Unipath, England). The yeast counts was carried out by (spread plate) on Potato Dextrose Agar (PDA) and incubated at 25°C for 72 h. Characteristic colonies appearing on the respective selective agar media were counted, multiplied by the dilution factor and expressed as $\log_{10}$ colony forming units per milliliter ($\log_{10}$ cfu mL^{-1}). The viable counts of yeasts and LAB were carried out according to (IDF, 1990).

Proximate Chemical Composition

Proximate chemical composition of fermented milk products were carried out to determine the contents of moisture, protein, ash, fat, lactose, Total Soluble Solids (TSS) pH and Titrable Acidity (TA) according to the AOAC (1990).

Determination of Organic Acids

Organic acids were determined according to the modified method of Marsili *et al.* (1981). A 1.00 g sample was added to 0.2 mL 0.5 M H_2SO_4 and 8 mL acetonitrile and mixed for 30 min. After centrifugation and filtration, the samples were analyzed using an Aminex HPX-87H HPLC column, held at 37°C, connected to a Perkin-Elmer HPLC (Perkin-Elmer, Norwark, CT, USA). H_2SO_4 (15 mM), at a flow of 0.4 mL min^{-1}, was used as the mobile phase. Organic acids were identified according to their retention times compared with standard solutions of the following acids: citric, orotic, pyruvic, succinnic, lactic, formic, acetic and uric acid (Sigma). The analysis was externally calibrated using mixed standard solutions in deionized water, prepared as for the samples.

RESULTS AND DISCUSSION

When these characteristics are compared with those of fresh milk samples, it was clearly seen that there was a noticeable decrease in pH values of the fermented milks (which ranged between 4.02±0.9 to 4.26±0.1 and an increase in titrable acidity (expressed as lactic acid %) which ranged between 1.75±0.05 and 1.95±0.15% Table 1. The greatest change occurred by the first culture combination (product A). The pH after 48 h in all the fermentation types was generally in close agreement to those of pH of robe (4.0) and garris (3.9) as determined by El-Hadi (2001) and El-Hadi *et al.* (2004), respectively. In addition, there was a marked drop in total soluble solids contents which ranged between 7.5±0.2 to 7.8±0.2 as well as lactose which ranged between 3.82±0.2 to 3.43±0.1%. The protein and ash contents were higher when compared with those of fresh milk. These changes are obviously due to fermentation. The fat content of the fermented milk samples were more or less similar in all fermented milk samples. The moisture percentage ranged between 90.4±1.4 to 91.2±1.6 %. These values are in close agreement to the moisture content of *Gariss* as reported by Mirghani (1994) who determined a value of 92.6%. Average values of ash and protein percentage were found to be comparable to Yoghurt and *Gariss* (Mirghani, 1994). Most of the chemical parameters were in close agreement with the data in the literature for many African fermented dairy products (Mutukumira, 1996; Steinkraus, 1996; Keller and Jordan, 1990; Isono *et al.*, 1994).

Table 1: Chemical composition of Rob samples obtained from different sources

Samples	pH	Moisture	Ash	Protein	Fat	Lactose	TSS	TA
	----------	----------	----------	----------	----------	----------	----------	(%) ----------
Fresh milk	6.7±0.05	88.5±1.3	1.2±0.03	3.47±0.07	3.3±0.01	3.4±0.10	11.8±1.2	0.14±0.08
A	4.02±0.2	90.4±1.4	0.81±0.02	3.56±0.2	3.58±0.08	3.43±0.1	7.65±0.1	1.82±0.02
B	4.04±0.5	90.3±0.7	0.85±0.03	3.67±0.07	3.25±0.1	3.34±.14	7.8±0.2	1.75±0.05
C	4.26±0.5	91.2±1.6	0.77±0.45	4.0±0.2	3.21±0.2	3.49±0.13	7.6±0.15	1.95±0.15

A, B and C are the fermented milk product samples

Table 2: Organic acids (ppm) of various fermented milk products after 48 h fermentation at 26°C

Parameters	PM	A	B	C
Citric acid	78.4	90.2	88	86
Orotic acid	77.6	88.5	92	84
Pyruvic acid	3.2	5.5	5.2	5.3
Succinic acid	387	450	430	480
Lactic acid	ND	9020	9445	9178
Formic acid	ND	41	78.6	68.5
Uric acid	ND	10.8	16	14.3
Acetic acid	542	1285	1355	1358

N:D Not Detected

Table 3: The viable LAB counts in HTST milk after 48 h fermentation with selected combinations of yeasts and LAB strains LAB count in combination with (log_{10} cfu mL^{-1})

LAB strains	Initial LAB	Final LAB	*Pichia membranefaciens*	*Candida famata*
L. plantarum	7.26	9.14	9.12	9.40
L. paracasei subsp. *paracasei*	7.47	9.33	9.38	9.30
L. fermentum	7.65	8.78	8.73	8.53

Table 4: The viable yeast counts in HTST milk after 48 h fermentation with selected combinations of yeast and LAB strains yeast count in combination with (log_{10} cfu mL^{-1})

Yeast strains	Initial yeast counts	Yeast counts	*L. plantarum*	*L. paracasei* subsp. *paracasei*	*L. fermentum*
Pichia membranefaciens	5.6	7.2	6.96	7.12	6.92
Candida famata	6.6	6.45	6.28	7.08	6.86

The organic acids detected during fermentation included citrate, orotate, pyruvate, succinate, lactate, formate, ureate and acetate (Table 2). Lactic, formic and uric acids were not detected in the pasteurized milk but detected in all fermented milk types. The concentration of the organic acids content in all fermented milk samples was due to fermentation. The concentrations of succinic, orotic and citric acids were high at the end of the fermentation. The concentrations of most of these acids were more or less similar in all fermented milk products. The increase in lactate in the fermented milk products was due to the consumption of lactose by the lactic acid bacteria and converting it to lactic acid. The presence of lactic and acetic acids could play a significant role in improving the shelf-life of the fermented products as they have a direct antimicrobial effects (Davidson, 1997). The antagonism is believed to result from the action of acids on the bacterial cytoplasmic membrane, which interferes with the maintenance of membrane potential and inhibits active transport (Blom and Mortvedt, 1991).

The counts of LAB strains in the fermented milk products were in the range 8.14 -9.51 log_{10} cfu mL^{-1} (Table 3). Both of *L. plantarum* and *L.paracasei* subsp. *paracasei* grew approximately two log cycle while *L. fermentum* grew approximately one log cycle and the viable counts were nearly similar in both single and culture combination. The viable counts of the three *Lactobacillus* strains were not changed by co-inoculation with yeast. The final viable counts of *L. paracasei* subsp. *paracasei* and *L. fermentum* were comparatively higher in culture combination with *Pichia membranefaciens* than in single culture. On the other hand *L. plantarum* had higher viable counts in culture combination with *Candida famata*. These LAB counts are comparable to those encountered with many other dairy starters (Teuber, 1995). The lower potentialities of *L. fermentum* to grow in the HTST milk could be due to presence of wild strains of lactic acid bacteria in the fermented milk. However, the higher counts of *L. fermentum* founded co-culture with *Pichia membranefaciens* suggest that the yeast stimulated growth of the LAB. The yeast could achieve this by providing essential metabolites such as pyruvate, amino acids and vitamins.

The final populations of yeast cultures were in the range of 6.45-7.12 log_{10} cfu mL^{-1} Table 4. *Candida famata* in co-culture with *L. plantarum* had a lower final population than in the original inoculum, suggesting that their viability may have been reduced at the end of the fermentation. Yeast

growth in milk products is attributed to the ability of the yeasts to utilise milk constituents, such as proteins, fat, lactose and citrate (Fleet, 1990). Other reports also attribute this growth in part to symbiosis with other microflora in the mixed culture (Koroleva, 1988). *Pichia membranefaciens* grew well in the HTST milk but showed faster growth in culture combination with *L. paracasei subsp. paracasei* attaining maximum populations of about 7.12 $\log_{10}$ cfu mL^{-1} after 48 h. When in co-culture, it attained the maximum populations after 48 h.

CONCLUSION

The study showed that fermentation of HTST milk improved its nutritional value by increasing most of the chemical components. In addition, the study showed that *Lactobacillus plantarum*, *Lactobacillus paracasae* and *Pichia membranefaciens* could grow mutually in culture combination and could have potential as a mixed starter culture. The higher final populations of some LAB strains in culture combination with yeasts, was indication of a beneficial effect of the yeasts on the LAB. However, in some cases co-inoculation did not result in a major effect on the final numbers of either the yeast or the LAB. Further study is needed to investigating the genetic blue-prints of the starter cultures used in the present study as that will provide new applications which can be beneficial to the processor and consumer.

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