POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
www.pan.olsztyn.pl/journal/
e-mail: joan@pan.olsztyn.pl
Pol. J. Food Nutr. Sci.
2007, Vol. 57, No. 3, pp. 295–300

BIOCHEMICAL EVALUATION AND MICROBIAL QUALITY OF RAS CHEESE SUPPLEMENTED WITH PROBIOTIC STRAINS

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Key words: Ras cheese, electrophoresis, probiotic cultures, microbial evaluation, biochemical properties

Starter consisting of *L. reuteri, L. casei and L. gasseri* was used either separate or mixed with *L. bulgaricus* and *S. thermophilus* to produce probiotic Ras cheese. In the control cheese use was made of a starter consisting of *L. bulgaricus* and S. *thermophilus*. The first starter produced cheese containing more soluble nitrogen, essential and non-essential amino acids and α and β-galactosidase, amino peptidase and dipeptidase enzymes than the cheese of the mixed starter. The control cheese contained less of both starters than the cheeses. This was manifested in more protein breakdown appearing in more intense bands on electrophoresis electrophoregram. Both starters inhibited the growth of coliform, staphylococcus bacteria, yeast and molds on their cheeses. Moreover, both starters could help in lactose intolerance.

INTRODUCTION

Ras cheese, the main traditional hard cheese in Egypt is manufactured in a high proportion under artisan conditions from raw cow's or mixture of cow's and buffalo's milk without using starter cultures and marketed when it has a queried sharp flavour closed to kefalotyic cheese after 3 to 6 months [Hofi *et al*., 1970; Scott, 1981; Phelan *et al.*, 1993]. Recent discoveries in several areas of bioscience support the hypothesis that, beyond nutrition, diet may modulate various human body functions. These new concepts have led to the introduction of functional foods that encompass a wide range of ingredients and functional aspects [Roberfroid, 1998]. Foods containing probiotic bacteria belong to the category of functional foods are described as" foods claimed to have a positive effect on health" [Stanton *et al*., 1998]. To date the most popular food delivery system for probiotic cultures has been freshly fermented dairy foods, such as yoghurt and fermented milks as well as unfermented milk and desserts with added cultures. Members of the genera *Bifidobacterium* and *Lactobacillus* are widely used as probiotic microorganisms in functional foods; but other nonpathogenic bacteria, including members of the genus *Enterococcus* and yeasts, are also used as probiotics [Sanders & Huis in't Veld, 1999; Corbo *et al.,* 2001]. Recently Fathi & Dabiza [2005] reported that probiotic bacteria improved the body and texture of Ras cheese. They also reported that the final products contained viable probiotic bacteria.

The incorporation of culture with beneficial effects into a functional food is successful when the cultures maintain viability until being consumed and if the added cultures do not adversely affect the composition, texture or sensory attributes of the product. Results from cheeses used as functional food are still limited [Corbo *et al.,* 2001]. Probiotic bacteria other than *Bifidobacteria* and *Lactobacilli* such as *Enterococcus faecium* and *Lactobacillus paracasei* of human origin were also successfully used to develop functional cheeses [Gardiner *et al.,* 1998, 1999]. Therefore the main objective of this study was to follow the changes in protein; microbiological quality and biochemical properties of probiotic Ras cheese.

MATERIALS AND METHODS

Starter cultures. *L. bulgaricus* and *S. thermophilus* were purchased from (CHR – Hansen's laboratory Copenhagen-Denmark) and *L. reueri* was isolated from Egyptian dairy products. *L. gasseri* was kindly donated by Dr. Saito Faculty of Biological Resource Science – Tokoku University, Japan.

Rennet. Ha-La rennet was obtained from CHR–Hansen's (Denmark).

Chemicals and enzymes were purchased from Sigma – Aldrich Company.

Apparatus. Vertical slab gel electrophoresis apparatus, Hoefer Scientific instruments Company Model 400 Serial No. 93-1117 was used in the study.

Cheese manufacture. Ras cheese was processed according to [Hofi *et al*., 1970]. The traditional cheese (control) was manufactured with traditional starter while the probiotic Ras cheese was manufactured with 50% and 100% replacement of normal starter with probiotic starter. Cheeses of the experimental starters were left uncoated, while the control

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cheese was coated with plastic containing 0.05% natamycin (CESKA WL 500.0400). Ripening was carried out at 12±2°C and 90% relative humidity for 4 months. Cheese was analysed fresh at 15, 30, 60, 90 and 120 days of ripening.

Microbiological analysis. Twenty grams of cheese were first homogenized in 180 mL of sodium citrate solution for 1 min. Subsequent serial dilutions were made in Ringer's solution. Nutrient agar was used for counting aerobic psychrotrophic bacteria at 7°C for 10 days and mesophilic bacteria at 32°C for 48 h, Baird parker agar supplemented with egg yolk tellurite for counting staphylococci at 37°C for 48 h, and violet red bile agar for counting coliforms at 30°C for 24 h [IDF, 1985]. Mesophilic, thermophilic lactobacilli and yeast and molds were determined according to [Corbo *et al*., 2001].

Media and supplements: All media used were purchased from Oxoid Basingstoke, UK.

The microbial composition of the starter. The traditional Ras cheese (control) was manufactured using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (1:1 v/v) as a traditional starter. The probiotic Ras cheese was manufactured using *L. reuteri* +*L. casei* +*L. gasseri* (1:1:1 v/v/v) as probiotic strains.

Analytical methods. Total and soluble nitrogen were determined by the Kjeldahl method according to the International Dairy Federation methods [IDF, 1993]. Amounts of D- and L-lactic acids and acetic acid were determined by means of an enzymatic kit (Boehringer-Mannhein, Milan, Italy).

Estimation of enzymatic activity. Cheese extracts were prepared according to Corbo *et al.* [2001]. Cheese water extract was dialyzed for 24 h against 0.05 mol/L phosphate buffer (pH 7.0) to remove salts and small peptides. The extract was sterilized by filtration through 0.22 μm pore size syrifil filter (Nucleopore, Coaster Corporation, Cambridge, MA). Enzymes activity was reported as units per gram of cheese.

The α and β-galactocidase activities were estimated by determining the rate of hydrolysis of o-nitrophenol-α-galactopyranosidase and o-nitrophenol-β-galactopyranosidase (Sigma-Aldrich), respectively. In this case, the water-soluble extracts were not filtered prior determination. The release of o-nitrophenol was determined at 420 nm, and the concentration was determined from a standard curve. One unit of enzyme activity was defined as the amount of enzyme that released 1 μm of o-nitrophenol/min.

Aminopeptidase (EC 3.4.11.1) activity was determined according to Gobbetti *et al.* [1997] using Leu-P-NA, Ala-p-NA, Arg-P-NA and Pro-P-NA as substrates. One unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance at 410 nm of 0.01/min at 37°C and pH 7.0. The pH and temperature used in this and subsequent analyses were selected as optimal for the enzyme activities in Ras cheese.

Dipeptidase (EC 3.4.13.11) activity was measured by the Cd- ninhydrin method [Folkertsma & Fox, 1992] by using Leu-Leu as a substrate. A unit of activity was defined as the

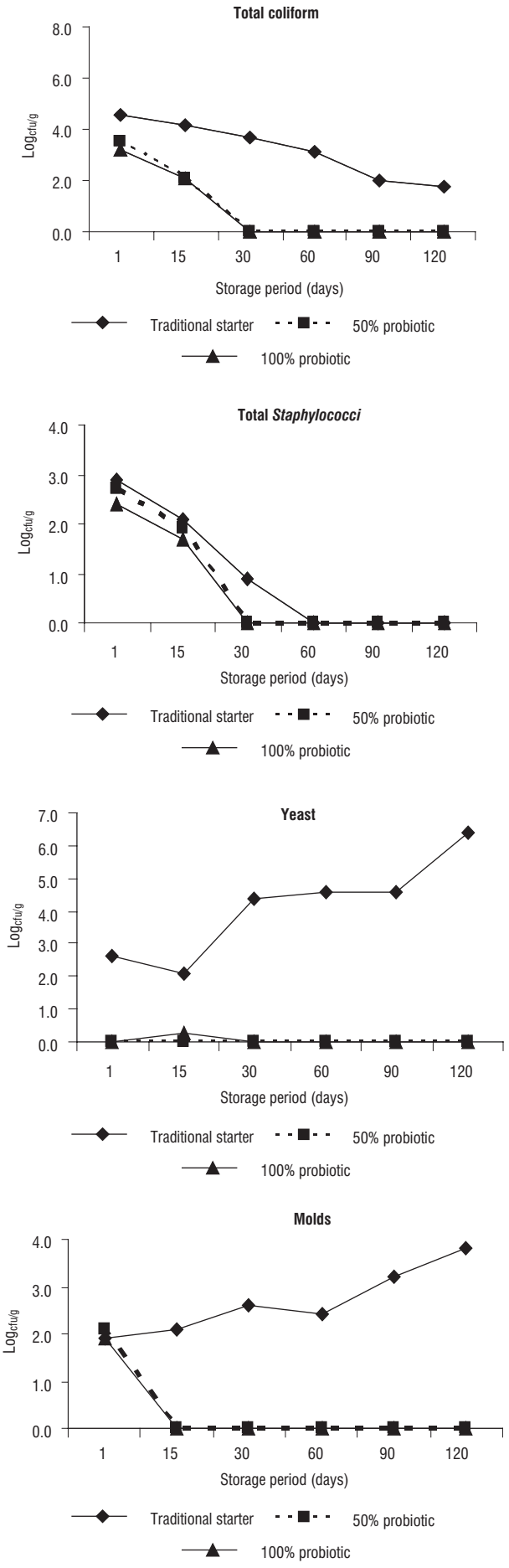


FIGURE 1. Microbiological quality of traditional and probiotic Ras cheeses.

amount of enzyme that produced an increase in absorbance of 0.1/min at 505 nm.

Assessment of amino acids. Cheese samples were hydrolysed according to the method of Millipore Cooperative [1987]. Amino acids were analysed with HPLC (Waters Assoc., USA).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The SDS-PAGE of milk protein and digested protein samples was carried out according to the method described by Laemmli [1970] using an SDS--Gel electrophoresis apparatus including glass plates, combs, spacer, casting device, gel chamber and a power supply unit (Pharmacia-LKB, Upsalla, Sweden).

RESULTS AND DISCUSSION

Microbial quality

Counts of different microbial groups in cheese during ripening are illustrated in Figures 1 and 2. Total coliforms and total staphylococci disappeared after 30 days and molds disappeared after 15 days from the probiotic cheeses. The cheeses were free from yeasts.

On the other hand, in the control cheese total staphylococci disappeared after 60 days, coliform persisted to the end of ripening (1.8 Log_{10} cfu), and yeasts and molds increased through ripening till the end with 6.4 and 3.8 Log_{10} cfu/g, respectively. Counts of mesophilic and thermophilic lactic acid bacteria in probiotic cheese showed a slight increase on the 15th day of ripening, then declined throughout the ripening period. These two groups were higher in the control cheese.

On the other hand, psychrotrophic bacteria were increased during ripening reaching a final count of 5.0, 4.2 and 4.2 Log_{10} cfu/g for traditional 50% and 100% probiotic cheeses, respectively, at the end of the ripening period. Yeasts and molds grew on the control cheese surface but probiotic cheeses showed no such a growth.

Results obtained showed the potential advantages of using the above probiotic strains to produce safe and healthy cheese.

In this respect, Osman & Abbas [2001] found that yeast; moulds and coliform bacteria were not detected in probiotic Ras cheese throughout ripening and storage periods. While, Shehata *et al.* [2004] found that yeast and mould, proteolytic, psychrophilic and viable spore forming bacterial counts significantly decreased along the ripening period of Ras cheese. They also found that Ras cheese containing *bifidobacteria* had the lowest viable undesirable bacterial counts of yeast and moulds counts during the ripening period. Mehanna *et al.* [2002] reported that lactic acid bacteria in soft cheese were slightly increased during storage, but moulds and yeasts were not detected in all probiotic cheeses.

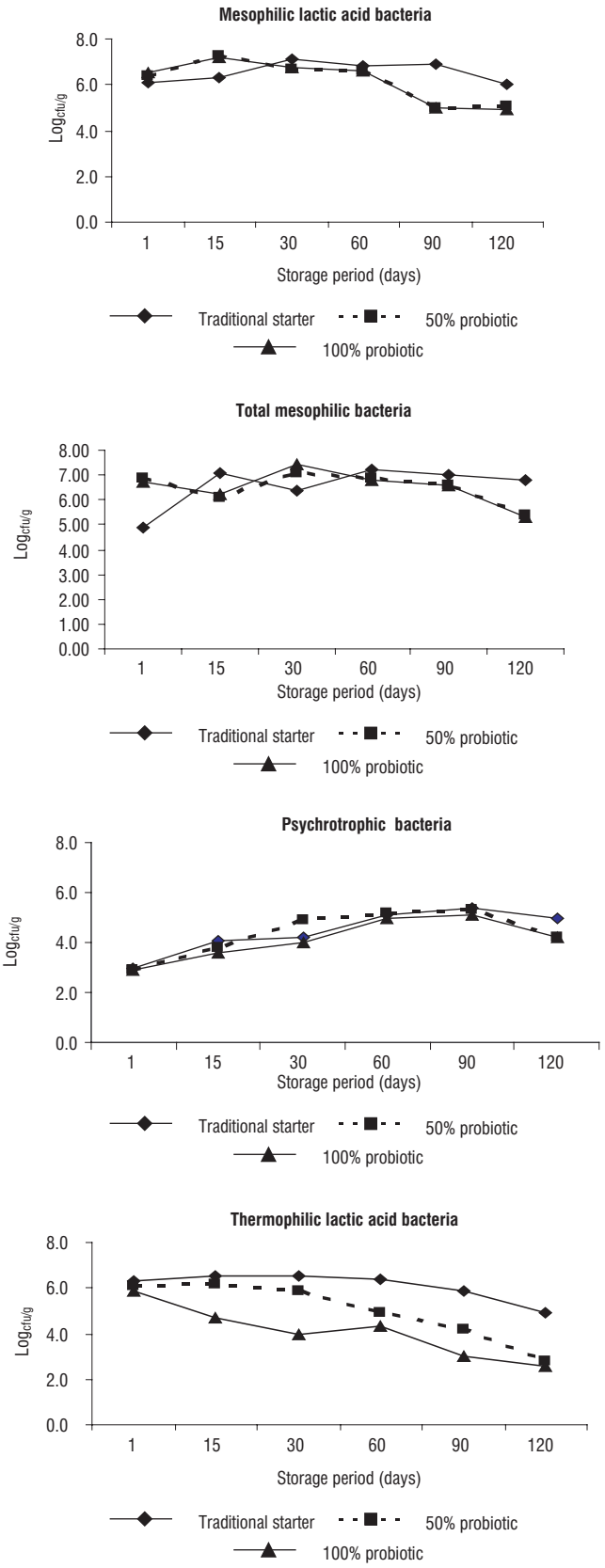


FIGURE 2. Microbiological quality of traditional and probiotic Ras cheeses.

Chemical analysis

Changes in total nitrogen content (TN) and protein degradation of Ras cheese made with traditional and probiotic strains as indicated by SN/TN ratio is presented in (Table 1). The probiotic Ras cheese with 50% replacement had nearly the same average amount of total nitrogen (TN) of the traditional Ras cheese. But probiotic Ras cheese with 100% replacement of probiotic strains contained higher TN. Protein breakdown in probiotic Ras cheese was higher than in traditional Ras cheese (control). Lactic acid and acetic acid contents were also higher than in the control cheese. The production of lactic acid and acetic acid during ripening of cheese mainly depended on the residual lactose in the curd.

Desjardins *et al.* [1990] found that the proteolysis of probiotic Ras cheese was higher than of the control.

TABLE 1. Chemical analysis of traditional and probiotic Ras cheeses after 120 days of ripening.

Analysis	Traditional cheese	Probiotic cheese	
		50%	100%
	Average	Average	Average
Total nitrogen (%)	16.01	16.19	18.02
Soluble nitrogen (%)	7.31	7.95	8.83
SN/TN (%)	7.4	7.61	7.72
Lactic acid (D+L)(g /kg)	10.04	10.61	11.21
Acetic acid (g /kg)	0.9	1.31	1.91

*Each value represents the average of two cheese samples.
** 50 and 100 % replacements of Normal starter with probiotic strains.

This increase could be attributed to the acidity and the starter culture used in probiotic Ras cheese. It is well known that higher acidity enhances the rennet action and the other acidic proteases enzymes [Gouda *et al.,* 1992; Metwally *et al*., 1984; Fox, 1969].

Enzymes activity

Data presented in Table 2 revealed that the contents of enzymes in probiotic Ras cheeses were higher than in the traditional Ras cheese and that the enzyme activities were dependent on the percentage of probiotic strains replacement.

TABLE 2. Enzyme specific activities (u/g) in traditional and probiotic Ras cheeses after 120 day of ripening.

Enzymatic activity	Traditional cheese	Probiotic cheese	
		50 %	100 %
α-Galactosidase	3.90	9.95	10.09
β-Galactosidase	44.01	69.52	72.69
Amino peptidase	24.85	41.26	42.01
Dipeptidase	56.45	87.92	86.27

*Each value represents the average of two cheese samples.
** 50% and100% replacements of Normal starter with probiotic strains.

Residues of dipeptidase and amino peptidase are important for proteolysis in cheese because they cause primary hydrolysis of caseins into peptides that are available for further degradation by enzymes produced from lactic acid cultures used.

The highest α and β-galactosidase activities were found when probiotic strains (*L. reuteri; L. casei* and *L. gasseri*) are used either individually or in a mixture with the traditional culture of control cheese. Therefore, the addition of probiotic strains to Ras cheese provide a lactose- free cheese. Moreover the high β-galactosidase activity, probably improves tolerance for other dairy products by individuals who suffer from lactose intolerance [Corbo *et al.,* 2001]. In addition the increased tolerance to dairy products that contain viable lactic acid bacteria is related to the intestinal lactose hydrolysis by enzymes that are released from some bacteria during transit or colonization of the small intestine [Kilara & Shahani, 1976; Kolars *et al.*, 1984].

Free amino acids content

A summary of individual free amino acids in traditional and probiotic Ras cheese at the ripening is shown in Table 3. The distribution of free amino acids contents in cheeses had changed with type of strains used and their concentrations. The 100% probiotic replacement yielded the highest concentration of free amino acids. The lowest levels were recorded with traditional cheese. Recommendable increases were observed for total essential and non essential amino acids with probiotic cultures.

TABLE 3. Formation of free amino acids in traditional Ras cheese and probiotic Ras cheese (mg/g cheese) after 120 days of ripening.

Amino acids	Traditional cheese	Probiotic cheese	
		50%	100%
Essential amino acids			
Leucine	21.61	22.01	23.21
Arginine	7.51	7.62	8.21
Lysine	6.41	6.45	6.9
Histadine	5.40	5.77	5.81
Phenyl alanine	10.7	11.2	11.6
Methonine	5.9	6.1	6.42
Valine	11.6	12.55	12.64
Total essential amino acids	**69.13**	**71.7**	**74.79**
Non essential amino acids			
Glutamic acid	48.6	48.81	48.88
Aspertic acid	19.9	19.71	19.81
Alanine	8.16	8.95	9.02
Asparagine	7.40	7.46	7.51
Serine	5.19	5.41	6.42
Tyrosine	14.61	14.98	15.6
Glycine	3.84	3.91	4.41
Proline	20.1	21.4	21.7
Total non essential amino acids	**127.8**	**130.63**	**133.35**

*Each value represents the average of two cheeses

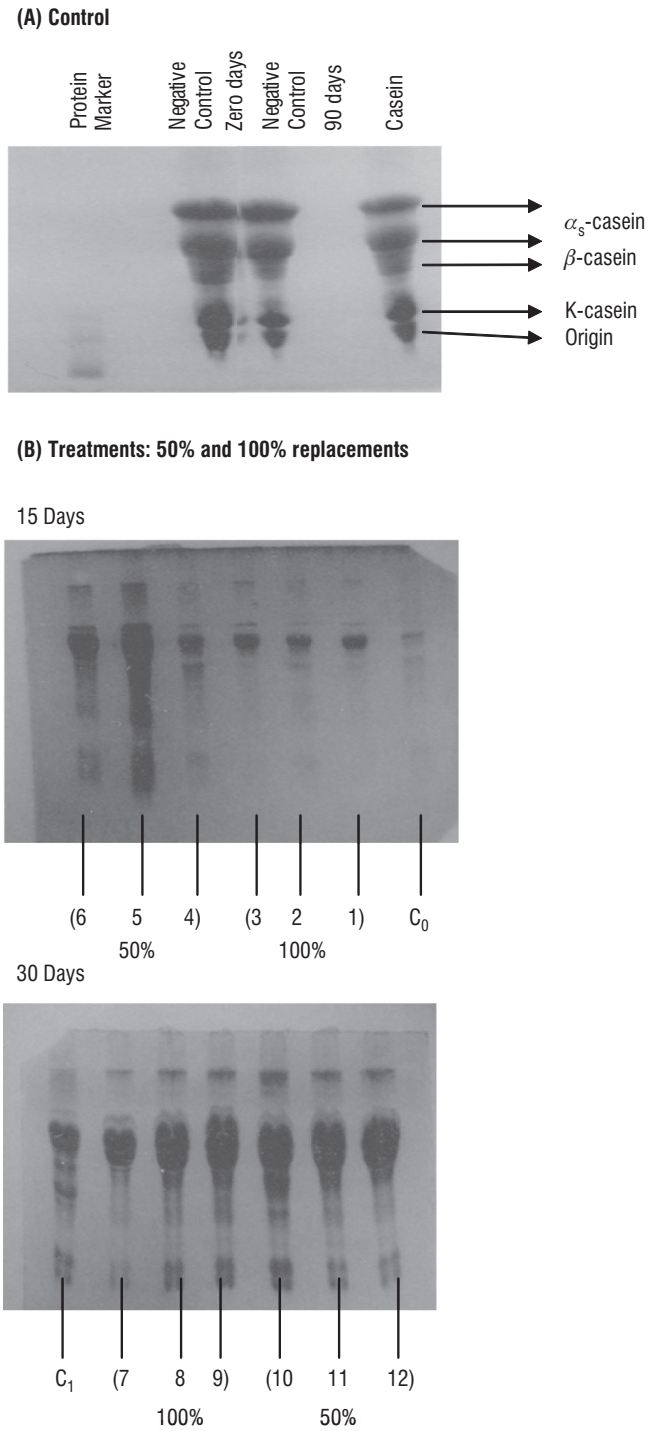
It is inferred that the amino acids are not formed by the action of rennet but by the enzymes released by lactic acid bacteria of the starter.

Glutamic acid, leucine, aspartic acid, tyrosine, valine and phenylalanine were generally in higher proportion in all cheeses. Similar results revealed that microbial strain of *L. casei–casei* L_2 A gave the best quality cheese with higher amounts of the total amino acids [Puchades *et al*., 1989].

Protein degradation by polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic patterns of fresh and ripened cheeses are illustrated in Figures 3 and 4. The intensity of casein fraction bands increased by ripening, indicating the increase in their concentration. The bands of the control cheese were less intense indicating that probiotic strains exhibited more proteolytic effect than the control strain. The fractions appeared as reported by El-Shibiny [1978], Mahran *et al.* [1988] and Shehata *et al.* [2004], κ-Casein in the slow moving region, and β-casein, α_s-casein in the fast moving region.

Similar observations were made by Ramos *et al.* [1988] who mentioned that fresh curd of Domiati cheese showed a very limited proteolysis. Regarding the probiotic Ras cheeses (Figure 3) shows the electrophoregram after 15 days of the ripening. The disappearance of κ-casein and the highest reduction of β-casein bands density and intensity were found in probiotic Ras cheese with 100% replacement. While probiotic Ras cheese with 50% replacement recorded the highest concentration in bands den-



FIGIURE 3. Electrophoretic pattern of traditional and probiotic Ras cheese: waxed (2, 5, 8, 9, 10, 11) unwaxed (1, 3, 4, 6, 7, 12).

sity and intensity especially in waxed cheese (slot 5). On the other hand, the lowest intensity of κ-casein and β-casein were observed in unwaxed cheese with 50% replacement. The intensive bands were α_s-casein in all cheese samples.

After 30 days of the ripening, there were observed differences in bands density and intensity for traditional and probiotic Ras cheese (Figure 3) as well as between waxed and unwaxed cheese. The highest protein degradation was observed in probiotic Ras cheese with 100% replacement (slots 10, 11, 12). On the other hand, a new band was observed in the fast moving region of all cheeses, being the highest density and intensity for probiotic Ras cheese with 100% replacement compared with traditional and 50% replacement.

After 60 days of the ripening period, the bands density and intensity were reduced in probiotic Ras cheese as compared to traditional Ras cheese especially in the case of β-casein bands. Waxed cheese recorded the same bands similar to the traditional Ras cheese (Figure 4).

After 90 days of the ripening period, the density and intensity of bands were reduced as compared with traditional Ras cheese

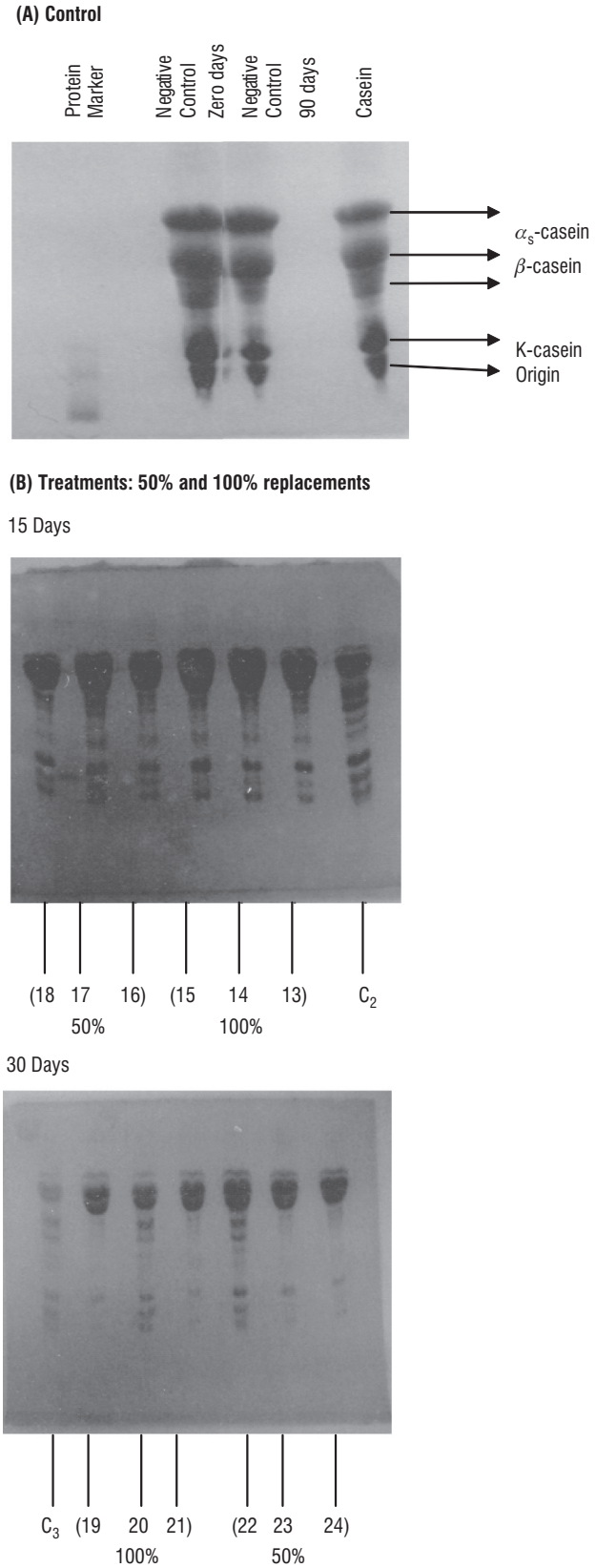


FIGURE 4. Electrophoretic pattern of traditional and probiotic Ras cheese: waxed (14, 17, 20, 22) unwaxed (13, 15, 16, 18, 19, 21, 23, 24).

and unwaxed probiotic Ras cheese. Whereas, some bands were disappeared between α_s-casein and β-casein region. Moreover the highest reduction in bands density and intensity were observed in β-casein region. While the highest concentration of bands were observed in waxed probiotic Ras cheese for 50% and 100% replacement as compared by control Ras cheese and unwaxed probiotic Ras cheese, and the major band of these degradation corresponds to α_s-casein in all types of cheeses (Figure 4).

Gouda [1987] reported that addition of sodium chloride to a solution of α_s and β-casein completely degraded by calf rennet (CR) in the presence of up to 6% NaCl proteolysis was slightly reduced by increasing the NaCl over 6%, and that in the absence of NaCl about 80% of β-casein was degraded by CR as well as that addition of NaCl drastically reduced the proteolysis of β-casein by CR. On the other hand, Gouda [1987] mentioned that reduction of pH increased the rate of proteolysis of both α_s and β-caseins.

CONCLUSIONS

Results obtained in the study showed that the probiotic Ras cheese with 100% replacement of probiotic strains could accelerate the ripening process, improved flavour, cheese quality and the microbiological quality of Ras cheese. Moreover, the production of probiotic Ras cheese will improve the marketability of cheese. Finally, Ras cheese could be considered as an excellent source of viable probiotic strains.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. El- Sayed Abd Alla, Food Toxicology and Contaminants; Prof. Dr. N. Tawfik and Prof. Dr. O. Sharaf, a group of dairy microbiology, National Research Centre, for useful comments and suggestions.

Financial support for this work was provided by the project No. 7040502-NRC.

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Received July 2006. Revision received in January and accepted February 2007.