Bifidobacterium bifidum in probiotic Edam cheese: influence on cheese ripening

Latha Sabikhi · M. H. Sathish Kumar · B. N. Mathur

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Abstract A study was conducted to determine the efficacy of Edam cheese as a carrier of probiotic bifidobacteria. Probiotic Edam cheese containing 10^7 viable cells of *Bifidobacterium bifidum* was monitored over a period of 3 months for ripening changes. Lactose in control as well as experimental cheeses was depleted within 15 days. Moisture decreased from 46.61 and 47.24 % to 42.06 and 42.46 % in control and experimental samples, respectively while pH in both the cheeses increased from ~5.20 to ~5.45. The free fatty acids increased from 2.23 % and 2.31 % on 0-day to 2.78 % and 2.83 % after 3 months, in control and probiotic cheeses, respectively. The 0-day and 3-month values of total volatile fatty acids in both samples were ~2.30 and ~2.95 ml NaOH (0.1 N) used per 10 g cheese, respectively. The soluble protein increased respectively from 5.42 and 5.30 % to 15.00 and 14.96 % after 3 months of storage in control and experimental cheeses. More of α -casein was degraded than β -casein after 3 months in both the samples. The study demonstrated that Edam cheese may be a suitable vehicle for delivering probiotic bifidobacteria to the end user.

Keywords Edam cheese · Bifidobacteria · Probiotic · Ripening · Soluble protein · Free fatty acids

Introduction

Newer forms and range of food products favouring nutritional and therapeutic aspects are being increasingly developed through R&D endeavours in response to the enhanced consumer awareness regarding food intake, nutrition and health. As a consequence of this consumer interest, there has been a phenomenal growth of foods containing these probiotic cultures, particularly in Western Europe, USA and Japan. Technological performance of the probiotic strains, effect on novel product development and viability thereof in dairy products during manufacture and storage, as well as stability during exposure to gastrointestinal conditions such as low pH and high bile concentrations are among the properties that have been widely studied and reported. The most widely studied probiotic strains are bifidobacteria and lactobacilli.

Bifidobacterium strains can survive intestinal transit and persist transiently within the colon (Fujiwara et al. 2001; Von Wright et al. 2002). The therapeutic roles of bifidobacteria in animal models and in human beings are due to four major mechanisms including modulation of the host immune system (Saavedra and Tschernia 2002), resistance to infectious diseases caused by rotavirus and other enteropathogens (Shu et al. 2001), control of inflammatory bowel diseases such as Crohn's disease, ulcerative colitis and pouchitis (Marteau 2002) and prevention of cancer (Yazakawa et al. 2001; Li et al. 2003).

Bifidobacteria need an anaerobic environment and neutral pH to survive and be viable at numbers required to provide therapeutic benefits (Boylston et al. 2004). This makes it difficult for several foods to provide the optimal environment for the growth of bifidobacteria. Cheese, however is an exception. Several studies have demonstrated the possibility of incorporating bifidobacteria into different types of cheeses including Cheddar (Dinakar and Mistry 1994; McBrearty et al. 2001; Ong et al. 2006), cottage

L. Sabikhi (✉) · M. H. S. Kumar
Dairy Technology Division, National Dairy Research Institute,
Karnal 132001 Haryana, India
e-mail: lsabikhi@gmail.com

B. N. Mathur
National Dairy Research Institute, Karnal 132001 Haryana, India
e-mail: bnmathur2003@yahoo.com

Present Address:
B. N. Mathur
Q-35, Radhasoamy Satsang, Dera Baba Jaimal Singh, Beas,
Amritsar 143204 Punjab, India

(Blanchette et al. 1996), Canestrato Pugliese (Corbo et al. 2001) and Crescenza (Gobbetti et al. 1998). Cheese is a good carrier of probiotics because of its low pH and oxygen level and high buffering capacity. Its texture and unique lipid content may also provide protection to probiotics during gastric passage (Stanton et al. 1998; Kailasapathy and Chin 2000; Boylston et al. 2004; Phillips et al. 2006). However, utilization of probiotics in the cheese presents some challenges: low moisture content; presence of salt; starter cultures competing for nutrients and developing acid and flavour during maturation. Effective incorporation of bifidobacteria into cheeses requires that the organisms maintain their viability throughout processing, without adversely altering sensory and biochemical characteristics. The presence of exogenous microflora not natural to cheese may introduce complex technological and product quality-related hurdles in the manufacturing process or during the ripening period.

Mild ripened varieties of cheeses such as Edam are better suited for the Indian palate as most Indians prefer them to sharp-flavored cheeses. This, coupled with the present dilemma of Indian traders who are forced to import special varieties of cheeses due to their unavailability within the country, warranted concerted R&D efforts on Edam cheese. In the current study, the ripening process of probiotic Edam cheese containing *Bifidobacterium bifidum* was monitored to characterize possible deviations from typical Edam cheese consequent to the incorporation of *B. bifidum*. The parameters studied during the current investigation to monitor ripening were residual lactose, moisture, pH, soluble protein (SP), secondary proteolysis and lipolysis via free fatty acids (FFA) and total volatile fatty acids (TVFA) content. The ripening studies were conducted over a period of 3 months.

Materials and methods

Pooled cow milk obtained from the herd of the cross-bred Karan-Swiss and Karan-Fries as well Sahiwal and Tharparkar cows maintained at National Dairy Research Institute, Karnal, India was used for the manufacture of Edam cheese. Freeze dried DVS culture (Type LD CH-11) for Edam cheese and Ha La Film (red colour, 85 cp viscosity and 0.5 % natamycin) for coating the cheese were acquired from M/s Chr. Hansen (Horsholm, Denmark). Rennet from *Mucor miehei* was procured from M/s. Sigma Chemicals (Missourie, USA). The ATCC 15696 strain of *B. bifidum* was obtained from the American Type Culture Collection (Virginia, USA). It was activated and maintained in Yoshioka medium (5.50 g yeast extract, 12.50 g peptone, 11.00 g glucose, 0.25 g KH_2PO_4 , 0.25 g K_2HPO_4 , 10.00 g sodium acetate, 0.10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.00 mg MnSO_4 , 5.00 mg FeSO_4 and in 1.0 l distilled water, pH-7.2, autoclaved. Ten μl of 10 % solutions of N-acetyl D-glucosamine and sodium thioglycollate [prepared separately and filter-sterilised using 0.45 μm filters] were added per 10 ml of broth/agar aseptically, at the time of inoculation/plating).

Preparation of Edam cheese

Control Edam cheese (CEC) was prepared following the standard procedure (Fig. 1) suggested by Scott (1986). Probiotic Edam cheese (PEC) was prepared by adding *B. bifidum* (ATCC 15696) @1 % along with Edam cheese starter and its proximate composition was analyzed as described by Sabikhi and Mathur (2002).

Fig. 1 Process diagram for Edam cheese

Lactose

Lactose was analysed by the colorimetric method using the phenol-sulphuric acid method described by Action (1977). Two grams of Edam cheese was blended well with 10 ml of 0.5 M NaOH and dispersed by mixing with 100 ml of water. The contents were shaken with 10 ml of 10 % (m/v) solution of $\text{ZnSO}_4 \cdot (7\text{H}_2\text{O})$ and the volume was made up to 200 ml. The solution was filtered through a fast filter paper, discarding the first five ml of the filtrate. One ml each of the filtrate and 5 % phenol solution was taken in test tubes and mixed thoroughly. From a calibrated dispenser, 5 ml of concentrated H_2SO_4 was run rapidly and directly into the solution and the tubes were shaken thoroughly. The tubes were kept in a boiling water bath for 10 min and subsequently cooled to 15–20 °C. The absorbance of each tube was measured using a spectrophotometer (Beckman Coulter, USA) at 490 nm in a 10 mm cell with distilled water as reference. The lactose concentration in the cheese sample was calculated using a standard curve.

Moisture and pH

Moisture in Edam cheese was determined by the gravimetric method (IS:SP:18 (Part XI) 1981). The pH of cheese was estimated using a Microprocess controlled pH Analyser (LabIndia, New Delhi, India) Version I.

Soluble protein

The soluble protein of cheese was determined following the method by Kosikowski (1982) with certain modifications. Three g of cheese was accurately weighed and mixed with a small amount of Sharpe's extraction solution (Sharpe's stock solution: glacial acetic acid –57.5 ml, sodium acetate –136.1 g, sodium chloride –47.0 g, calcium chloride –8.9 g, volume made up to 1,000 ml with distilled water. Sharpe's extraction solution made by diluting the stock solution 1:3 with distilled water) at 40 °C in a mortar. The contents were ground to a paste and transferred to a 100 ml volumetric flask. The volume was made up with Sharpe's extraction solution. The flask was kept in a water bath at 50° to 55 °C for 1 h with occasional shaking. It was then filtered through Whatman filter paper No. 42. Ten ml of filtrate was distilled and the total soluble nitrogen was estimated by the microKjeldahl method.

Electrophoretic pattern of cheese proteins by polyacrylamide gel electrophoresis (PAGE) of Edam cheese

PAGE of the cheese samples was carried out by method of Weber and Osborne (1969) after modifications using a Midicell™ EC350 Electrophoretic Gel System (EC Apparatus Corporation, Florida, USA). The gel was prepared dissolving 8 g of acrylamide (Sigma Chemicals, Missouri, USA) and

28 g urea in Tris-citrate buffer (pH-8.65) in 100 ml of solution. Just before pouring the gel, 100 µl of TEMED and 100 mg ammonium persulphate crystals were added to the material and mixed thoroughly. To 0.5 g of grated sample (Edam cheese or freshly prepared case in which served as the control) 2.0 ml of 0.2 M sodium barbital buffer (prepared in 6 M urea, final pH 8.6) followed by one drop of bromophenol blue solution (0.1 %w/v) was added. Ten µl of the prepared sample was subjected to electrophoresis until the brown borate boundary moved about 8 cm past the insertion line (about 6 h). The gel was stained and destained as per the method suggested by Creamer (1991). The gel was dipped in the staining solution (Coomassie brilliant blue R –1.0 g, isopropanol –500 ml, acetic acid glacial –200 ml, volume made up to 2 l with water) for 30 min followed by two successive washes in the destaining solution (isopropanol –200 ml, acetic acid glacial –200 ml, volume made up to 2 l with water) for 30 min each. The destained gel was intensified in 10 % trichloroacetic acid for 24 h before photographing and interpreting the results.

Free fatty acids (FFA)

FFA were analysed by the method described by Deeth and Fitz-Gerald (1976). Grated cheese sample (~ 3 g) was ground to a paste with 5 ml distilled water and mixed with 10 ml of extraction mixture containing isopropanol, petroleum ether and 4 N H_2SO_4 in the proportion of 40:10:1. An additional 6 ml of petroleum ether was added and the sample tempered at 40 °C for 10 min. The two layers were allowed to separate (5–10 min) after shaking vigorously for 20 s. An aliquot of the upper layer was titrated against 0.02 N methanolic KOH solution using 1 % methanolic α -naphtholphthalein as the indicator. The FFA was calculated as 5.64 xv/w (where v = volume in ml of 0.02 N KOH used for titration, x = ratio of the total solvent layer to the aliquot used for titration, and w = weight in g of the cheese sample) and expressed as % oleic acid (% OA).

Total volatile fatty acids (TVFA)

TVFA were determined by the method described by Kosikowski (1982). The volatile fatty acids liberated from the cheese were collected and estimated quantitatively by titrating against a standard alkali. Approximately 10 g sample of cheese was ground to a paste with 25 ml of 10 % sulphuric acid. The contents were transferred to an 800 ml Kjeldahl flask and the pestle and mortar were washed with a total volume of 50 ml of the acid and the washings were transferred to the Kjeldahl flask. The solution in the flask was shaken with 35 g of magnesium sulphate. After adding 250 ml glass distilled water, the contents were steam distilled to collect 280 ml distillate. This distillate was titrated against 0.1 N NaOH using phenolphthalein as indicator. The

insoluble volatile fatty acids were collected from the condenser by washing it with 25 ml of neutral alcohol and were titrated in a similar manner. The sum of the two readings gave the total volatile fatty acids in terms of ml of 0.1 N NaOH used per 10 g of cheese.

Statistical analysis

Data were recorded as mean $\pm$ S.E. of four independent replicates carried out on different days with freshly prepared cultures, cheese and chemicals. Two-way analysis of variance was carried out using SPSS Version 10 and the significant difference between treatment means compared at 5 % level.

Results and discussion

PEC contained 41.83 % fat in dry matter (FDM), 24.96 % protein, 3.49 % ash and 10^7 viable cells of *B. bifidum*.

Fig. 2 Biochemical changes in probiotic Edam cheese during ripening. Error bars indicate the SE of mean ($n=4$). CEC: control Edam cheese, PEC: probiotic Edam cheese

Lactose

Lactose concentration in the control as well as the experimental cheeses was very low even in fresh cheese and was quickly metabolised within a fortnight of ripening. On the 15th day, no residual lactose could be detected in the cheese samples. This was confirmed on monitoring the lactose content for a further period of 5 days at the end of which the carbohydrate remained undetectable. Figure 2a presents the depletion of lactose in Edam cheese. The probiotic cheese had less lactose than the control sample throughout the experimental period. The statistical analysis of the two samples (Table 1) revealed that control and probiotic cheeses did not vary significantly in their lactose contents during ripening.

The lactose content in the cheese at 0-day was in agreement with several early reports on this (Huffman and Kristoffersen 1984; Daigle et al. 1999). Daigle et al. (1999) reported that on the first day after manufacture, lactose

Table 1 Analysis of variance: ripening changes in Edam cheese

Sources of variation	DF	Lactose	Moisture	pH	FFA ^a	TVFA ^a	Soluble protein ^a
Treatments	1	22.45	0.87	4.27E-03	1.15E-02	1.87E-02	0.12
Ripening period	3	2957.83*	26.28*	3.38E-02*	0.37*	0.58*	111.14*
Interaction (treatment × period)	3	5.51	4.9E-02	3.35E-05	1.31E-03	0.17	2.34E-02
Error	16	6.32	0.11	1.88E-05	4.60E-03	6.00E-03	3.50E-02

^a On dry matter basis; * significant at $P < 0.05$

was completely hydrolyzed in cheeses made with bifidobacteria. Similar results are also reported by Corbo et al. (2001) who found only traces of lactose in Canestrato Pugliese cheese with bifidobacteria whereas on the 56th day, the amount of lactose was ~1.7 g/kg in the traditional cheese. The marginally lower residual lactose content in the PEC in the current study may be attributed probably to its higher bacterial load (Sabikhi and Mathur 2002). Bifidobacteria can utilise carbohydrates such as glucose, galactose, lactose and fructose as carbon sources and the major products of fermentation are acetic and lactic acids in the ratio 3:2 (Marshall and Tamime 1997; Naidu et al. 1999). Similar results were reported by Kasimoglu et al. (2004) who found that the lactic acid content in probiotic Turkish white cheese was higher than control cheese.

Moisture and pH

Moisture content decreased progressively during the ripening period for both control and probiotic Edam cheeses (Fig. 2b). Moisture in the experimental Edam cheese remained marginally higher than that in the control Edam cheese throughout the ripening period. The 0-day moisture in the cheese samples was 46.61 and 47.24 % for CEC and PEC, respectively. These values reduced progressively to 43.47 and 43.72, 42.67 and 42.92 and 42.06 and 42.46 % respectively after 1, 2 and 3 months of ripening. The most discernible decrease in the moisture content of both types of Edam cheese was during the first month, after which, the rate of loss of moisture diminished. The analysis of variance (Table 1) of the ripening changes in the control and probiotic Edam cheese indicated that there was no significant difference in the moisture content of both the cheeses. The pH of both control as well as probiotic cheeses increased progressively and more or less uniformly throughout the ripening period of 3 months, as depicted in Fig. 2c. The pH in the experimental cheese remained marginally lower than that in the control cheese throughout ripening. From an initial value of 5.25, the pH of CEC increased to 5.31 and 5.36 through the first and second months of ripening to a final pH of 5.43 after 3 months. The corresponding values for the PEC were 5.23, 5.27, 5.33 and 5.40. There was no significant difference in the pH of both the cheeses.

Rao and Singh (2007) found that a lower pH in the cheese matrix leads to higher moisture retention in the cheese during ripening. Alternatively, higher moisture content facilitates improved starter activity during initial stages of manufacture and hence, a quicker fall in the pH. McBrearty et al. (2001) reported that the addition of *B. lactis* Bb-12 resulted in a slightly higher level of moisture in the experimental Cheddar cheese, corroborating the present findings. The current results on pH of Edam cheese agree with the observations of Lawrence et al. (1987) who described that the pH of Dutch type cheese increased from ~5.1 to ~5.3–5.9 depending on the age of the cheese and the season of manufacture. Bifidobacteria are known to produce lactic and acetic acids in the ratio 2:3 (Marshall and Tamime 1997). Higher bacterial load results in faster drop in pH, owing to higher metabolic activity (Gardiner et al. 1998; Kasimoglu et al. 2004). In the present study, although the extra probiotic bacterial load in PEC led to a marginally lower pH, the pH drop was not significant as bifidobacteria are slow and weak acid producers.

Soluble protein

The most appreciable increase in the soluble protein (SP) was observed during the first month of ripening for both control as well experimental Edam cheese, after which the rate of increase was more or less uniform. Figure 2d demonstrates the SP content in the control and experimental Edam cheese samples on dry weight basis. The initial SP content in the control cheese was 5.42 % which increased considerably to 12.17 % during the first month of ripening. The rate of dissolution of the protein thereafter was much lower, the values being 13.88 and 15.00 % after 2 and 3 months of ripening, respectively. A similar trend was observed in the case of PEC, where the SP of 5.30 % at 0-day increased to 11.84, 13.81 and 14.96 % after 1, 2 and 3 months of ripening, respectively. The SP contents of both kinds of cheeses did not vary significantly (Table 1).

The higher SP content of CEC despite the lower bacterial load may be owing to the low proteolytic profile of bifidobacteria (Klaver et al. 1993). The 3 month old cheese (control and probiotic) was slightly bitter, which suggests the formation of certain bitter peptides during proteolysis.

Gardiner et al. (1998) reported that while proteolysis during Cheddar cheese ripening was influenced by the adjuncts of *Lactobacillus* at the level of free amino acids level, cheese flavor, texture, and appearance were not affected. In another study (Gardiner et al. 1999) probiotic strains of *Enterococcus faecium* increased the proteolysis in Cheddar cheese, but the sensory evaluation showed no significant difference in flavor/aroma and body/texture scores between control and experimental cheeses. McBrearty et al. (2001) incorporated two bifidobacteria strains in Cheddar cheese and found that *B. longum* BB536 did not adversely affect cheese composition. During early ripening, more extensive proteolysis and improved flavor were observed in the *B. lactis* Bb-12 cheese compared with the control cheese. Ong et al. (2006) reported that in Cheddar cheese containing *Lactobacillus acidophilus*, *L. casei*, *L. paracasei* and *Bifidobacterium* spp. did not affect the level of water-soluble nitrogen (primary proteolysis), but led to high concentration of free amino acids by a secondary proteolysis during ripening. Bergamini et al. (2006) obtained similar results with probiotic lactobacilli (*L. acidophilus* and *L. paracasei* subsp. *paracasei*), with no influence on primary proteolysis in a semi-hard cheese.

PAGE

The electrophoretic profile of casein in CEC and PEC after 3 months of ripening is shown in Fig. 3. The degradation of control (pure casein), CEC and PEC is shown in lanes 1, 2 and 3, respectively. At the age of 3 months, α - as well as β -casein in both CEC and PEC were degraded. However, the extent of hydrolysis of α -casein was higher as evidenced by the larger amount of β -casein left intact after 3 months. There was no apparent difference in the breakdown pattern of β -casein in both the CEC and PEC. However, there was a

slightly higher breakdown in the α -casein in the PEC than in CEC. This difference in the proteolytic pattern of the α -casein does not translate itself to variations in the physico-chemical or sensory attributes in both the cheeses.

Dinakar and Mistry (1994) found in an early study that bifidobacteria did not alter proteolysis in Cheddar cheese. In bacterially ripened cheeses, β -casein is more resistant to proteolysis than α_{s1} -casein (Fox et al. 1993). Ong et al. (2006) reported that though there was no influence on the primary proteolysis in Cheddar cheese containing *Lactobacillus acidophilus*, *L. casei*, *L. paracasei* and *Bifidobacterium* spp., the degree of secondary proteolysis indicated by the concentration of free amino acids was significantly higher in probiotic cheeses. The results of the current study are similar to those of Ong et al. (2006) who reported that hydrolysis of α_s -casein increased consistently in probiotic cheeses during 6 months of ripening.

Free fatty acids

FFA (% OA) in CEC as well as PEC increased progressively during ripening (Fig. 2e). There was a sharp increase in the FFA contents after 2 months of storage in both the samples. FFA on the 0-day in CEC and PEC, respectively was 2.23 and 2.31 % (OA) as against the corresponding values of 2.30 and 2.33 % (OA) after 1 month of ripening. During the subsequent periods (months 2 and 3), CEC had an FFA content of 2.35 and 2.78 % (OA) as against the values of 2.37 and 2.83 % in PEC. There was no significant difference in the FFA values of both the cheeses on 0-day, 1, 2 or 3 months of ripening as shown in Table 1.

A certain concentration of FFA in the correct balance is necessary for optimum flavor, but an excess of FFA or an incorrect balance leads to off-flavors (McSweeney and Fox 1993) especially in mild cheese varieties. The present results

Fig. 3 PAGE analysis of Edam cheese (Lane 1- pure casein, Lane 2- CEC, Lane 3- PEC). CEC: control Edam cheese, PEC: probiotic Edam cheese

are in agreement with the report of Corbo et al. (2001) who found no change in the FFA content when Canestrato Pugliese hard cheese was supplemented with bifidobacteria.

Total volatile fatty acids

Total volatile fatty acids (TVFA) produced in both the control and the probiotic Edam cheeses exhibited a similar trend as the FFA, increasing progressively during ripening (Fig. 2f). In both CEC and PEC, the TVFA content remained unchanged at 2.26 ml and 2.34 ml, respectively during the first month of ripening, increasing thereafter, to 2.33 ml and 2.91 (CEC) and 2.35 and 2.95 ml (PEC) after 2 and 3 months. After the second month, the TVFA values in both the cheeses increased at a greater rate. The analysis of variance (Table 1) indicated that the TVFA contents in both the cheeses did not vary significantly.

Volatile organic compounds such as acids, carbonyls, lactones, H₂S, methane, dimethylsulphide etc., originating from fat and protein compounds of cheese are responsible for cheese aroma and contribute to both background and characteristic flavor (Manning and Moore 1979). Kondyli et al. (2003) found that in low-fat Kefalograviera-type cheese made with commercial adjunct starters of containing lactobacilli and lactococci, the control as well as experimental cheeses had comparable quantities of volatile components.

Conclusion

The results of the experiments indicated that the addition of bifidobacteria did not negatively influence the ripening process of Edam cheese. Therefore, *B. bifidum* is a suitable adjunct that could complement the normal Edam cheese starter to enhance the probiotic attributes of the cheese. Edam cheese can be, thus, an effective vehicle for the delivery of probiotic bifidobacteria to the consumers.

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