

## A New Probiotic Cheese with Antioxidative and Antimicrobial Activity

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### ABSTRACT

The aim of our study was to develop an original probiotic cheese based on the Estonian open-texture, smear-ripened, semisoft cheese “Pikantne.” Cheese was produced by two methods using cheese starter cultures (Probat 505) in combination with 0.04% of probiotic *Lactobacillus fermentum* strain ME-3 ( $10^9$  cfu/mL) with high antimicrobial activity and antioxidative properties. The probiotic *Lactobacillus* was added into milk simultaneously with starter cultures (cheese A) and into drained curd (cheese B). After addition of probiotic *L. fermentum* ME-3, the cheese composition, flavor, and aroma were comparable to the control cheese (score values = 4.5, 4.2, and 3.7 for control cheese, cheese A, and cheese B, respectively). Cheese A, which had good sensory properties, was chosen for further testing of viability and probiotic properties. The probiotic strain was found to withstand the technological processing of cheese, surviving and sustaining moderate antimicrobial and high antioxidative activity throughout ripening and storage (the ripened cheese contained approximately  $5 \times 10^7$  cfu/g viable ME-3 cells), although the viability of the ME-3 strain incorporated into the cheese showed a slight decrease between d 24 and 54 after cheese preparation. Semisoft cheese “Pikantne” serves as a suitable carrier of antimicrobial and antioxidative *L. fermentum* ME-3.

**(Key words:** antimicrobial activity, antioxidative activity, lactobacilli, probiotic cheese)

**Abbreviation key:** LA = linolenic acid, Mn-SOD = Mn-superoxide dismutase, OHEL = obligately heterofermentative lactobacilli, TAA = total antioxidative activity.

### INTRODUCTION

Probiotics have been defined as live microbial food supplements that benefit human health (McFarland,

2000; Salminen, 2001). Viable lactic acid bacteria of probiotic foods have several scientifically established and/or clinically proved health effects, such as reduction and prevention of diarrheas of different origin, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and antitumorigenic activities (McFarland, 2000; Andersson et al., 2001; Salminen, 2001). Moreover, some studies have shown that certain lactic acid bacteria possess antioxidative activity (Kaizu et al., 1993; Peuhkuri et al., 1996; Kullisaar et al., 2002). They are able to decrease the risk of accumulation of reactive oxygen species in a host organism and could potentially be used in probiotic food supplements to reduce oxidative stress. In a previous study (Kullisaar et al., 2002), it was reported that *Lactobacillus fermentum* strain ME-3 (DSM 14241) has high antimicrobial and antioxidative activity. In healthy volunteers, it has been demonstrated that the consumption of fermented milk containing this *Lactobacillus fermentum* strain exhibited antioxidative and antiatherogenic effects (Kullisaar et al., 2003).

The suitability of different cheeses as carriers for antioxidative *Lactobacillus* strains has not been evaluated. As usual, cheese has certain advantages for a carrier state of probiotic organisms compared with the other, more acidic dairy products. There are different types of probiotic cheeses available on the market worldwide. Bifidobacteria are the most widely used probiotic additives in cheese (Dinakar and Mistry, 1994; Gomes et al., 1995; Daigle et al., 1999). However, there are relatively few scientific reports concerning lactobacilli of human origin as probiotic cheese additives (Gomes et al., 1995; Gardiner et al., 1998; Ross et al., 1999).

Different methods have been described for incorporation of probiotic *Lactobacillus* additives into cheese. Probiotics, as well as other nutritional supplements (antioxidants, vitamins, herbs, etc.), have been added to shredded natural cheese (U.S. Pat. 6090417). Bacteria with probiotic properties could be included with cheese starters (NLAB, 2002) added directly to cheese milk or to the curd before hooping (Gardiner et al., 1998; Ross et al., 1999).

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Probiotic bacteria have many desirable properties, such as safety, bile and acid resistance, adherence to human intestinal cells, colonization of the human gut, and production of antimicrobial substances. Being of human origin is considered to be of great importance (Lee and Salminen, 1995; Saarela et al., 2000). Nevertheless, potential problems may arise when trying to introduce a probiotic strain of human origin to dairy products. A probiotic strain should withstand the manufacturing process without loss of viability or negative effect on the sensory properties of the food product. The strain and the claimed properties should maintain stability in the food product during processing and also during subsequent storage (Lee and Salminen, 1995; Sanders and Huis in't Veld, 1999; Saarela et al., 2000), which could pose a problem for strains of human origin.

A large number of viable organisms are required in order to exert a probiotic effect in the food product. It is postulated that an active probiotic food should contain at least  $10^5$  cfu/g, and the food should be consumed daily in order to achieve a beneficial effect (Lee and Salminen, 1995). Therefore, it is of great importance to control the stability of probiotic numbers and properties in cheese.

The aim of our study was to develop a tasty original probiotic cheese based on the original Estonian open-texture, smear-ripened, semisoft cheese "Pikantne." The suitability of the probiotic *L. fermentum* ME-3 as a cheese additive was tested and its ability to retain viability and antioxidative and antimicrobial potential in the cheese environment was evaluated.

## MATERIALS AND METHODS

### Origin and Properties of Microbial Strain

The probiotic *Lactobacillus* strain *L. fermentum* ME-3 was previously isolated from the gastrointestinal tract of a healthy child (Sepp et al., 1997; Mikelsaar et al., 2002). The strain was identified by API CHL 50 System (bioMérieux, Marcy l'Etoile, France) and by internal transcribed spacer PCR using the reference strain *L. fermentum* ATCC 14931 (Annuk et al., 1999). The *L. fermentum* ME-3 has been deposited in the culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSM 14241). The patent application has been submitted to the Estonian Patent Agency (Application No. 0356/01PV), as well as to International Bureau of WIPO (Application No. PCT/EE02/00006).

The cells and cell lysate of *L. fermentum* ME-3 have a strong antioxidative potency. The cells produce Mn-superoxide dismutase (**Mn-SOD**) ( $0.859 \pm 0.309$  U/mg of protein), contain reduced glutathione ( $9.95 \pm 3.30$   $\mu$ g/mL), and scavenge hydroxyl radicals (~75%). In addition, the cells of ME-3 have high total antioxidative

activity (**TAA**) values ( $29 \pm 1.0\%$ ) (Kullisaar et al., 2002). *Lactobacillus fermentum* ME-3 has been tested for production of  $H_2O_2$  in a qualitative assay as well as by a quantitative method (Kullisaar et al., 2002). The base value of  $H_2O_2$  production in intact cells was  $31 \pm 26$   $\mu$ g/mL.

### Cheese Manufacture

A probiotic cheese containing *L. fermentum* strain ME-3 has been developed at the Department of Microbiology of Tartu University in cooperation with a small cheese manufacturing plant (Vana-Kuuste Dairy Oy) located in the southern part of Estonia. The probiotic cheese was prepared on the basis of Estonian "Pikantne" cheese.

Freeze-dried cheese inoculant Probat 505 (Wisby, Denmark), containing *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, and *Leuconostoc mesenteroides* ssp. *mesenteroides* was used as a starter. Cheese was made with 30 L of pasteurized (70 to 76°C, 20 to 25 s) whole milk. Probiotic lactobacillus was incorporated into cheese in two different manners. In the case of cheese A, 0.04% of probiotic strain ME-3 suspension ( $10^9$  cfu/mL) in 0.9% NaCl was added into the pasteurized cow milk, together with 0.5% cheese starter and  $CaCl_2$  (25 g per 100 kg) before rennet (12 g/tonne of milk) (Maxiren, Gist-Brocades, The Netherlands) coagulation for 35 min at 35°C. The curd was cut and cooked to 39°C. The procedure lasted approximately 100 min. The acidity of curd was pH 6.4–6.5 at the end of the procedure. After cooking, the curd was placed into the mold to drain off the whey. In the case of cheese B, the same portion of probiotic strain (0.04% of suspension of  $10^9$  cfu/mL) was added after curd draining. The cheese made without probiotic additive served as a control.

Cheese blocks (3 kg each) were turned from time to time for approximately 210 min in order to allow the curd to mat together. Thereafter, cheese was removed from the mold, salted in 18% brine at 9°C, extracted from the brine bath, allowed to dry for 2 d, and were left to ripen at 12°C for 30 d at a relative air humidity of 85 to 90%. Throughout ripening, cheese blocks were turned around and rubbed with 5 to 7% brine in order to support the uniform formation of smear on the surface of the cheese. After ripening, the cheese blocks were cleaned from the smear, allowed to dry (3 d), covered with paraffin for 2 s at 120 to 150°C, and stored for 30 d at 6°C (Table 1). Three replicates of experimental cheeses A and B were made.

### Sensory Evaluation of Cheese

Estonian standard EV ST 616-92 was applied for cheese grading. Evaluation was based on four features,

**Table 1.** Procedure of preparation probiotic cheese.

Step	Concentration	Start time	Duration	Temperature (°C)	Samples taken
Pasteurization		1st day	20–25 s	70–76	
Starter addition	0.5%	1st day			
Cheese A: probiotic addition	0.04%	1st day			
CaCl <sub>2</sub>	25 g/100 kg	1st day			
Rennet addition	12 g/t				
Rennet coagulation		1st day	35 min	35	
Cooking/cutting		1st day	100 min	39	
Draining			210 min		
Cheese B: probiotic addition	0.04%	1st day			
Molding		1st day			
Brine salting	18%	1st day	24 h	9	
Drying		2nd day	3 d		
Ripening		5th day	30 d	12	10th, 24th day
Cleaning from smear		35th day			
Drying		35th day	3 d		
Paraffin covering		38th day	1–2 s	120–150	38th day
Storage		38th day	30 d	6	54th, 66th day

such as outer appearance, flavor and aroma, consistency of body, color, and texture, resulting in a maximum of five points for each characteristic. According to this system, there are four classes for cheese, such as excellent (5.00 to 4.50 points), standard (4.00 to 4.49 points), II class (3.50. to 3.99 points), and III class (under 3.50 points). Cheese A, cheese B, and control were graded in a blind manner after 30 d of ripening by five specialists from a local cheese manufacturing plant and the Department of Food Technology of the Estonian Agricultural University. The arithmetical mean of the character point was determined.

The composition of cheese was analyzed in the laboratory of the Veterinary and Food Administration in Tartu, Estonia (Table 2).

### Microbial Analysis of Cheese Milk and Probiotic Cheese

Milk was analyzed after pasteurization and before cheese preparation. Samples for microbiological analyses were aseptically taken from milk and from the center of the cheese. The cheese samples were homogenized, serial dilutions of milk and homogenized cheese

were prepared with 0.9% NaCl solution, and 0.1 mL of each dilution was spread onto the de Man-Rogosa-Sharpe (MRS) agar medium (Oxoid Ltd., Basingstoke, Hampshire, U.K.). The plates were incubated at 37°C for 2 d in a variable atmosphere incubator (IG 150, Jouan, France) with the following microaerobic atmosphere CO<sub>2</sub>/O<sub>2</sub>/N<sub>2</sub>: 10/5/85.

For determination of the cheese lactoflora, the colonies of different morphology grown on MRS were selected (Mikelsaar et al., 2002; Annuk et al., 2003). The provision identification of cocci was based on the microscopical analyses after Gram staining and vancomycin susceptibility. The *Lactobacillus* spp. isolates were identified according to five tests: carbohydrate fermentation patterns, gas formation from glucose, hydrolysis of arginine, catalase activity, and vancomycin resistance.

The survival of the strain ME-3 in cheese and its counts per gram during the ripening and storage were analyzed on d 10, 24, 38, 54, and 66 after the cheese preparation. Putative *L. fermentum* ME-3 colonies were selected on the basis of colony morphology (white, convex colonies with regular edges), microscopical evaluation after Gram staining (regular, nonspore forming, gram-positive plump rods, variable in length, mostly occurring in parallel pairs), negative catalase reaction, growth at low temperatures, and lysozyme production and gas production from glucose. This latter aspect is the main property of obligately heterofermentative lactobacilli (OH<sub>EL</sub>), distinguishing this fermentation group from obligately homofermentative and facultatively heterofermentative lactobacilli (Lencner et al., 1984; Kandler and Weiss, 1986). Lysozyme production positively distinguishes *L. fermentum* from other species of the OH<sub>EL</sub> group (Lenzner and Lenzner, 1982).

**Table 2.** Composition of cheese containing probiotic *Lactobacillus fermentum* ME-3.

Cheese composition	Concentration per 100 g
NaCl	1.5%
Carbohydrates	—
Fat (in dry matter)	50%
Protein	29 g
Vitamin A	0.26 mg
Vitamin B <sub>2</sub>	0.3 mg
Vitamin C	1.6 mg



Changes in the antagonistic activity towards enteropathogenic microbes, as well as antioxidative activity of the reisolates compared with the pure culture of ME-3, were investigated on d 24, 38, and 66 after the cheese preparation.

### Antimicrobial Activity

Antagonistic activity of ME-3 against cheese starter lactic acid bacteria and ME-3 as well as its reisolates from cheese against *Escherichia coli* K12, *E. coli* ATCC 700336, *E. coli* ATCC 700414, *Shigella sonnei* ATCC 25931, *Staphylococcus aureus* B46, *Salmonella enteritidis* ATCC 13076, and a clinical isolate of *Salmonella* Typhimurium was assessed using a streak line procedure (Annuk et al., 2003). A single line of lactobacilli culture, grown in MRS broth for 48 h, was seeded in the middle of a modified MRS agar plate. Target bacteria were seeded in duplicate perpendicular to the streak line of lactobacilli. The width of the zone of inhibition (mm) of the target bacteria extending from the culture line of lactobacilli was measured.

### TAA

*Lactobacilli* were grown in MRS broth overnight and were pelleted by centrifugation at 4°C (1500 rpm for 10 min), washed with isotonic saline (4°C) and resuspended in isotonic saline (Sigma, St. Louis, MO). The suspension was adjusted to  $10^9$  cfu/mL.

The TAA of *L. fermentum* ME-3 and the reisolates from cheese (cheese A) was assessed by using a lipid peroxidation test described elsewhere (Kullisaar et al., 2002). The TAA of the sample was expressed as the inhibition percentage of the linolenic acid (LA) peroxidation by the sample. The high numerical value (%) indicates the high total antioxidative activity of the sample. Peroxidation of LA-standard in isotonic saline served as a control.

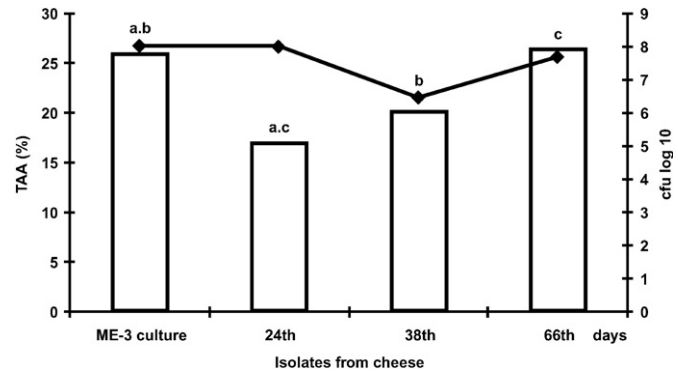
### Statistical Analysis

The changes in antagonistic activity and TAA of the initial ME-3 culture and ME-3 reisolates in different times from cheese were compared by Mann-Whitney rank sum test. The computer program Sigma Stat for Windows 2.0 (Jandel Corp., San Rafael, CA) was applied. Differences were considered statistically significant when  $P < 0.05$ .

## RESULTS

### Sensory Evaluation

Cheeses A, B, and the control cheese were described to be of commercial grade with respect to sensory crite-



**Figure 1.** Changes in total antioxidative activity (TAA) and microbial cell counts ( $\log_{10}$  cfu/g) of the probiotic additive *Lactobacillus fermentum* ME-3 in vitro and in reisolates from cheese (cheese A) during the 66-d period after preparation.

Changes in cell counts of the probiotic additive are indicated by  $\blacklozenge$ .

<sup>a</sup>A significant difference in TAA of ME-3 culture/cheese isolate on d 24;  $P = 0.006$ .

<sup>b</sup>A significant difference in ME-3 culture/cheese isolate on d 38;  $P = 0.03$ .

<sup>c</sup>A significant difference in isolate on d 24/cheese isolate on d 66;  $P = 0.01$ .

ria after 1 mo of ripening. Both cheese variants with probiotic additive were found to have flavor and texture comparable to the control cheese (the original cheese, “Pikantne”). The score value of the control cheese was 4.5 points. Cheese A achieved a grade of 4.2 points; cheese B scored 3.7 points due to its harder consistency and slightly bitter taste. Cheese A was chosen for further testing of its viability and probiotic properties (antimicrobial and antioxidative activity) because of its better sensory properties.

### Survival of the Strain in Cheese

The cheese milk contained different unidentified cocci (total counts  $7 \times 10^3$  cfu/mL) and two *Lactobacillus* species such as *L. plantarum* ( $3 \times 10^2$  cfu/mL) and *L. casei* ( $10^2$  cfu/mL).

In the ripe control cheese, the numbers of the lactic acid cocci reached  $6 \times 10^8$  cfu/g. The counts ( $8.5 \times 10^7$  cfu/g of cheese) of different cocci in the probiotic cheese were not significantly different from the control cheese. In the control and probiotic cheeses, the counts of *L. plantarum* were similar  $5 \times 10^8$  cfu/g, yet for *L. casei*, the counts were somewhat lower in ripe probiotic cheese ( $5 \times 10^6$  cfu/g vs.  $5 \times 10^7$  cfu/g, respectively). ME-3 survived in cheese A during 66 d of the trial. However, during d 24 and 54, some decline ( $P < 0.05$ ) was noted, yet the numbers of probiotic lactobacilli gained the initial level ( $5 \times 10^7$  cfu/g) to d 66 (Figure 1).

**Table 3.** Antagonistic activity of *Lactobacillus fermentum* ME-3 and changes in the antagonistic activity of the reisolates from cheese during the ripening period, expressed as inhibition zone values (mm) on agar.

Target bacteria	Inhibition zone values (mm) mean $\pm$ SD				
	ME-3	Cheese isolates of ME-3			Probiotic cheese
		24th day	38th day	66th day	
<i>Staphylococcus aureus</i> B46	19.5 $\pm$ 1.3	14.5 $\pm$ 1.0	15.1 $\pm$ 1.4	14.7 $\pm$ 6.5	9.3 $\pm$ 2.4
<i>Escherichia coli</i> K12	23.5 $\pm$ 2.4	19.2 $\pm$ 1.2	18.7 $\pm$ 4.1	15.8 $\pm$ 5.2	14.0 $\pm$ 2.1
<i>Escherichia coli</i> ATCC 700336	24.0 $\pm$ 1.4	18.5 $\pm$ 3.5	18.1 $\pm$ 1.4	15.0 $\pm$ 5.8	12.3 $\pm$ 2.7
<i>Escherichia coli</i> ATCC 700414	21.5 $\pm$ 0.7	15.2 $\pm$ 0.9	17.3 $\pm$ 1.0	21.2 $\pm$ 7.2	11.8 $\pm$ 2.5
<i>Salmonella</i> Typhimurium	18.3 $\pm$ 4.6	19.2 $\pm$ 5.0	16.8 $\pm$ 3.2	14.8 $\pm$ 5.8	11.1 $\pm$ 2.4
<i>Salmonella enteritidis</i> ATCC 13076	24.5 $\pm$ 0.7	19.0 $\pm$ 1.1	18.7 $\pm$ 2.1	14.5 $\pm$ 5.6	13.5 $\pm$ 3.1
<i>Shigella sonnei</i> ATCC 25931	20.5 $\pm$ 0.7	21.0 $\pm$ 7.4	17.2 $\pm$ 3.8	20.2 $\pm$ 6.1	13.0 $\pm$ 3.1
Average for gram-negative bacteria	22.0 $\pm$ 1.6	18.7 $\pm$ 3.2	17.8 $\pm$ 2	16.9 $\pm$ 6	12.6 $\pm$ 2.7

### Antagonistic Activity

*Lactobacillus fermentum* ME-3 revealed no antimicrobial effect on cheese starter lactic acid bacteria (data not shown). Compared with the original culture of ME-3, the reisolates of the probiotic additive revealed some decrease in antagonistic activity against all of the tested pathogens. The base values of antagonistic activity of ME-3 and cheese are described in Table 3. *Lactobacillus fermentum* ME-3 expresses some decreased antagonistic activity against both gram-positive and gram-negative pathogens during the ripening period ( $P < 0.05$ ) and storage ( $P < 0.05$ ), apart from *E. coli* ATCC 700414 and *Shigella sonnei* ATCC 25931, which were suppressed at the same level on d 66 as by the original pure culture of ME-3. The antimicrobial activity of the ripe probiotic cheese was weaker than that of the reisolated probiotic additive.

### TAA

It was noted that the increasing tendency of TAA was expressed by the reisolates of the strain throughout the trial as follows: 17% on d 24 and 20% on d 38. At the end of the experiment (d 66), the TAA of the probiotic *Lactobacillus* cells gained approximately the same value as the pure culture of ME-3 (26.3 vs. 26%, respectively) (Kullisaar et al., 2002) (Figure 1).

### DISCUSSION

The main target point of the probiotic *Lactobacillus* is the human gastrointestinal tract, and the cheeses may serve only as probiotic carriers. Taking advantage of probiotics for their antimicrobial and antioxidative properties is expected to gain popularity for use in humans. Different varieties of cheese have been used as probiotic delivery vehicles (Blanchette et al., 1996; Gobetti et al., 1998; Gomes and Malcata, 1998). The most thoroughly investigated cheese type used as a probiotic

carrier is Cheddar cheese (Dinakar and Mistry, 1994; Stanton et al., 2001). There is little data for semisoft or soft smear-ripened cheeses (Antosson et al., 2003) as probiotic vehicles. In this study, we have proved that the Estonian "Pikantne" cheese is a good carrier for the probiotic *L. fermentum* strain, ME-3. "Pikantne" belongs to the Tilsit-type cheese variety. It was chosen as a carrier of the probiotic additive because its ripening period is relatively short (the ripening time of "Pikantne" is commonly 20 to 30 d, depending on the weight of cheese blocks), and the ripening starts from the surface. The inner part of cheese is quite stable for a relatively long period, and the probiotic strain has chance for adaptation to the environment of cheese. Other advantage was the lack of antagonistic activity between the probiotic additive and the cheese starter (lactic acid bacteria). The small private dairy was chosen for the experimental probiotic cheese preparation due to its suitable size and location near Tartu University.

The probiotic strain, ME-3, was found to withstand the cheese manufacturing process and to survive during ripening and storage without negative effects on the quality of cheese. *Lactobacillus fermentum* ME-3 had a negligible effect on cheese composition, flavor, and aroma. The probiotic-containing cheese A was considered to be of commercial grade, and the sensory analysis indicated that it was slightly different compared with the control cheese. In spite of both methods of incorporating ME-3 into cheese being successful, adding ME-3 into milk together with cheese starters should be the preferred method in the industrial manufacture of cheese since it is an easier method of achieving higher cell counts. When added to the curd, probiotic additive could be partly drained off with whey.

There are relatively few reports concerning *L. fermentum* as a secondary flora in cheese (Smith and Cunningham, 1962; Cogan et al., 1998; Beresford et al., 2001), although some other species of the OHEL group (e.g., *L. brevis*, *L. danicus*) have been isolated from

cheese, including Estonian semihard cheese (Kask, 2003). We found that *L. fermentum* ME-3 was distinguishable from the nonstarter lactic acid bacteria of cheese since the cheese milk did not contain any obligately heterofermentative lactobacilli. Therefore the traditional microbiological methods (Kandler and Weiss, 1986) used for primary identification of cheese lactobacilli were sufficient to confirm its presence of the probiotic additive, *L. fermentum* ME-3.

When inoculated into cheese milk together with starter microbes, ME-3 multiplied in cheese, sustaining cell counts of  $5 \times 10^7$  cfu/g, which satisfied the suggested therapeutic minimum (Lee and Salminen, 1995), as well as the recommended concentration of probiotic in food ( $10^7$  or even  $10^8$  cfu/g) (Reid, 2001; Stanton et al., 2001). Besides, different nutritional conditions inside curd grains could be crucial for the survival and probiotic properties of the added *Lactobacillus* strain. The probiotic cheese environment did not contain remarkable amounts of carbohydrates (Table 2). Nevertheless, nonstarter lactobacilli could use sugars released via enzymatic hydrolysis of  $\kappa$ -casein and several other compounds present in cheese environment as potential energy sources after lactose is consumed by starter strains during the first 24 h of ripening. On the other hand, arginine could be used as an energy source by heterofermentative lactobacilli (Laht et al., 2002). Heterofermentative *L. fermentum* ME-3 is able to use arginine as an energy source. In any case, the proteolytic activity of ME-3 needs further investigation.

The high antimicrobial activity of lactobacilli is associated with the production and synergistic activity of organic acids and  $H_2O_2$ . The production of antimicrobial compounds is shown to be dependent on the growth environment (Annuk et al., 2003). The antagonistic activity of lactobacilli against both gram-negative and gram-positive target bacteria is dependent on the fermentation group of the lactobacilli, representatives of the OHEL group being the strongest antagonists (Annuk, 2002). Compared with other *L. fermentum* strains, ME-3 is a relatively efficient producer of lactic and acetic acids (Annuk, 2002). The production of  $H_2O_2$  by lactobacilli is mainly strain-specific, but there is also a correlation between fermentation group and  $H_2O_2$  production: lactobacilli from the OHEL group are the second-strongest producers of  $H_2O_2$  (Annuk et al., 2003).

We revealed that the original culture of *L. fermentum* ME-3 had a high antimicrobial effect against the enteropathogenic pathogens *E. coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Shigella sonnei*, although a slightly decreased antimicrobial activity of ME-3 isolates from ripened cheese and during shelf life was noted. The antimicrobial activity of ripe cheese was predictably lower than of ME-3 isolates from ripe

cheese. It is possible that it was influenced by the lower concentration of probiotic additive in cheese and the cheese protein matrix surrounding the *Lactobacillus* cells also has a weakening effect on the antimicrobial activity.

“Pikantne” is manufactured from pasteurized milk and is mostly safe for consumers. According to the Estonian Health Protection Inspectorate, no outbreaks due to consumption of the milk products have been reported since 1997. However, the probiotic additive, ME-3, has the potential to suppress pathogens, occasionally contaminating cheese curd.

Some lactic acid bacteria are able to decrease the risk of reactive oxygen species accumulation in host organisms, and could potentially be used as oxidative stress-reducing probiotic food supplements. The original culture of ME-3 expresses high TAA values (Kullisaar et al., 2002). In healthy volunteers, it has been demonstrated that the consumption of fermented milk containing *L. fermentum* ME-3 exhibited antioxidative and antiatherogenic effects (Kullisaar et al., 2003). When incorporated into cheese, the ME-3 cells probably undergo a stress caused by the cheese-making process and a decline in cell counts, TAA, and antimicrobial properties is noted. The ability to tolerate low temperatures distinguishes ME-3 from other *L. fermentum* strains. This enables the strain to survive and multiply at relatively low temperatures during cheese ripening and even lower temperatures during storage. However, the most interesting finding of our study was the established association between the germination activity of ME-3 and its probiotic properties. It could be proposed that the functional properties of ME-3 need time to adapt to the unusual cheese environment. The cells surviving cheese making start to multiply almost in parallel with growth of TAA, and after 2 mo (66 d after cheese preparation), were able to reach the same TAA values as the original ME-3 culture (Figure 1). Nevertheless, the health benefits of the probiotic cheese must still be demonstrated in further clinical studies.

## CONCLUSIONS

The present study shows that the *L. fermentum* strain, ME-3, is suitable for use as a novel probiotic cheese supplement. The strain sustained its probiotic properties: antioxidative and antimicrobial activity. We demonstrated that Pikantne, the Estonian open-texture, soft cheese, proved to be an appropriate probiotic delivery vehicle for ME-3.

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