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Storage stability of fermented milk with probiotic monoculture and transglutaminase

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Abstract: The effect of microbial transglutaminase on selected physicochemical and organoleptic characteristics and viability of probiotic bacteria in fermented milk inoculated with probiotic monoculture (*Lactobacillus acidophilus* LA 5 or *Bifidobacterium bifidum* BB 12) was analysed. Four types of samples were prepared: (1) fermented milk inoculated with *Lactobacillus acidophilus* LA 5, (2) fermented milk inoculated with *Bifidobacterium bifidum* BB 12, (3) fermented milk produced from milk previously treated with mTGase and inoculated with *Lactobacillus acidophilus* LA 5, (4) and fermented milk produced from milk previously treated with mTGase and inoculated with *Bifidobacterium bifidum* strain BB 12. The samples were analysed after the 1st, 7th and 14th day of storage at 5 ± 1°C. It has been found that the use of microbial transglutaminase for the production of fermented milk inoculated with monoculture affected its viscosity, hardness, acetaldehyde content and increased the viability of probiotic bacteria. The enzyme activity resulted in an significant decrease in the titratable acidity of the experimental products, positively affected viscosity, the viability of probiotic bacteria and the organoleptic properties of fermented milk.

Keywords: acidity; enzyme; fermentation; hardness; viscosity

Fermented milk is considered by nutritionists as having high nutritional value and positive bioactive effects, usually reinforced by the addition of prebiotic ingredients and probiotic bacteria. The genera *Lactobacillus* and *Bifidobacterium* are used the most often as probiotic bacteria in this type of products (Nowak *et al*. 2010) but the fermented milk should contain at least 10^6 CFU of labelled (*i.e.*, probiotic) bacteria per millilitre (Wang *et al*. 2012). Probiotic bacterial cells do not have the ability to typically ferment milk. A clot produced by them is usually very soft and short-lasting, especially in case of a single probiotic strain (Zarębа *et al*. 2008).

A recently used method for shaping the texture of fermented milk is based on the application of transglutaminase (protein-glutamine amine γ-glutamyltransferase). This enzyme catalyses acyl transfer reactions between γ-amide groups of glutamine in proteins, as donors of the acyl groups, and primary amines, which are acceptors of these groups. Enzyme actions cause changes in the structure of proteins and peptides, of which the most important

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is the formation of crosslinks that alter the ability to retain water, solubility, viscosity, elasticity, smell or colour of the product. Microbial transglutaminase (mTGase) is a single polypeptide chain with a molecular weight of 38,000 Da, consisting of 331 amino acids, with the isoelectric point at a pH of 8 to 9. Although improvement in the rheological characteristics of probiotic fermented milk can be achieved by using mTGase, the possible effects of this treatment on the viability of probiotic strains should be kept in mind. In the subject literature, there is no comprehensive information on the effect of cross-linking enzyme on the quality of fermented milk inoculated with probiotic monoculture and on the viability of these strains in the end-product. Therefore, in this study we assessed the impact of mTGase on the organoleptic characteristics, acidity, hardness, viscosity and acetaldehyde content in fermented milk containing probiotic monoculture such as *Lactobacillus acidophilus* LA 5 and *Bifidobacterium animalis* ssp. *lactis* BB 12.

MATERIAL AND METHODS

Materials. The raw material was homogenised (15 MPa, 55°C) and pasteurized at a high temperature (85°C, 30 s) cow's milk, containing 3.2% fat, 3.0% protein and 4.8% lactose.

Probiotic cultures. The experimental fermented milk was produced not with the use of conventional starter cultures commonly applied in the production of various kinds of milk-fermented beverages but only with probiotic freeze-dried DVS type monocultures of *Lactobacillus acidophilus* LA 5 and *Bifidobacterium animalis* ssp. *lactis* BB 12 manufactured by Chr. Hansen (Hoersholm, Denmark). The strains *B. animalis* ssp. *lactis* BB 12 and *L. acidophilus* LA 5 are among Chr. Hansen's best clinically documented probiotic strains. They have been tested in numerous clinical studies and have demonstrated health benefits in relation to gastrointestinal health. Reviving and activating starters were performed as per the instructions of Technological Production Starter, developed by Bielecka (1984) in non-fat milk (0% fat).

Transglutaminase. Activa MP® (E.C. 2.3.2.13) (Ajinomoto Co. Inc., Japan) was used as an additive during the production of two variants of fermented milk. This enzyme preparation, recommended for cross-linking of milk proteins, is composed of Ca^{+2} independent transglutaminase derived from the microorganism *Streptoverticillium mobaraense* (1%) and 99% of maltodextrin. It was used in the original form, without any further purification (Dmytrów *et al*. 2010).

Fermented milk – processing conditions. The pasteurized milk was heated to 40°C and divided into 4 batches (each of 4000 ml). Two of them (2 × 4000 ml) were enriched with 0.02% of Activa MP® and then were incubated for 2 h at 40°C. After the incubation, the processed milk was heated at 80°C for 1 min to inactivate the enzyme. After cooling to 40°C, each batch of milk treated with mTGase was inoculated with 2.5% of activated starter containing single probiotic strains, *i.e., L. acidophilus* LA 5 and *B. animalis* ssp. *lactis* BB 12, respectively. The other two batches of milk were inoculated only with the listed potentially probiotic strains (variants without the addition of mTGase). Incubation of all variants of fermented milk was carried out at 42°C. The end of the fermentation was indicated by the pH and fermentation curve set in the culture specification. The samples were cooled down to 5 ± 1°C and stored at the same temperature for 14 days. Four types of samples of fermented milk were obtained: inoculated with *L. acidophilus* LA 5 (LA), inoculated with *B. animalis* ssp. *lactis* BB 12 (BB), treated with mTGase and inoculated with *L. acidophilus* LA 5 (LA-TG), and treated with mTGase and inoculated with *B. animalis* ssp. *lactis* BB 12 (BB-TG). The samples were tested after 1st, 7th and 14th days of storage at 5 ± 1°C.

Physicochemical analysis. The following characteristics were analysed in the samples: acetaldehyde content using diffusion method with hydrochloride hydrazine in Conway Chambers (Lees & Jago 1969), titratable acidity in Soxhlet Henkel degrees (AOAC, 2000) and active acidity were measured with a pH meter (IQ 150; Spectrum Technologies, UK). The analysis was performed in 6 replicates.

Hardness measurement. Texture profile analysis was performed using a TA.XT Plus texture analyser with a computer set (Stable Micro System Ltd., UK). The samples were penetrated with a pressure of 1G and a velocity of 5 m/s up to a depth of 25 mm. The diameter of the aluminium pin was 20 mm. The analysis was performed at 10°C in 6 replicates.

Viscosity measurement. Viscosity measurement of stirred fermented milk was carried out at 20 ± 1°C using the measurement system composed of double gap concentric cylinders in TA Instruments AR 2000 rheometer. The apparent viscosity of the

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samples (2 ml) was determined in a range of share rate 1–400 s^{-1} maintaining the constant temperature of the sample (Peltier module) during the experiment. The average value of apparent viscosity at a shear rate of 2.68 s^{-1} was obtained. All viscosity measurements were performed thrice.

Microbiological analysis. Microbiological analysis included the determination of the cell number of the probiotic microflora in the fermented milk by the classical plate method with two parallel independent replicates for three replicates of each of the analysed samples (EN ISO 6887-5:2010). The MRS agar medium purchased from Merck (Merck KGaA, Germany) was used for the cultures. The plates were incubated at 37°C/72 h using an anaerobic jar equipped with Anaerocult A (Merck KGaA, Germany). The results were converted to the number of colony-forming units per 1 g of the product (CFU/g).

Organoleptic assessment. Organoleptic evaluations were conducted by participants (n = 9) trained in the organoleptic evaluation of fermented milk, according to ISO (1998). The appearance, taste, smell and consistency of samples were evaluated on a 5-point scale where 1 was the worst score and 5 was the best score. The evaluation was carried out in a room that was free of any foreign odours; each panellist had a separate test stand and distilled water to rinse their mouths. All physicochemical analysis and organoleptic assessment were performed in three series.

Statistical analysis. The statistical analyses were carried out by 2-way ANOVA with repeated measures and tests to determine the differences in 2 dependent and independent means (Student's t-test). Organoleptic assessment was analysed by means of the Kruskal-Wallis test. The pairwise comparisons were done by means of the post-hoc Dunn's test. The statistical significance of all the tests was P = 0.05.

RESULTS AND DISCUSSION

The obtained results confirmed a statistically significant increase in the titratable acidity of all the variants of samples (Table 1) – the largest in the sample LA, and the smallest in BB. mTGase activity resulted in an significant decrease in the titratable acidity of the experimental products. When comparing the first and last days of the storage period, a decrease in the pH of all the variants of fermented milk was found – the most significant in the case of the sample BB. Domagała & Wszołek (2008) reported

Table 1. Quality properties of analysed fermented milk during cold storage for 14 days at 5 ± 1°C

Property	Storage time (day)													
	LA			BB			LA-TG			BB-TG				
	1	7	14	1	7	14	1	7	14	1	7	14
Organoleptic evaluation (points)	4.74^{A}	4.75^{B}	4.63^{E}	4.70^{a}	4.63^{c}	4.50^{e}	4.88^{B}	4.75^{B}	4.70^{F}	4.75^{b}	4.70^{d}	4.63^{f}
Titratable acidity (SH°)	32.07 ± 0.16^{A}	35.73 ± 0.46^{C}	38.80 ± 0.35^{E}	30.50 ± 0.16^{a}	32.27 ± 0.80^{c}	33.20 ± 0.20^{e}	29.60 ± 0.69^{B}	32.60 ± 0.46^{D}	35.40 ± 0.35^{F}	28.30 ± 0.61^{b}	30.67 ± 0.39^{d}	32.10 ± 0.42^{f}
pH	4.6 ± 0.02^{A}	4.52 ± 0.03^{C}	4.38 ± 0.01^{E}	4.62 ± 0.02^{a}	4.48 ± 0.02^{c}	4.40 ± 0.02^{e}	4.60 ± 0.01^{B}	4.56 ± 0.02^{D}	4.35 ± 0.00^{F}	4.60 ± 0.00^{b}	4.44 ± 0.01^{d}	4.29 ± 0.02^{f}
Acetaldehyde (mg/l)	2.17 ± 0.07^{A}	1.53 ± 0.09^{C}	1.13 ± 0.07^{E}	3.38 ± 0.34^{a}	0.71 ± 0.47^{c}	0.62 ± 0.02^{e}	2.19 ± 0.01^{B}	1.33 ± 0.99^{D}	0.94 ± 0.07^{F}	2.04 ± 0.23^{b}	0.99 ± 0.13^{d}	1.11 ± 0.14^{f}
Hardness (N)	0.35 ± 0.01^{A}	0.42 ± 0.02^{C}	0.46 ± 0.04^{E}	0.40 ± 0.06^{a}	0.57 ± 0.01^{c}	0.58 ± 0.03^{e}	0.50 ± 0.04^{B}	0.52 ± 0.15^{D}	0.80 ± 0.03^{F}	0.48 ± 0.05^{b}	0.54 ± 0.09^{d}	0.85 ± 0.25^{f}
Viscosity (mPa/s)	0.414 ± 0.02^{A}	0.394 ± 0.01^{C}	0.449 ± 0.01^{E}	0.249 ± 0.02^{a}	0.309 ± 0.03^{c}	0.394 ± 0.02^{e}	0.613 ± 0.01^{B}	0.745 ± 0.01^{D}	0.623 ± 0.02^{F}	0.324 ± 0.01^{b}	0.448 ± 0.03^{d}	0.591 ± 0.02^{f}

$^{A–F}$means for samples containing *L. acidophilus LA 5* (LA and LA-TG) with different uppercase letters in the same raw are significantly different ($P < 0.05$); $^{a–f}$means for samples containing *B. animalis* ssp. *lactis* BB 12(BB and BB-TG) with different lowercase letters in the same raw are significantly different ($P < 0.05$); LA – milk fermented by *L. acidophilus* LA 5; BB – milk fermented by *B. animalis* ssp. *lactis* BB 12; LA-TG – milk treated with mTGase and fermented by *L. acidophilus* LA 5; BB-TG – milk treated with mTGase and fermented by *B. animalis* ssp. *lactis* BB 12

https://doi.org/10.17221/22/2019-CJFS

that an important factor influencing the quality of fermented milk is an appropriate choice of starter cultures. The type of starter and its composition affect the dynamics of souring. However, it is believed that probiotic bacteria are not able to ferment milk properly (Korbekandi *et al.* 2011). These reports are in contradiction with the results obtained in our work. The appropriate clot and an increase in titratable acidity were observed in all the experimental samples. This phenomenon can be explained partly by the fact that the lack of nutrients in the medium and weak proteolytic activity of probiotics lead to cell death and autolysis, causing an increase in β-galactosidase activity of the injured bacterial cells. Fernandez *et al.* (1998) have made similar observations writing about the enhanced activity of this enzyme in damaged cells of *L. acidophilus*. On the other hand, Usajewicz (2008) wrote that bacteria of the genus *Bifidobacterium* metabolize glucose, galactose and lactose. They cause heterofermentative lactic fermentation in which glucose is metabolised in the fructose-6-phosphate pathway often called 'Bifidobacterium shunt'. The final products of fermentation are lactic acid and acetic acid in the ratio of 2:3. The author of the work also claimed that *L. acidophilus* is one of the thermophilic homofermentative lactobacilli fermenting hexoses to lactic acid, D(–) or DL, but not metabolizing pentoses. Cow's milk, in addition to lactose, also contains traces of other free sugars, which allow some probiotics to conduct acidification of the environment.

We observed that samples prepared with mTGase had significantly lower acidity than those without mTGase. It can be explained by the fact that mTGase, as a result of protein cross-linking, limits the access of starter bacteria to amino acids and low molecular weight peptides, thus limiting their growth and the amount of lactic acid produced. Özer *et al.* (2007) found that the growth of starter bacteria is reduced in the presence of mTGase. Milanowić *et al.* (2007) reported a decrease in pH value of fermented milk produced with this enzyme and significant differences in the pH values of the samples containing mTGase and the control sample. Accordingly, in our study it was observed that a significant effect of mTGase on the acidity of samples may also result from the fact that the structure of fermented milk reinforced by the enzyme protects the probiotic cells (Korbekandi *et al.* 2011).

A statistically significant decrease in the acetaldehyde content was observed in all the products (Table 1). The acetaldehyde is metabolised to ethanol (as a result of alcohol dehydrogenase activity), resulting in the loss of its contents. Among the probiotics used for the production of fermented milk, *L. acidophilus* has the unique potential to produce acetaldehyde from the various components, that is, carbohydrates, amino acids (*e.g.*, threonine) and nucleic acids (Østile *et al.* 2003). It is believed that bifidobacteria do not produce the key carbonyl compounds, particularly acetaldehyde (Tamime *et al.* 2005). In our experiment, fermented milk produced with the use of mTGase was characterized by better organoleptic features. The positive impact of mTGase on the organoleptic properties of the experimental products was reported by Lorenzen *et al.* (2002) who stated that transglutaminase works as a factor enhancing the consistency and structure of fermented milk. The results of the rheological analysis demonstrated a significant increase in the hardness of all the tested fermented beverages. We can explained it by the fact that the milk protein gel becomes generally harder, probably because the protein-protein binding becomes stronger. Lorenzen *et al.* (2002) confirmed that the gel hardness of fermented milk containing mTGase is almost twice higher than the gel hardness of sample made from milk without the addition of this enzyme. During storage of the fermented milk, the viscosity of each sample increased only slightly. It is clear that mTGase positively affected viscosity when compared to the samples without mTGase (Table 1). Tratnik *et al.* (2006) reported that mTGase addition resulted in obtaining samples with greater amount of protein particles, increased the number of particles that bind water molecules and increased viscosity due to a greater water-binding capacity.

Our study showed that all the tested variants of fermented milk were characterized by normative content of live probiotic bacteria (at least 10^6 CFU/g) and during the storage, a general decrease in their number was observed, however, to a level not exceeding the therapeutic minimum (Table 2). The use of mTGase resulted in increased viability of probiotic bacteria and in case of beverages produced without the use of this enzyme a greater number of viable cells was observed in fermented milk containing *L. acidophilus* LA 5. Sady *et al.* (2007) explained the decrease in the number of probiotics by the increase in the acidity of the samples during storage. They concluded that among the evaluated species of microorganisms (*S. thermophilus, L. acidophilus* and *Bifidobacterium* sp.), the biggest decrease in the number of viable cells was observed for *Bifidobacterium* sp. The decrease in the viability of *L. acidophilus* in yoghurt produced from cow's milk

https://doi.org/10.17221/22/2019-CJFS

Table 2. Survival rate of probiotic bacteria (CFU/g) in fermented milk stored refrigerated for 14 days

Sample	Storage time (day)		
	1	7	14
LA	1.5×10^{8Ab}	1.3×10^{8Bb}	1.0×10^{8Cb}
BB	1.3×10^{8Ad}	3.5×10^{7Bd}	1.2×10^{7Cd}
LA-TG	4.0×10^{8Aa}	2.3×10^{8Ba}	2.0×10^{8Ca}
BB-TG	2.2×10^{8c}	1.8×10^{8Bc}	5.1×10^{7Cc}

$^{A–C}$means with different uppercase letters in the same raw are significantly different ($P < 0.05$); $^{a–d}$means with different lowercase letters in the same column are significantly different ($P < 0.05$)

or other milk-based products with a pH of 4 to 5 has been observed in many studies (Zaręba *et al*. 2008; Buriti *et al*. 2010; Mituniewicz-Małek *et al*. 2014). It is known that the main factors affecting the viability of *Lactobacillus* sp. in food are the probiotic strains, the inoculum concentration, the fermentation time, the decrease in the medium pH value, the presence of oxygen or hydrogen peroxide, the concentration of bacterial metabolites and the storage temperature (Donkor *et al*. 2006). Neve *et al*. (2001) observed an increase in bacterial cell viability in fermented milk produced with mTGase during cold storage.

CONCLUSIONS

Transglutaminase belongs to the functional additives that are finding increasing application in food processing. Our study proved that the use of mTGase in the production of potentially probiotic fermented milk allows to improve the organoleptic characteristics, viscosity, and hardness, and also increases the viability of probiotic bacteria. However, being aware that many properties of the finished product may substantially depend on the metabolic abilities of individual strains, the results should not be generalized, and our study is merely a prelude to further analyses related to the attempts to designate the strains that will prove themselves most effective in such products.

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