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Enterococcus faecium strain used as an adjunct culture in a starter for kashkaval cheese plays important role to proteolytic processes and release of bioactive peptides during ripening

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ABSTRACT

In the present study the potential of preliminary selected strain *Enterococcus faecium* used as an adjunct culture in starter for kashkaval cheese was evaluated. The strain *Enterococcus faecium* MH3 was selected among 17 isolates on the basis of its high level of proteolytic and peptidase activities. The active proteolytic system of *E. faecium* MH3 leads to high level of transformation of caseins to end products. A high degree of transformation of β-casein and αs1-casein in the initial stage of ripening (up to 15 d) and during storage up to 90 d was determined by Urea-Polyacrylamide gel electrophoresis (exceptionally high degree of degradation – 91% and 83% for αs1-casein and β-casein). The results reveal that the new starter combination, including strain *Enterococcus faecium* MH3 as a nonstarter culture, can be successfully used to enhance proteolysis, shorten the ripening period and improve the quality of hard cheeses. Kashkaval produced using *Enterococcus faecium* MH3 in the starter contains significantly more free amino acids and increased concentration of low molecular weight peptides. The inhibitory activity of low molecular weight peptides against angiotensin-converting enzyme was significantly higher than those of common kashkaval cheese. The aim of the present work was to study the possible use of *Enterococcus faecium* strain as an approach towards enhancing the proteolytic processes during Kashkaval cheese ripening, acceleration of the ripening process and improving the quality of the end product.

Keywords: *Enterococcus*, Kashkaval, bioactive peptides

Introduction

Enterococci are widespread inhabiting different habitats as soil, food, water and gastrointestinal tract of humans and animals. Genus *Enterococcus* currently comprises more than 20 species. *Enterococcus faecalis*, *E. faecium* and *E. durans* are predominant species most frequently found in dairy products (Hassanzadazar et al., 2014). Their important role in cheese making and contribution to the promotion of the sensory characteristics is due to proteolytic and lipolytic activity, citrate utilization and aromatic volatile compounds production in various traditional cheeses in the Mediterranean countries. Usually enterococci are non-starter microflora in the cheese but there are some advantages including enterococci in the cheese starter (Leuschner et al., 1999). Various probiotic bacteria mainly lactobacilli and bifidobacteria groups and recently enterococci genera are used in functional foods (Bulajic & Mijacevic, 2004). The use of probiotics or their antimicrobial compounds in foods are new approach for controlling of food borne pathogens such as *L. monocytogenes* (Simova et al., 2009). Because of inhibitory effects of enterococci strains on *Listeria and Staphylococcus* genera, they would be potential candidate for protective culture. They could be considered as additional

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biopreservative hurdles for the listeria growth inhibition in fermented foods and be of practical use in food industry. Due to the significant proteolytic activity of certain strains enterococci (Garcíaa et al., 2002) they can contribute to the formation of bioactive peptides with inhibitory activity to the enzyme responsible for the elevated blood pressure – angiotensin-converting enzyme (ACE).

Kashkaval cheese is a Bulgarian semi-hard type cheese made with starter containing mainly Lactobacillus bulgaricus and Streptococcus thermophilus strains. The aim of this study was to determine the role of high proteolytic *E. faecium* strain included in the Kashkaval cheese starter to the proteolytic processes during ripening and ability to release bioactive peptides with ACE-inhibitory properties.

Materials and Methods

Bacteria

Enterococci were isolated as explained by Simova et al (14). The species belonging was determined by Arbitrary Ribosomal DNA Restriction Analysis (ARDRA) and 16S rDNA sequencing (Rodtong & Tannock, 1993; Giannou et al., 2008). The strain identity of enterococci was determined by Pulsed Field Gel Electrophoresis (PFGE) (Giraffa et al., 2000; Dimitrov et al., 2005). PFGE was performed on CHEF-DR II system (Bio-Rad Laboratories) for 24 h at 5.5 V cm^{-1} with a pulse time ranging from 5 s to 25 s at 14 °C.

λ-Ladder DNA (Sigma, Pulse Marker 50-1000 kbp, Poole, UK) and λ-DNA HindIII digests (Sigma, Pulse Marker 0.1-200 kbp, Poole, UK) were used as a molecular weight standards.

Cheese making

Two Kashkaval cheese batches were made using two different starters: starter containing only *L. bulgaricus* and *S. thermophilus*, and starter containing *Enterococcus faecium* MH3 strain in addition to *L. bulgaricus* and *S. thermophilus*. Kashkaval cheese was made according to Bulgarian State Standard BDS 14-2010.

Proteolysis

The nitrogen fractions of the cheese samples, including pH 4,6-soluble nitrogen (SN) and soluble nitrogen in 12% trichloroacetic acid (TCA) considered as non-protein nitrogen (NPN), were obtained by the method of Sousa and McSweenly (Sousa & McSweeney, 2001). Urea-polyacrylamide gel electrophoresis (PAGE) of the pH 4,6-insoluble fractions of cheese was performed using a vertical slab gel unit (Hoefer) according to the method of Mayer et al. (Mayer et al., 1998). The gels were stained with Coomassie Briliant Blue G250.

Free amino acids were determined after precolumn derivatization with phenyl-isothiocyanate using Pico.Taq method (Cohen et al., 1984). One gram cheese was mixed with 2 ml 30% methanol in 0.3N hydrochloric acid containing 50 nmol norvaline as internal standard. The mixture was homogenized, kept in refrigerator for thirty minutes, and after that centrifuged at 10000 g for 5 minutes. Five hundred microliters from the supernatant were transferred to ultrafiltration cartridge with cut-off 5000 Daltons (Microcon YM-5, Milipore). The cartridges were centrifuged for 30 minutes at 12000 g. Twenty five microliters from the filtrate were transferred to small Pyrex tubes (6x50 mm) and the samples were evaporated under vacuum. After that the derivatization was conducted according to the Pico.Taq method (WARERS) using specially designed reagents and HPLC column for free amino acids. Ten microliters were injected into the column and the analysis was performed using HPLC equipment SHIMADZU 10A. The UV detection was at 254 nm.

Assay of ACE-inhibitory activity

ACE activity was determined by the method of Cushman and Cheung (Cushman & Cheung, 1970) with some additional modifications. One gram of cheese homogenised in 5 ml phosphate buffer, pH 7,0) was subjected to purification through reverse-phase cartridge (Waters C18ec). Following washing with water the peptides were eluted with 5 ml 60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluate was freeze dried and reconstituted in 1 ml 0.1% TFA. The substrate Hip-His-Leu was dissolved in 100 mM Na-borate buffer (pH 8.3) to concentration in assay mixture of 6 mM. The final concentration of NaCl was 300 mM. To 190 mkl of substrate solution 20 mkl purified supernatant or peptide fraction were added and the reaction was initiated by 40 mkl of ACE enzyme solution (0.1 U/ml).

Results and Discussion

On the basis of the analysis of peptidase activities, *E. faecium* MH3 is a strain with a full-formed proteolytic system – remarkably strong aminopeptidase (Leu-ρNA and Lys-ρNA), high dipeptidase and tripeptidase activities towards Leu-containing substrates and high activities towards

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Pro-containing substrates (Dimitrov, 2012). The peptidases present in *E. faecium* MH3 refute the perceived arguments in favor of the proteolytic potential of *E. faecium* and its usage as a nonstarter culture, i.e. to enhance proteolysis in cheeses. The 25-days old Kashkaval A surpassed the indicators of good ripeness: degree of proteolysis – 28.9%, depth of proteolysis – 35.7%, which, up to 90 days of age in Kashkaval B (control), did not reach the levels of Kashkaval A – 24% degree of ripeness and 27.8% depth of proteolysis. The nitrogen soluble at pH 4.6 in percent of the total nitrogen, and the nitrogen soluble in 12% TCA in % of the total nitrogen increased to 20.5% and 23.2%, respectively (Figure 1).

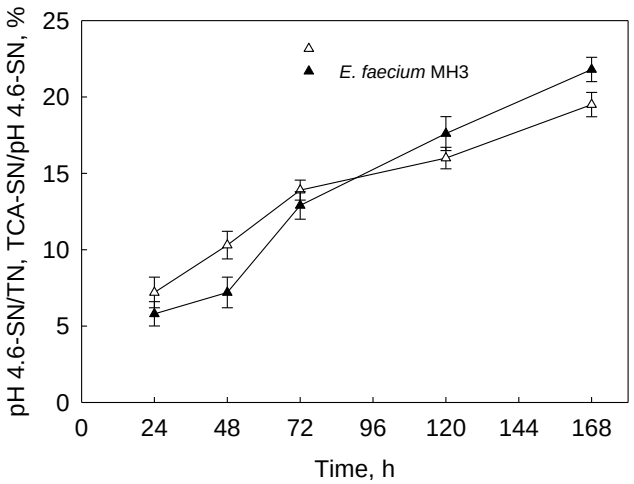

Figure 1. *Change of nitrogen soluble at pH 4.6 in % of the total nitrogen (empty symbols), and of nitrogen soluble in 12% TCA in % of the nitrogen soluble at pH 4.6 (filled symbols) in single strain cultures E. faecium MH3*

The levels of pH 4.6-SN/TN and of TCA-SN/pH 4.6-SN in the *E. faecium MH3* culture are a consequence of the high peptidase activity of the strain and the faster rate of transition of pH 4.6 soluble nitrogen into 12% TCA soluble nitrogen (pH 4.6-SN/TN – 19.5% and TCA-SN/pH 4.6-SN – 21.8%) (Figure 1).

The results from the UREA-PAGE electrophoresis of the casein fractions in Kashkaval A and Kashkaval B (control) are given in Figure 2. The degree of Kashkaval A maturity at each of the four stages from the start of ripening was significantly higher than that in the respective ripening periods of Kashkaval B. The results reveal that the new starter combination, including strain *E. faecium* MH3 as a nonstarter culture, can be successfully used to enhance proteolysis, shorten the ripening period and improve the quality of hard cheeses. They are proof that highly active proteolytic systems participate in the transformation of caseins to end products. A high degree of transformation of β-casein and αs1-casein in the initial stage of ripening (up to 15 days) and during storage up to d 90 (exceptionally high degree of degradation – 91% and 83% for αs1-casein and β-casein) is an indication of action of highly developed proteolytic systems of *E. faecium* MH3.

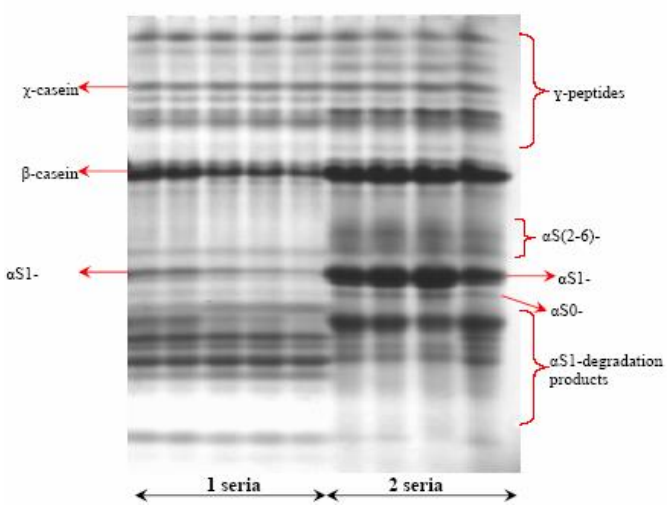

Figure 2. *UREA-PAGE of casein fractions in Kashkaval A (starter culture L. bulgaricus + S. thermophilus + E. faecium MH3) (1 series) and in Kashkaval B (S. thermophilus + L. bulgaricus) (2 series).*

The high peptide content in Kashkaval A as early as 15 days of age indicates a high rate of transformation of the high-molecular weight peptides into lower molecular weight peptides. Typical indications of hydrolysis of the non-casein fractions were obtained: 1) Equalization between the amount of the high-molecular weight peptides and the sum of the amounts of low- and medium-molecular weight peptides as early as 15 days, after which the low- plus medium-molecular weight peptides prevailed over the high-molecular weight peptides up to 60 days. This is the first time that such results about hard cheeses have been documented. 2) The levels of low-molecular weight peptides are higher in Kashkaval A and twice as much as the 60-days old controls (Figure 3).

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In the Kashkaval A hydrophilic peptides increased from 17 % at 30-days old cheese to 26% at 60 days of age, which correlated with the active transformation of pH 4.6-soluble nitrogen into 12% TCA-soluble nitrogen and with the active release of amino acids. The results point to high aminopeptidase activity acting in Kashkaval A, produced by the proteolytic *E. faecium* MH3 caused formation of hydrophilic peptides by 15 days of ripening, thus increasing the transformation activity in the soluble nitrogen fraction to end products – free amino acids.

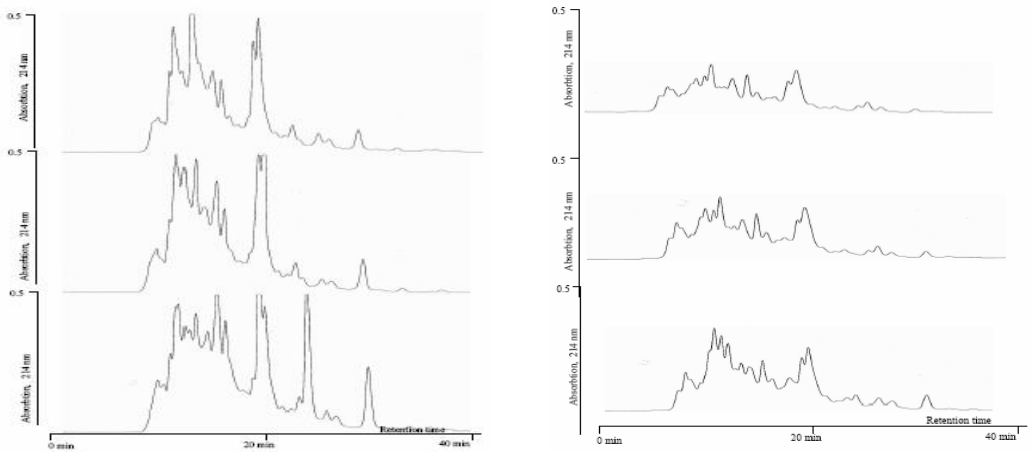

Figure 3. *HPLC-chromatograms of pH4,6 soluble peptides with molecular mass lower than 10 kDa during ripening of Kashkaval A (left) at 15, 30, 60 days (up to down), and Kashkaval B (right).*

The concentration of free amino acids in Kashkaval A is six times higher than its counterpart in Kashkaval B (Figure 4). Even on 15 days from the beginning of ripening, the concentration of free amino acids in Kashkaval A was definitely higher than that in Kashkaval B on 60 days. This indicates that cheese ripening can be greatly accelerated by using the nonstarter strain *E. faecium*. The amino acid content in Kashkaval A correlated mainly with the amino acid profile of strain *E. faecium* MH3. The high activity of amino acid release by *E. faecium* MH3 (89.61 mg/100 g) caused accumulation of a significant amount of free amino acids in ripe Kashkaval A – from 781.40 mg /(100 g) to 1278.92 mg/(100 g) at 30 and 60 days of age, respectively (Figure 4).

The ACE-inhibitory activity of the Kashkaval made with *E. faecium* strain MH3 was remarkable higher than those made with common starter containing L. bulgaricus and S. thermophilus strains only – 73% and 38%, respectively.

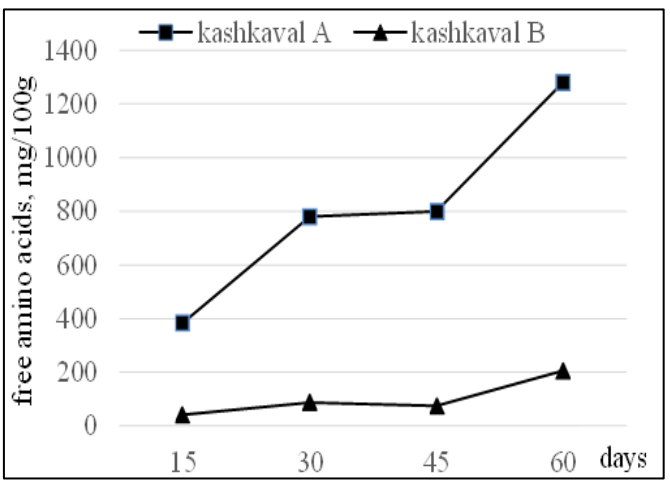

Figure 4. *Change in the concentration of free amino acids in Kashkaval A and in Kahkaval B as function of ripening period.*

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Conclusion

This paper proposes a new approach to enhance and accelerate proteolysis in cheeses (Kashkaval cheese in particular) with the aim of improving and shortening ripening time and improving the product quality. A new starter culture was designed with the nonstarter highly proteolytic bacteriocinogenic strain *E. faecuim* MH3 added to the starter culture for Kashkaval cheese manufacture, thus fulfilling three aims: (i) acceleration of ripening of Kashkaval cheese; (ii) maximal utilization of the proteolytic enzyme potential of the starter; (iii) release of bioactive peptides with ACE-inhibitory properties. The degradation of the hydrophobic peptides from the early stage to the end of ripening and the formation of hydrophilic peptides is an evidence of the efficient action of the nonstarter culture *E. faecium* MH3. The exceptionally high concentration of free amino acids in Kashkaval cheese with *E. faecium* MH3 strain in the ripening period (from 378.27 mg/100 g to 1278.92 mg/100 g, on 15 and 60 days, respectively), is an indicator of proteolysis and of high nutritional and biological value of Kashkaval cheese.

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