ABSTRACT
Functional foods hold a great promise for future trends in human nutrition. Consumption of milk and milk products has a pronounced probiotic effects together with the expected modification of allergenic properties of milk due to the process of fermentation. The proteolytic system of lactic acid bacteria consists a cell wall bound proteinase and several intracellular peptidases, and can contribute to the liberation of bioactive peptides. Food-derived bioactive peptides are claimed to be health-enhancing components, which can be used for functional food.

In this study the attention was focused on homemade milk products from the Balkan region and also from Armenia and Iraq, as a source of newly potential starters for application in the food industry. In this context the aim of this study was to screen the proteolytic activity of newly isolated strains, by determination of the optimal conditions of proteolysis for reducing the milk proteins allergenicity. The proteolytic activity of 50 lactic acid strains was tested against major milk proteins. Some of the studied strains showed relatively high proteolytic activity towards α-casein, α-lactalbumin and β-lactoglobulin reducing their concentration proved by the HPLC analysis and Tricine-PAGE. Hydrolysis of milk protein generated peptides with antimicrobial effect against Escherichia coli, Staphylococcus aureus, Listeria innocua, Enterobacter aerogenes, Enterococcus avium and Salmonella choleraesuis.

Consequently to obtain a maximal effect of the product, a combination of strains with different properties could be used as starters in milk industry.

Keywords: casein, proteases, whey proteins, allergy

Introduction
Lactic acid bacteria (LAB) are characterized by their high demand of essential growth factors such as peptides and amino acids. However, milk does not contain sufficient free amino acids and peptides to allow growth of LAB (1,2). Therefore, these LAB posses a complex system of proteinases and peptidases which enable them to use milk casein as a source of amino acids and nitrogen. The first step in casein degradation is mediated by cell wall located proteases, which cleave casein to olygopeptides. Further degradation to smaller peptides and amino acids that can pass through the cell membrane is performed by peptidases (3).

The proteolytic activity of LAB has been studied extensively and proteolytic enzymes have been isolated and characterized. A variety of LAB isolated from fermented milk products have been previously reported as displaying beneficial functions for humans, including antimicrobial (3, 4), antitumor (5, 6, 7), and antimutagenic activities (6, 8), as well as effects on modulating the immune system (9, 10), lowering cholesterol levels (11), and reducing lactose intolerance in the host (12). The milk allergy has been classified according to the onset of symptoms as immediate or delayed type. The milk allergy seems to be manifested by tree major proteins found in milk: α-lactalbumin, β-lactoglobulin and caseins.

In these report, we present an evaluation of proteolytic
activity and formation of bioactive peptides of various strains isolated from traditional milk products during cultivation in milk.

**Materials and methods**

**Bacterial Strains and Culture Conditions**

A total of 30 LAB isolated from three different types of homemade yoghurts and cheeses were used. The strains were from the collection of the Department of General and Industrial Microbiology, Faculty of Biology. All the used strains were identified to belong to genera *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Enterococcus*. The strains with prefix “BG”, “I” and “A” correspond to strains isolated from Bulgarian, Iraqi and Armenian milk products, respectively. Initial identification of all the strains was performed by API 50CHL system (BioMerieux, France), according to the manufacturer’s instructions. These isolates were subcultured in MRS broth (Merck) and kept frozen at -20°C in MRS supplemented with 10% glycerol. For the present study the strains were cultivated in the milk 10%, and in 5% whey proteins (Whey Protein Concentrate WPC 80, Interfood BV, Holland).

SDS-PAGE of caseins and whey proteins was performed according to Laemmli and stained with Coomasie R 250.

**Reversed Phase-High Performance Liquid Chromatography (RP-HPLC)**

RP-HPLC was used to analyze the digestion of total milk proteins. The samples were separated with a Nucleosil C18 column (250 x 0.3 mm i.d., Macherey-Nagel, Hoerdt, France) using a Waters (Milford, MA, USA) HPLC system with the following conditions: flow-rate, 0.3 mL/min; solvent A, trifluoroacetic acid (TFA) 0.11% (v/v) in water; solvent B, acetonitrile/water/TFA, 70/30/0.09 (v/v/v). Elution was performed with a linear gradient of solvent B from 0 to 50% over 10 min, then 50 to 80% in 20 min; column was then rinsed with 100% of solvent B. Detection was performed with a Waters 996 photodiode array detector at 220 nm.

**Antimicrobial Assay**

Antimicrobial assay was performed as previously described (13) by the well diffusion method by using soft 0.8% agar. The activity of the collected samples was checked against *E. coli* NBIMCC 3398, *Listeria innocua* F (ENITIAA, Ecole Nationale des Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France), *Staphylococcus aureus* NBIMCC 746, *Enterococcus aerogenes* NBIMCC 3699, *Enterococcus avium* NBIMCC 1278 and *Salmonella choleraesuis* NBIMCC 3591.

**Results and Discussion**

The initial screening of the strains tested on skim milk agar for their proteolytic activity showed that all the strains have a proteolytic activity and generate a clear zone even after 4 h of incubation (data not shown). The strains were grown in 10% skim milk for 3 hours. Samples 20 μl were taken and applied for SDS-PAGE analysis. The results are presented on fig.1a, 1b and 1c. The different strains tested display low proteolytic activity against caseins (Fig. 1). As observed by SDS-PAGE caseins were degraded by the different strains in the comparison with the control milk. It should be noted that only 3 strains (*Streptococcus thermophilus* BG 1, *Pediococcus pentosaceus* I 1 and *Enterococcus faecium* A 1) showed high capacity of the hydrolysis of the caseins in milk.

![Fig. 1a. SDS-PAGE in samples obtained after 3 h cultivation of the strains in 10 % skim milk. 1-control, 2-Lb.bulgarius BG2, 3.Lb.brevis BG3, 4-Lb.brevis BG6, 5-Lb. pentosus BG8, 6- Str.thermophilus BG1, 7-Lb.bulgarius BG 7, 8-Str. thermophilus BG7, 9-Ent. faecium BG12](image)

![Fig. 1b. SDS-PAGE in samples obtained after 3 h cultivation of the strains in 10 % skim milk. Lanes: 1- Lb.plantarum, 2-Lb.pentosus I1, 3- Lb.pentosus I2, 4—Lb.plantarum I6, 5-Lb.brevis I2, 6- BLG and LAC, 7-P.pentosaceus I3, 8-P. pentosaceus I5, 9-Ent.faecium I6, K-control](image)
that within the first 3 hours of incubation the peaks of caseins disappeared mostly completely and a great number of more hydrophilic peaks appeared (Fig. 2).

From these presented results also was clear that the studied strains hydrolyzed whey protein fraction of the used milk. Therefore in our further study we have cultivated the strains in a whey based medium for 20 hours without lactose. The obtained results were shown on Fig. 3a (Bulgarian strains), 3b (Iraqi strains) and 3c (Armenian strains).

It could be noted that most of the strains display relatively high proteolytic activity against α-lactalbumin. The strains *Lb. bulgaricus* BG 1, *Lb. bulgaricus* BG 2 and *Lactobacillus pentosus* I2 showed the highest proteolytic activity against α-lactalbumin. Proteolysis of β-lactoglobulin after 20 hours cultivation of the different strains tested was also shown on Fig. 3a, 3b and 3c. All the strains tested were able to reduce to some extent the amount of β-lactoglobulin. But existed difference between the origin of the strains. The better hydrolysis were strain *Lb. bulgaricus* BG 2, *Lactobacillus pentosus* I2 and less active were strains from Armenia.

A clinical study using α-lactalbumin enriched infant
formula showed an activity against enterophatogenic \textit{E. coli} NBIMCC 3398. The action might be related to peptides that are released from \( \alpha \)-lactalbumin during digestion. Existed also data about action of trypsin, chymotrypsin resulted in antibacterial peptides. These peptides were mostly active against gram positive and weaker effects against gram negative (15). In our research was shown that some of the strains which utilized to greater extent \( \alpha \)-lactalbumin had activity against \textit{E. coli} NBIMCC 3398 and \textit{Listeria innocua} F. (Fig. 4a and Fig. 4b)

![Fig.4a](image1)

**Fig.4a** Antibacterial activity against \textit{E. coli} in cell free fraction after cultivation in whey based medium. 5- \textit{Lb.bulgaricus} BG2, \textit{Str.thermophilus} BG1, 7- \textit{Lb.pentosus} I2.

![Fig.4b](image2)

**Fig.4b** Antibacterial activity against \textit{L.inoccua} in cell free fraction after cultivation in whey based medium. 1- \textit{P.pentosaceus} I3, 2- \textit{Ent.faecium} A18

Whey presents a rich and heterogeneous mixture of secreted proteins with wide ranging nutritional, biological and food functional attributes. In our study we used whey products as a source of energy and food for lactic acid bacteria. The expand utilization of whey proteins will rely on exploitation of individual whey proteins. That’s why the bioactive properties of \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin and derived peptides from them are of great interest. Extensive hydrolysis of milk proteins to small peptides or even amino acids is used to make dairy proteins for commercial hypoallergenic milk products.

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REFERENCES