Investigation of the Proteolytic Potential of Isolates of the Genus *Lactobacillus* Obtained from Homemade Bulgarian Cow, Sheep, Buffalo yoghurt and lactic acid products, for the Hydrolysis of Whey Proteins using Tris-Tricine-SDS Polyacrylic Amide Gel Electrophoresis

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Abstract

The main objective of the present study is the proteolytic potential of Lactobacillus isolates for degradation of whey proteins. To implementation this goal, the following tasks were set: Isolation of lactobacilli strains from cow and sheep, buffalo yoghurt and lactic acid products, characteristics of newly isolated strains, survival at different temperatures, acidification of the nutrient medium, monitoring the production of biologically active peptides, study of the proteolytic activity of the strains, hydrolysis of basic whey proteins, hydrolysis of whey proteins (β-lactoglobulin and α-lactalbumin). Of the 30 samples used, 27 lactobacilli strains were isolated to pure cultures. Antimicrobial activity of strains R4.2 against E. coli. The studied strains have proteolytic potential and some of the studied strains can hydrolyze whey proteins as α-lactalbumin and β-lactoglobulin. Low molecular weight molecular moieties are formed after cultivation in the presence of α-lactalbumin analysed using Tris-Tricine PAGE.

***Key words**: Lactobacillus,* Bulgarian homemade cow yogurts, Tris-Tricine-SDS polyacrylic amide gel electrophoresis, whey proteins.

Introduction

Milk provides the newborn with the necessary substances for its survival immediately after birth (Juan, P., 2002). Milk components are water, carbohydrates, fats, proteins, vitamins and minerals.

The development of the dairy industry today is directly dependent on the development and extended knowledge of food microbiology and structure, functional and biological properties of the various components of milk. Milk proteins, in particular those of cow's milk, have been intensively studied over the years. They are repre-

sented by caseins and whey proteins. Milk proteins are the first foreign proteins to be consumed in large quantities by children (Järvinen et al., 2001) and there is evidence of milk allergy in children under certain conditions (Prioult et al., 2005; Jakobsson et al., 1979; Bock et al., 1987; Hùst et al., 1988; Saarinen et al., 1999; Halken et al., 2000; Gerrard et al., 1986).

The probiotic properties and proteolytic activity of lactic acid bacteria have been intensively studied in recent years. (Ehn et al., 2005; Booth et al., 1990; Wohlrab and Bockelmann, 1993; Bockelmann et al., 1996; Law and Haandrikman, 1997). A complex system of proteinases and peptidases that makes them possible to develop them in milk. It is well known that bacterial proteases hydrolyze milk proteins during the fermentation process, which can reduce its allergenicity (Nentwich et al., 2004) and to the formation of peptides with different biological activity (opioid, immunomodulatory, antimicrobial, antihypertensive), and to the release of important amino acids that increase the nutritional value and quality of the lactic acid product.

Functional foods, in turn, are a promising alternative means of supporting human health and improving the quality of life. Traditionally, fermented dairy products, that have a beneficial effect on human health have been found to contain bacterial strains belonging to the genera Lactobacillus, Streptococcus and Bifidobacterium (Prasad et al., 1998; Dunne et.al., 1999). Lactobacillus is one of the most important for the dairy industry. This group is extremely important and useful for people, as they give specific qualities to the fermented product (taste, aroma, texture) and contribute to its preservation. In addition, some lactobacilli are used as probiotics to affect and balance the natural microflora of the human digestive tract.

Homemade yogurts have a wide variety of lactic acid bacteria. Therefore, they can serve as a source of new potential starter crops, which are of great interest to the lactic acid industry. The selection of new strains with specific properties, increases the diversity of the final product and overcomes the industrialization of lactic acid products with the use of mass commercial starters. Careful selection of strains with diverse characteristics, leads to diversity in the specific qualities of the final product, as lactic acid bacteria, including strains of the genus Lactobacillus, are an important part of the enzymatic process. The choice of starter culture can be made after its detailed taxonomic determination, careful analysis of physiological characteristics, technological stability of the strain and assessment of its health impact on the consumer.

Whey proteins

Whey proteins represent approximately 20% of the total protein content in cow's milk. They are typical globular proteins that completely denature at 90 °C for 10 minutes. They have a secondary, tertiary and quaternary structure. They are not phosphorylated and are not sensitive to calcium. All whey proteins have intramolecular disulfide bridges, that stabilize their structure (Fox, 2001). The two main proteins of lactoserum are β-lactoglobulin and α-lactalbumin. Other proteins, immunoglobulins, serum albumin, lactoferrin, lactoperoxidase, alkaline phosphatase, catalase, sulfhydryl oxidase and plasmin are found in very small amounts.

β-lactoglobulin

β-lactoglobulin (BLG) occupies about 50% of the total content of lactoserum proteins in milk. It is a major protein in the milk of ruminants and pigs, but is absent in humans. Its molecular weight is 19kD and is composed of 162 amino acid residues. It has two internal disulfide bonds and one thiol group and exists as a non-covalently linked dimer.

α-lactalbumin

α-lactalbumin (αLAC) occupies about 25% of the total content of lactoserum proteins in milk. Its molecular weight is 14kD and is composed of 123 amino acid residues.It contains eight cysteine groups, that form internal disulfide bonds and four tryptophan residues. α-Lactalbumin has a well-ordered secondary structure and a compact spherical tertiary structure. This protein connects with Ca и Zn. Its tertiary structure is stabilized by the binding of Ca^{2+} ions (Brew and

Grobler, 1992). In thermal denaturation at pH thermalna < 4.0 causes the release of bound calcium. (Kuwajima et al., 1986).

Material and Methods

Isolation of microorganisms

The isolation of lactobacilli strains from home-made lactic acid products is performed, after classical microbiological approaches. MRS broth and MRS agar medium were used (MRS broth, Merck, KGaA, 64271 Darmstadt Germany), suitable for isolating lactobacilli strains. After preliminary enrichment of the culture in 0.5% skim milk. (Merck, KGaA, Darmstadt Germany) for 16–18 h and a series of dilutions, the culture was cultured on agar MRS medium. From each petri dish with MRS agar medium, colonies from all samples were randomly selected and the selected strains were subsequently cultured in 42 °C MRS broth.

Physiological characteristics of the strains
Coagulation of milk

The isolated strains were seeded in 10% skimmed milk. The seeded strains were cultured at 42 °C under anaerobic conditions. The reading of the result was done after 24 h and 48 h.

Degree of acidification of the nutrient medium

The degree of acidification of the nutrient medium from the isolated strains was determined

Table 1. Strains isolated from cow, sheep, buffalo yoghurt and lactic acid products

№	Strains	Source	Nutrient medium for cultivation	Cultivation temperature
1	K2	cow yogurt	MRS	42 °C
2	K3	cow yogurt	MRS	42 °C
3	K5	cow yogurt	MRS	42 °C
4	K6	cow yogurt	MRS	42 °C
5	K14	cow yogurt	MRS	42 °C
6	K15	cow yogurt	MRS	42 °C
7	K19	cow yogurt	MRS	42 °C
8	K20	cow yogurt	MRS	42 °C
9	K21	cow yogurt	MRS	42 °C
10	K22	cow yogurt	MRS	42 °C
11	K24	cow yogurt	MRS	42 °C
12	K26	cow yogurt	MRS	42 °C
13	K27	sheep's yogurt	MRS	42 °C
14	O42	sheep's yogurt	MRS	42 °C
15	O43	sheep's yogurt	MRS	42 °C
16	O45	sheep' s yogurt	MRS	42 °C
17	O47	sheep' s yogurt	MRS	42 °C
18	B5	buffalo yogurt	MRS	42 °C
19	B7	buffalo yogurt	MRS	42 °C
20	B8	buffalo yogurt	MRS	42 °C
21	R.21	Lactic acid products	MRS	42 °C
22	R.22	Lactic acid products	MRS	42 °C
23	R.31	Lactic acid products	MRS	42 °C
24	R.32	Lactic acid products	MRS	42 °C
25	R4.1	Lactic acid products	MRS	42 °C
26	R4.2	Lactic acid products	MRS	42 °C
27	R4.3	Lactic acid products	MRS	42 °C

[*] *K – cow yogurt; O – sheep's yogurt; B – buffalo yogurt; R – Lactic acid products*

after 24 h cultivation of the isolates at 37 °C in sterile skim milk.The measurement was made using a pH meter Microprocessor pH 211, Hanna Instruments.

Homofermentativity and heterofermentativity test

The test strain was seeded on MRS broth with 5% glucose and no meat extract. The medium makes it possible to determine whether the strain tested produces glucose gas. The reading of the result was performed after 24 h, 48 h and 72 h. A positive result is considered to be the pushing of the cap from starvation agar to the top of the tube due to the gas released. If the result is negative, the cap remains in the home position.

Growth at different temperatures

The ability of the tested strains to grow at different temperatures was accounted for by culturing them in MRS-broth medium and incubating them at temperatures of 25 °C, 30 °C, 33 °C, 37 °C and 42 °C. Prior to the experiment, all tested strains were cultured in MRS broth for 16–18 hours and 10% inoculum was used. The tolerance of the isolated strains to different temperatures was reported at 24 h and 48 h. Growth was determined by optical density at a wavelength of 590 nm, against the control of MRS medium and control of the strain at 0 h on a spectrophotometer Shimadzu UV-1202 UV - VIS (Shimadzu Corporation).

Antibacterial activity

The analysis for antibacterial activity of the tested strains was performed by the method of diffusion in agar (Tagg и McGiven).The activity of the strains was determined according to test cultures (*E.coli*, *Listeria innocua, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*), as 100 µl of the neutralized cell-free supernatants, obtained after culturing the strains in MRS medium under standard conditions are dropped into pre-prepared wells in agar, inoculated with the appropriate test culture. The activity is reported in mm zones after 24-hour development of the test culture at 30 °C.

Screening for proteolytic activity

Initially, the isolated strains were tested for proteolytic activity using the agar diffusion method. Milk agar medium was used for this purpose. The test strains were cultured in MRS broth, incubated at 42 °C for 16–18 hours. A 100 µl sample of them was placed in a well in the middle of milk agar and incubated at 42 °C for 16–18 hours. The presence of enlightenment in the area around the well was considered to be a manifestation of proteolytic activity on the strains.

Investigation of the proteolytic activity of isolated strains to casein and whey proteins using polyacrylic amide gel electrophoresis

Polyacrylic amide gel electrophoresis (PAGE) is used, as a method of protein separation. It is due to the charge of the protein molecule and allows the successful separation of proteins according to their molecular mass. Electrophoretic separation of proteins according to their molecular weight is performed under denatured conditions, for this purpose, the sample is treated with sodium dodecyl sulfate (SDS-PAGE). On the other hand, the use of beta mercapto ethanol (β-ME) in PAGE aims to break the disulfide bonds in the studied proteins. The proteolytic activity of the isolated strains against whey proteins was determined on SDS-PAGE

Proteolytic activity to whey proteins

The ability of the isolated strains to hydrolyze the proteolytic whey proteins alpha-lactalbumin and beta-lactoglobulin was tested by culturing them on MRS medium with the corresponding protein added to it. The strains were cultured using whey protein concentrations – 0.05%. Prior to the experiment, the test strains were cultured in MRS broth at 42 °C for 16–18 hours. A 10% inoculum of the test medium was inoculated from the cultures and the strain was cultured at 42 °C in a thermostat. Samples taken (30 [mu] l of supernatant) were diluted with 90 [mu] l of SDS-PAGE sample buffer. The samples thus prepared were thermally treated at 100 °C for 3 minutes, after which they were analyzed electrophoretically using SDS-PAGE.

SDS-polyacrylic amide gel electrophoresis was performed with 12% polyacrylic amide separation gel and 3% polyacrylic amide concentrating gel on a HOEFER Mighty Small SE 245 apparatus. SDS-PAGE migrating buffer is used. The samples were migrated to 10 mA in the concentrating gel and at 20 mA in the separating gel, then stained for 1 hour in Kumasi Blue R-250 staining solution and decolorized for 2 h in a decolorizing solution.

Electrophoretic analysis of whey fraction with Tris-Tricine-SDS PAGE Tris-Tricine-SDS polyacrylic amide gel electrophoresis by the Schagger method was used to analyze low molecular weight whey proteins. This electrophoresis is used to separate low molecular weight proteins. For this purpose, 16.5% polyacrylic amide separating gel and 7% polyacrylic amide concentrating gel (Table 3) of a HOEFER Mighty Small SE 245 apparatus were used according to the method of Schagger (1987). Tris-Tricine-SDS-PAGE migration buffer was used. The samples were migrated to 125 volts and then stained with silver nitrate according to Blum (1987), Table. 4.

Results and Discussion

Isolation and morphological characterization of strains isolated from domestic yoghurts

Following classical microbiological approaches, lactobacilli strains were isolated from various

Table 2. Composition of SDS-PAGE concentrating and separating gel

Components	Concentrating gel	Separating gel
Acrylamide 40%	0.200 ml	1.500 ml
Tris 2M pH 8.8		0.665 ml
Tris 0,5 M pH 6.8	0.300 ml	
SDS 10%	25 µl	40 µl
H_2O	20000 ml	1.835 ml
Ammonium persulfate 10%	20 µl	40 µl
Themed*	4 µ	8 µ

* *N-, N' tetramethylenediamine*

Table 3. Composition of Tricin concentrating and separating gel – SDS-PAGE

Components	Concentrating gel	Separating gel
Acrylamide 40%	0.5 ml	1 ml
Tris 3M SDS 0,3% pH 8,45	0.62 ml	1.33 ml
Glycerol	-	0.55 ml
H_2O	1.4 ml	1.14 ml
Ammonium persulfate 10%	20 µl	40 µl
Themed*	2 µ	4 µ

* *N-, N' tetramethylenediamine*

Table. 4. Blum staining

Fixation	40% ethanol 10% acetic acid 50% H_2O	1 > H
Flushing 1	30% ethanol 70% H_2O	2 x 20 min
Flushing 2	H_2O	20 min
Sensitization	0.1 ml 10% $Na_2S_2O_3$ 50 ml H_2O	1 min
Flushing 3	H_2O	3 x 20 sec
Coloring	100 mg $AgNO_3$ 10 µl 37% formaldehyde 50 ml H_2O	20 sec

lactic acid products. From the samples used, 27 lactobacilli strains were isolated to pure cultures. For this purpose, MRS medium and temperature 42 °C were used. Based on the studied morphological characteristics, the strains were determined to belong to the genus Lactobacillus.

Physiological characteristics of strains isolated from domestic yogurts

The presented results of the conducted physiological studies are summarized by at least three independent experiments.

Catalase reaction

The experiment showed, that the isolated strains gave a negative reaction for the presence of catalase, which is typical for all lactic acid bacteria.

Homofermentativity and heterofermentativity test

The formation of glucose gas, as an indicator of homofermentativity and heterofermentativity, is determined on a Gibson medium. The strans were seeded with 1% inoculum in tubes with 5 ml medium. After inoculation, pour about 2 ml of cooled starvation agar into the tubes. Cultivated at 26 °C for 5 days. Gas formation is counted when lifting the cap from starvation agar.

Coagulation of milk

The experiment showed that, all tested strains, have the ability to coagulate milk. Inoculated in skim milk, they form a dense coagulum with a nail.

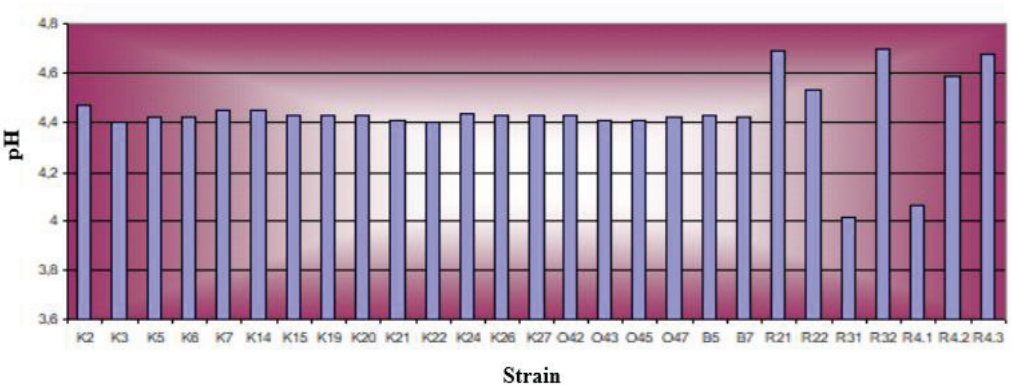


Fig. 1. Acidification of the nutrient medium after 24 h incubation of the strains in MRS medium

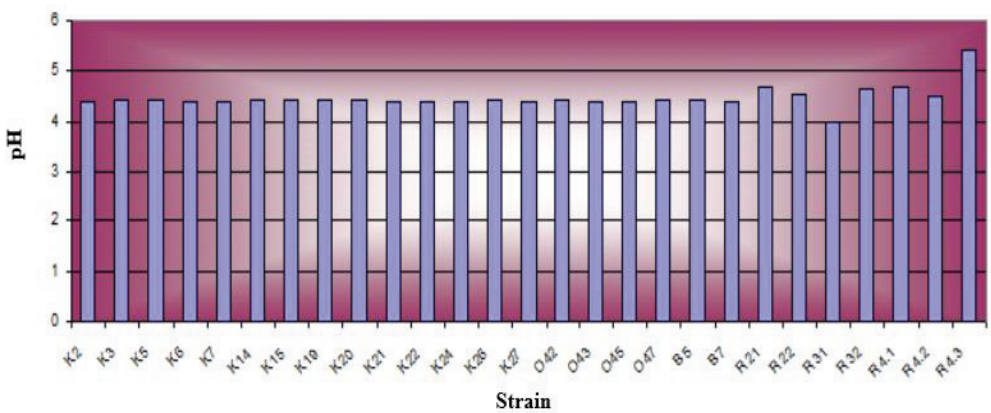


Fig. 2. Acidification of the nutrient medium after 24 h of incubation of the strains in MRS medium in the presence of 0.1% casein

Degree of acidification of the nutrient medium

The degree of acidification of the medium from the isolated strains is shown in Fig. 1, 2, 3 and 4.

All tested strains acidified the nutriend medium after 24 h incubation in the range between pH 3.98 and pH 5.42. Strain K21 and R21 acidify MRS medium least at pH 5.78 and 5.83, and in medium with casein strain R21 with pH 4.92. When using an additional nitrogen source such as α-lactalbumin and β-lactoglobulin, the weakest acidification of the medium is characteristic of strains R21, R32 and R4.3. The strongest acidification is in strain R31 (MRS medium) to 4.1, in medium with casein 4.02, in the presence of α-lactalbumin 3.98 and in β-lactoglobulin 4.01.

The degree of acidification of the medium by the starter microorganisms is an important parameter of the enzymatic process. Not excessive acidification of the nutrient medium, is preferred in the manufacturing process, since according to (Zanatta and Basso, 1992) and (Beal et al., 1999), this provides a more homogeneous coagulum structure and higher viscosity of the final product. But over-acidifying the product, on the other hand, could impair its taste. pH < 8 is necessary to ensure the formation of a stable gel of coagulated milk protein (Rasic et al., 1978). The resistance of lactobacilli to the increased acidity of the environment and to other adverse factors is associated with the synthesis of specific proteins. It was found that lowering the pH to 4.75 resulted in the expression of 3 (heat shock

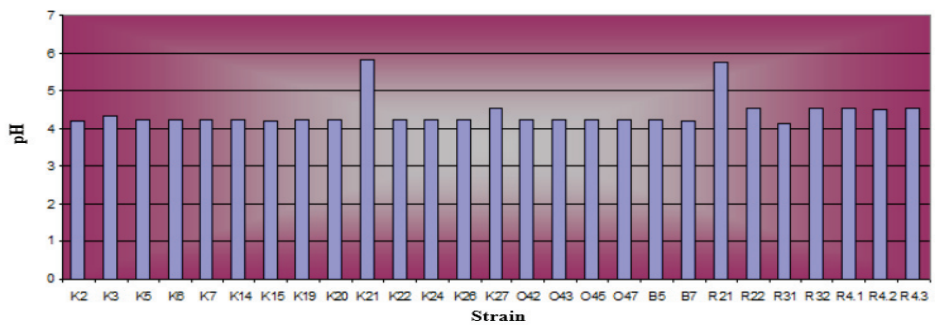


Fig 3. Acidification of the nutrient medium after 24 h incubation of the strains in MRS medium in the presence of 0.05 mg α-lactalbumin

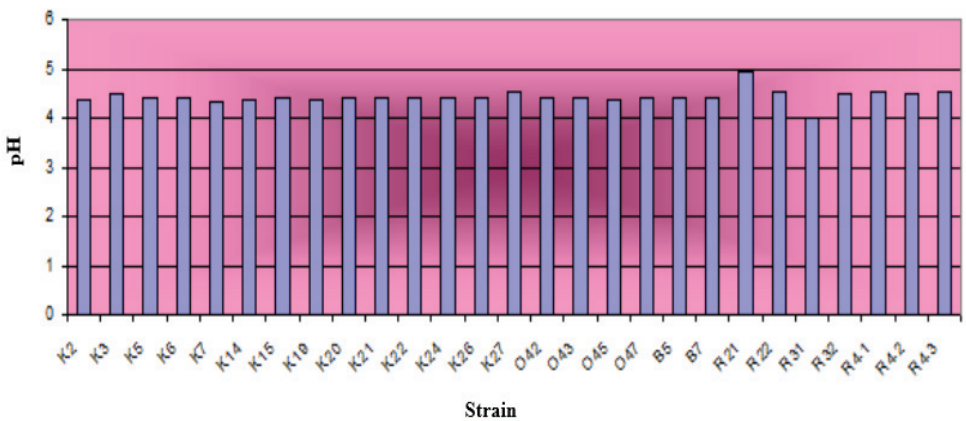


Fig. 4. Acidification of the nutriend medium after 24 h incubation of the strains in MRS medium in the presence of 0.05 mg β-lactoglobulin

proteins) proteins (GroES, GroEL, and DnaK) in Lactobacillus bulgaricus (Lim et al., 2000), and lowering the pH to 4.5 resulted in the synthesis of 9 proteins (14.1 to 56.2 kDa) in Lactobacillus acidophilus (Lorca and Font de Valdez, 2001).

Growth at different temperatures

The ability to grow in a certain temperature range is an important feature in the identification of lactic acid bacteria. The study of their growth at temperatures of 25 °C, 30 °C, 33 °C, 37 °C and 42 °C. The results of the experiment are summarized in Fig. 5. A, B, C.

The obtained results showed that the isolated strains tolerate equally temperatures from 30 °C to 42 °C, shown in Fig. 5. A and Fig. 5. B. The results of Fig. 5. C, show good survival of the strains at all temperatures studied. Strains that can tolerate high and low temperatures is specific to them and this can be successfully used in the stages of the fermentation process of milk that require exposure to high temperatures. It is well known that in response to exposure to stressful conditions, as heat shock and cold, the protective mechanisms for bacterial adaptation are activated, which include the synthesis of specific proteins, increasing the content of certain specific fatty acids, increasing the content of unsaturated fatty acids in membrane phospholipids (Wang et al., 2005; Melilli et al., 2004; Lim et al., 2000; Fernandez et al., 1990; Gomez et al., 2005). However, all these properties are specific to individual strains and they determine their specific technological characteristics.

Determination of the specific growth rate of the studied strains

The specific growth rate characterizes a bacterial culture only under defined specific conditions and is not a quantitative indicator for the taxonomic determination of the strain.

Antibacterial activity

The results obtained from the experiment for antibacterial activity of neutralized supernatants of the strains against the test cultures of E. coli

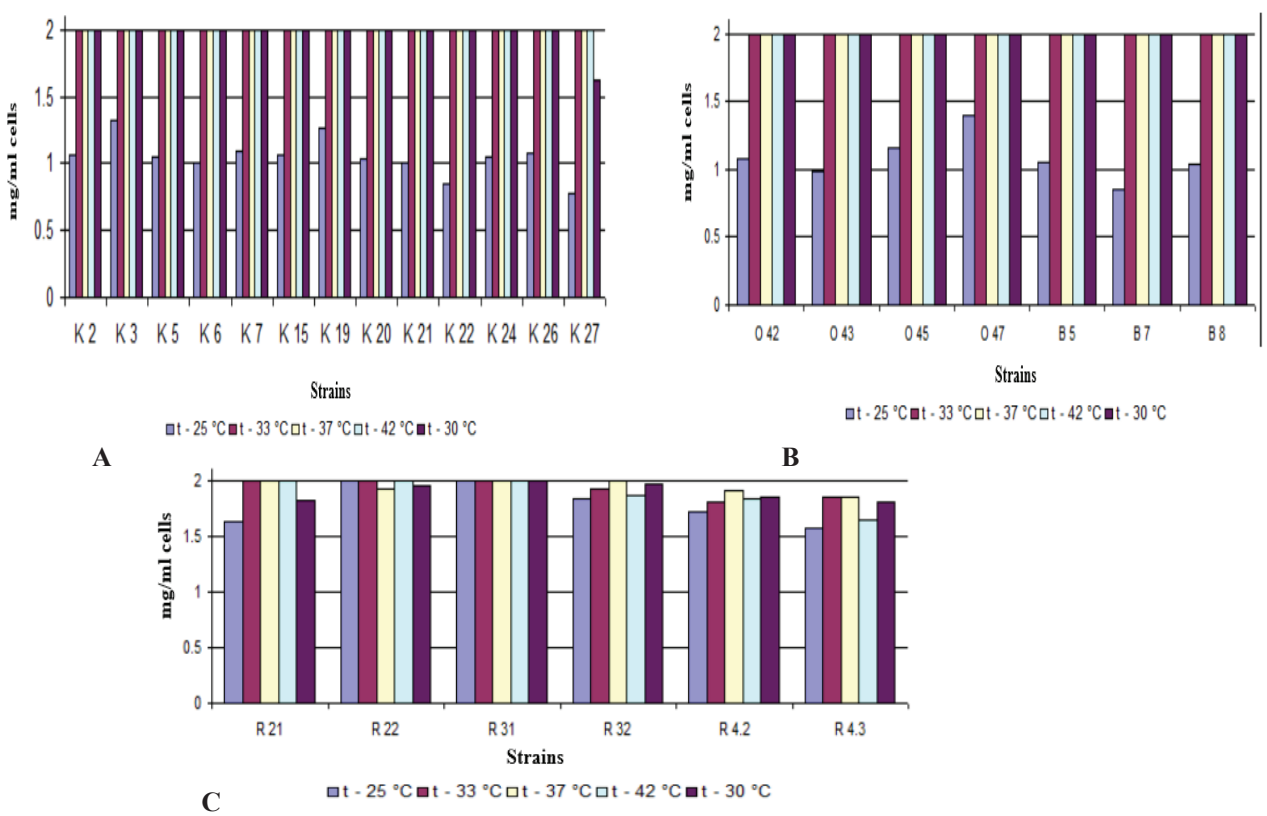


Fig. 5. A, B, C Growth at different temperatures of the studied strains

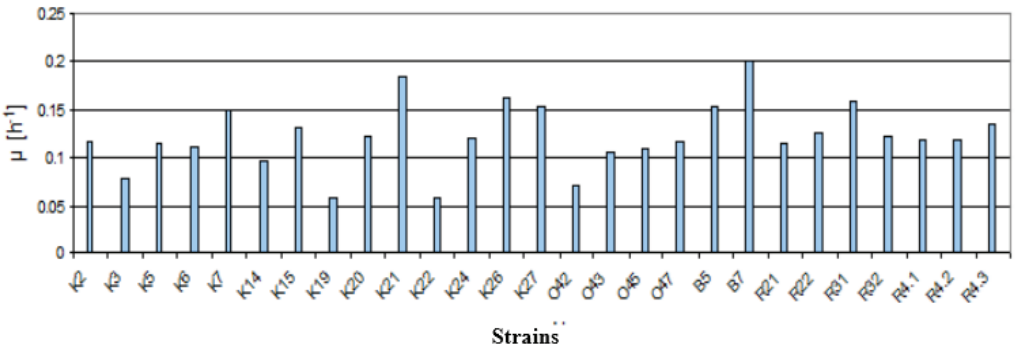


Fig. 6. Specific growth rate of isolated strains in MRS medium at 37 °C

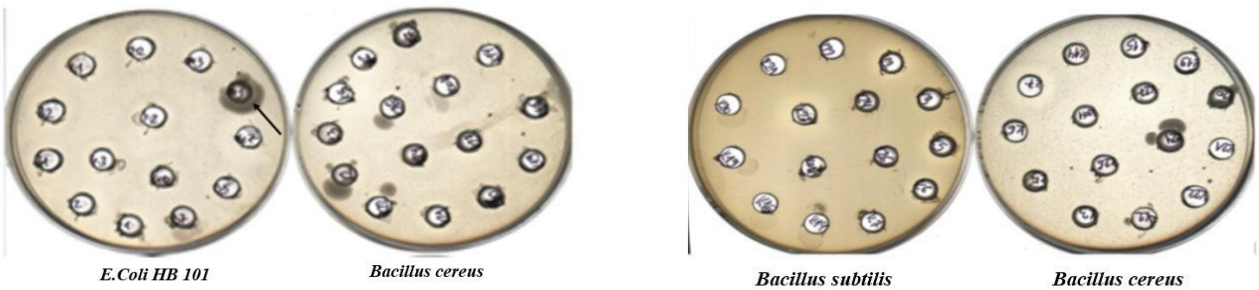


Fig. 7. Antibacterial activity of the strains against test cultures of *E.coli* HB101, *Bacillus subtilis*, *Bacillus cereus and Bacillus subtilis*

HB101, Bacillus subtilis and Bacillus cereus are presented in Fig.7.

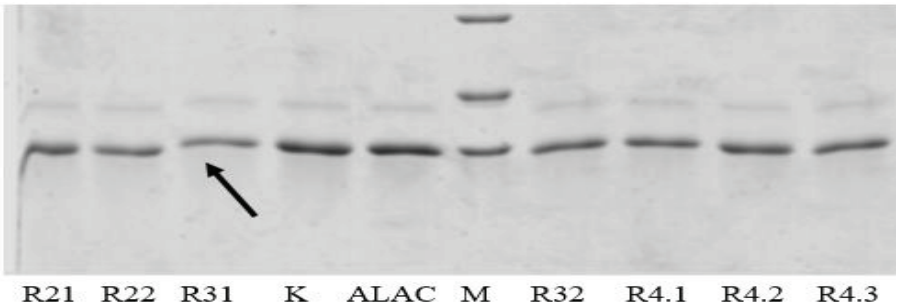
The obtained results showed, that strain R4.2 have antibacterial activity against *E. coli* HB101.

Investigation of the proteolytic activity of isolated strains to whey proteins

Whey proteins (ALAC and BLG) are well known to be strong allergens (Wal, 1998). They are peptides with a compact structure. The strongest of these is considered to be the BLG protein. (Host and Halken, 1998). Betalactoglobulin is a globular protein and its stable spatial conformation shows high resistance to digestion, which in a sense explains its high allergenicity (Prioult et al., 2005). It has been found that the hydrolysis of BLG by digestive enzymes reduces its allergenicity (Asselin et al., 1989), but also masks latent allergenic peptides that are recognized by specific IgE from the serum of allergic patients (Selo et al., 1999).

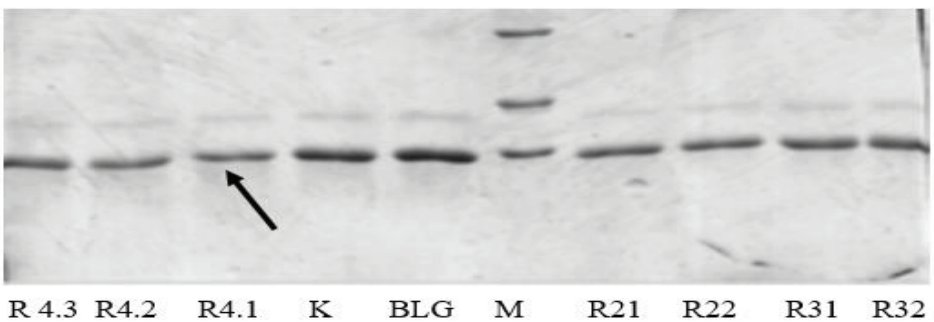
Three tryptic peptides from BLG have been identified as the major allergen epitopes (41–60; 102–124; 149–162) (Selo et al., 1999). In the digestive tract, these peptides can become targets for peptidases of endogenous probiotic bacteria colonizing the small intestine (Pessi et al., 1998). In addition to the hydrolysis of milk proteins during digestion, they can also be hydrolyzed by proteinases of starter cultures during fermentation, which creates an additional opportunity to reduce their allergenicity. The hydrolysis of whey proteins from the tested strains is shown electrophoretically in Fig. 8 and Fig. 9. In Fig. 8 presents the results obtained after 24 h cultivation of MRS strains + 0.05% α-lactalbumin.

The obtained results indicate, that the tested strains have a relatively intense pro-



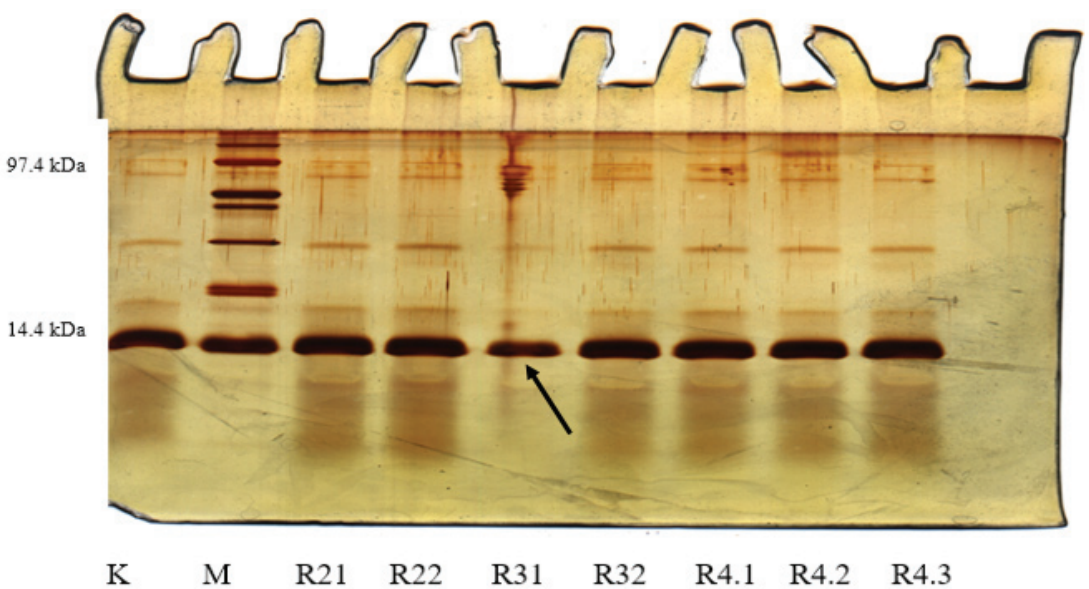
K – control MRS with 0.05% α-lactalbumin, M – marker R-strains

Fig. 8. SDS-electrophoresis, 12% gel samples obtained after 24 h of culturing the strains in the presence of 0.05% α-lactalbumin



K – control MRS with 0.05% α-lactalbumin, M – marker R-strains

Fig. 9. SDS-PAGE, 12% gel samples after 24 h culture of the strains in the presence of 0.05% β-lactoglobulin



K – control MRS with 0.05% α-lactalbumin, M – markers from 14.4 kDa to 97.4 kDa, R – strains

Fig. 10 Tris-Tricine PAAGE 16.5% gel samples with 0.05% α-lactalbumin obtained after 24 hours of culturing the test strains.

teolytic activity against α-lactalbumin. The results show that only one strain R31 has the most intense proteolytic activity against α-lactalbumin.

In Fig. 9 presents the results of SDS-PAGE of samples taken 24 h from the cultivation of strains of 0.05% β-lactoglobulin. The results obtained show, that the studied strains degrade β-lactoglobulin, but to a lesser extent than α-lactalbumin.

The results shown in Fig. 9 show that only one type of R4.1 may but in small quantities have activity against β-lactoglobulin. The results show that the tested strains hydrolyze β-lactoglobulin less than α-lactalbumin.

Analysis of the whey fraction, obtained after culturing the strains using Tris-Tricine PAGE.

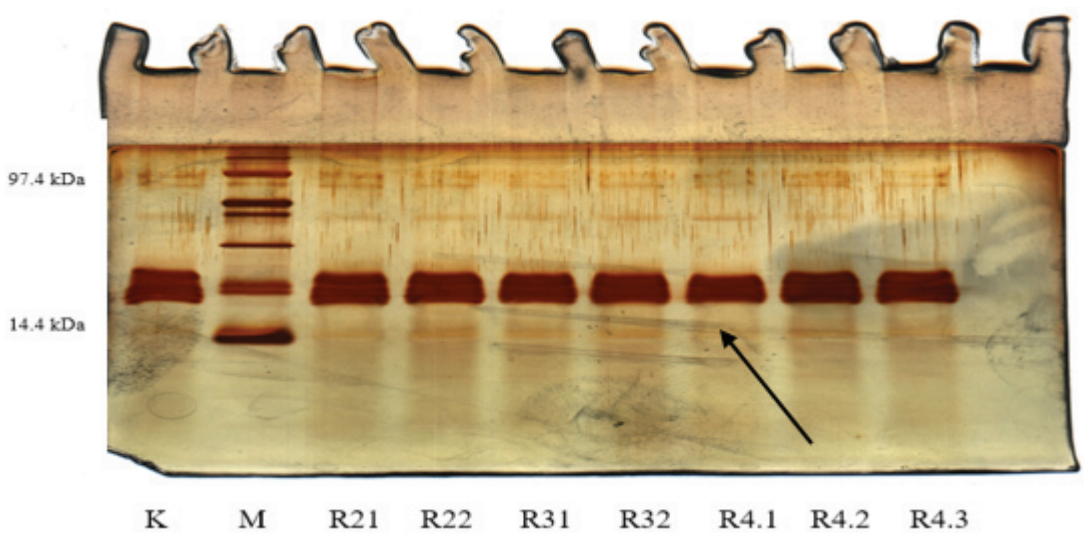
In order to monitor the possible formation of biologically active peptides from the tested strains, a fraction, obtained after 24 hours of culturing the strains in MRS with 0.05% α-lactalbumin and β-lactoglobulin was analyzed electrophoretically on Tris-Tricine PAGE. The results obtained from the electrophoretic analysis are presented in Fig. 10 and Fig. 11.

From the Fig. 10 it can be seen that strain R31 uses a significantly more intensively present additional source of nitrogen α-lactalbumin. Which is confirmed by the previous results obtained by SDS PAGE (Fig 8).

The results show that strain R4.1 uses a significantly more intensively present source of nitrogen β-lactoglobulin.

Conclusion

27 strains of lactic acid bacteria were isolated from lactic acid products. According to physiological and biochemical characteristics, the isolated strains were assigned to the genus Lactobacillus. The strains are characterized in terms of specific growth rate, influence of temperature, acidification of the environment. Antimicrobial activity of strain R4.2 against E. coli. Screening for proteolytic activity of the isolated strains was performed. The proteolytic activity of bacterial strains to α-lactalbumin and β-lactoglobulin was analyzed using SDS-PAGE and Tris-Tricine PAGE. Some of the studied strains hydrolyze the whey proteins α-lactalbumin and β-lactoglobulin.



K – control MRS with 0.05% β-lactoglobulin, M – marker from 14.4 kDa to 97.4 kDa, R – strains

Fig. 11. Tris-Tricine PAAGE of MRS with 0.05% β-lactoglobulin obtained after 24 hours of culturing the test strains.

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