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**Technology for obtaining freeze-dried bioactive products
based on protein hydrolysates**

ABSTRACT

*of a dissertation for the acquisition of an educational and
scientific degree "Doctor"*

Field of higher education: 5. Technical sciences

Professional direction: 5.12. Food technologies

Doctoral program: "Technology of biologically active substances
(incl. enzymes, hormones, proteins)"

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The dissertation work was discussed and directed for defense at a meeting of the Extended Scientific Council at the "Cryobiology and Biologically Active Substances" Department, Institute of Cryobiology and Food Technologies, Sofia, held on 05/09/2024.

The dissertation is written on 185 pages and contains 49 tables and 43 figures. The cited literature includes 243 sources, of which 22 are in Cyrillic and 221 are in Latin.

The numbering of sections, figures and tables does not correspond to those in the thesis.

The public defense of the dissertation work will take place on 2024. from hours in the Meeting Hall of the ICFT, Sofia 1407, 53 Cherni Vrah Blvd.

I. INTRODUCTION

The increased interest in rational and healthy nutrition creates prerequisites for the search for new technological solutions in the production of food and nutritional supplements. A current trend in this field is the preparation and use of protein hydrolysates of plant and animal origin as sources of bioactive peptides. Hydrolyzed milk protein is a food that is quickly and completely absorbed by the human body with a biostimulating effect, and cow's milk protein is the most important protein source used in the preparation of hydrolysates. The growing application of protein hydrolysates from milk and milk products in the form of protein supplements or clinical dietary formulas requires optimization of technologies for their production.

It is in this topical area that the research in the current dissertation is focused, which is dedicated to the development of a technology for obtaining new lyophilized bioactive products based on hydrolysates from fresh cow's milk and whey.

The focus of the development contains the following innovative elements:

- Use of fresh cow's milk and whey as a waste product as protein substrates for enzymatic hydrolysis and their conversion into products with high added value.
- Optimization of methods for enzymatic hydrolysis of casein and whey proteins in order to obtain bioactive hydrolysates and peptides. Establishing the influence of technological parameters - type, time of action and concentration of proteolytic enzymes on the degree of hydrolysis of protein substrates.
- Establishing the effect of adding strains of lactic acid bacteria *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447; *Lactobacillus gasseri* NBIMCC 2450 and *Lactocaseibacillus rhamnosus*

NBIMCC 1013 to hydrolysed protein substrates over the duration of the fermentation process and the survival of the probiotic bacteria.

- Determining the optimal parameters of the freeze-drying process and applying cryoprotectants to increase the survival of bacterial cells in the fermented milk and whey hydrolysates after freezing and lyophilization.
- Creation of an original technology for the production of bioactive products based on milk hydrolysates.

The use of protein hydrolysates as a substrate for fermentation with strains of lactic acid bacteria is poorly studied and available scientific information is scarce. The conducted experiments prove that milk hydrolysates obtained by enzymatic hydrolysis and subsequent lactic acid fermentation are a suitable basis for obtaining food products containing bioactive peptides that can have a positive impact on metabolic functions of the body. The addition of lactic acid bacteria strains to milk hydrolysates also contributes to improving their functional properties, i.e. turns them into a means of maintaining the balance and restoring the basic microflora of the human digestive system.

II. CONCLUSIONS FROM THE LITERATURE REVIEW

From the literature review, the following general conclusions can be formulated:

- ✓ Protein hydrolysates have been found to be of interest to the food industry due to their beneficial effects on human health. The enzymatic hydrolysis process is applied to obtain foods enriched with low molecular weight protein components and free amino acids, which are preferred in some diets. Enzymatic protein hydrolysates containing peptides with a characteristic amino acid composition are preferred for specialized clinical nutrition.
- ✓ Through hydrolysis of proteins from animal or plant sources, bioactive peptides can be generated. The health benefits of peptides are

expressed in antihypertensive, antimicrobial, antioxidant, immunomodulating and mineral-binding effects.

✓ Cow's milk protein is the most important protein source used in the development of hydrolysates intended for nutritional therapy. Hydrolyzed milk protein is a protein food that is quickly and completely digestible by the human body and has a biostimulating effect. Casein and whey proteins have been found to release peptides in three ways: through digestive enzymes, through the fermentation process, and through the action of proteolytic enzymes derived from microorganisms or plants.

✓ The quality of raw milk and whey and their dry matter content is crucial for the quality and properties of the final product. The use of whey to obtain a hydrolyzate with high biological value has not yet been sufficiently studied and is of interest to science and practice.

✓ Probiotic lactic acid bacteria to create the functional product from milk hydrolyzate must be in a symbiotic relationship with each other during their co-cultivation. Lactic acid bacilli exhibit higher proteolytic activity than streptococci. Co-cultivation between thermophilic streptococcus and lactic acid rods is expressed in the production of more free amino acids, especially essential ones, which are necessary and stimulate faster multiplication of streptococci. In turn, they release more formic acid in the reproduction process and thus positively affect the development of lactobacilli. The selected combinations of lactic acid bacteria strains must be resistant to the conditions in the human gastrointestinal tract and retain their characteristic morphological qualities for a longer time.

✓ The monitoring and reporting of the technological parameters – pH, temperature and time of action of the enzymes are of primary importance for obtaining a hydrolyzate with biologically active peptides. The viability and number of lactic acid probiotic bacteria depend on the correct and timely control of the fermentation process.

- ✓ The application of the modern method of technological processing - sublimation drying is a prerequisite for obtaining a functional probiotic product with a high titre of viable microflora, long-term storage and a maximally pronounced beneficial effect on the human body.

III. GOAL AND OBJECTIVES

PURPOSE

The aim of this thesis is to develop a technology for obtaining new lyophilized bioactive products based on hydrolysates from fresh cow's milk and whey.

TASKS

- ✓ Selection of different proteolytic enzymes for obtaining protein hydrolysates as a source of biologically active peptides and determination of their enzyme activity.
- ✓ Selection of a suitable substrate for carrying out a hydrolysis process and obtaining protein hydrolysates.
- ✓ Conducting experiments with different strains of microorganisms, in order to ensure specific characteristics in the final fermented product in a controlled and predictable manner.
- ✓ Carrying out fermentation experiments with selected strains of lactic acid bacteria in media with milk and whey hydrolyzate.
- ✓ Carrying out the sublimation drying process.
- ✓ Research on the quality indicators of the obtained functional products - organoleptic, biochemical and microbiological indicators, with a view to determining their health benefits.

IV. MATERIALS AND METHODS

1. Materials

The experimental part and the research were carried out during the period 2019 - 2022 at the Institute of Cryobiology and Food Technologies and the Central Laboratory for Chemical Testing and Control (CLHCIC) at the Food Agency - Sofia.

1.1 Biological material

1.1.1 Cow's fresh milk - collective milk from the farm of IPZZ, Troyan.

1.1.2 Whey from cow's milk - the research and experiments were carried out with collective whey obtained from the production of white brine cheese from the farm of IPZZ, Troyan.

1.1.3 Proteolytic enzymes:

Papain (Papain, Technical Powder) purchased from MP Biomedicals, LLC, cat. No. 102566, storage at a temperature of 2-8°C - of plant origin. The optimal activity of papain occurs at pH 5.8–7.0. The effective temperature range is 35-55°C.

Bromelain (Bromelain from pineapple steam) purchased from SERVA Electrophoresis GmbH, cat. No. 15250, storage at a temperature of 2-8°C - of plant origin. The optimal pH for bromelain enzymatic reaction is 6–8.5 for most of its substrates, and the effective temperature range is 40–65°C.

Chymosin (CHY-MAX EXTRA chymosin yeast) purchased from Admix LTD, batch 3478838, storage at 2-8°C - of animal origin. The conditions for the optimal course of the enzyme reaction are pH 5.5-6, and the temperature is 36-40°C.

1.1.4 Strains of microorganisms

- ✓ *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447
- ✓ *Lactobacillus gasseri* NBIMCC 2450
- ✓ *Lactocaseibacillus rhamnosus* NBIMCC 1013

The monocultures were purchased from the National Bank for Industrial Microorganisms and Cell Cultures in lyophilized form (NBIMCC).

1.1.5 Prebiotic components:

- ✓ Locust bean rubber (E410), batch number 4720/A02, company "Orion-Mateev" EOOD

- ✓ Pectin - company "Orion-Mateev" EOOD
- ✓ Sucrose - company "Orion-Mateev" EOOD

1.2 Food environments

1.2.1 Fresh cow's milk and whey from cow's milk - medium for carrying out enzymatic hydrolysis, sterilized at 121°C for 6 min.

1.2.2 MRS broth (de man, Rogosa, Sharpe, Oxoid) - medium for recovery, activation and inoculum of the probiotic strains *L. plantarum*, *L. gasseri* and *L. rhamnosus*.

1.2.3 MRS agar (Merck, Germany) for counting colonies after fermentation and during storage after lyophilization of *L. plantarum*, *L. gasseri* and *L. rhamnosus* strains, medium pH 6.8; sterilization at 121°C for 15 min.

1.2.4 PCA (Himedia) - to determine the total number of microorganisms.

1.2.5 Tryptone Bile X-glucuronide agar (Merck, Germany) - are isolation and enumeration of *Escherichia coli*.

1.2.6 Baird-Parker agar (Himedia) - isolation and enumeration of *Staphylococci*.

1.2.7 Sabouraud agar with dextrose (Himedia) - for cultivation and enumeration of molds and yeasts.

1.2.8 Fermentation medium - 10% milk or whey hydrolyzate, 2% lactose, 0.5% yeast extract, 4% glucose and dH₂O. pH of the medium 6.8; sterilization at 121°C for 6 min.

1.2.9 Cryoprotectant for lyophilization:

- ✓ 3% sucrose solution and 2% pectin solution
- ✓ 0.1% Locust bean gum solution (E410)

2. Methods of analysis

2.1 Organoleptic assessment

The organoleptic evaluation of the starting raw materials whey from cow's milk and fresh cow's milk was carried out according to the indicators: appearance, color, taste and aroma (BDS - 15612-83). The

developed bioactive products based on whey and milk hydrolyzate were evaluated organoleptically according to the 9-point Hedonic scale.

2.2 Physicochemical methods

- ✓ Determination of dry fat-free residue of whey and cow's milk - on flow analyzer "Milkoscan Conbfifoss 5000" and Milkotester.
- ✓ Determination of dry matter of whey and cow's milk - on flow analyzer "Milkoscan Conbfifoss 5000" and Milkotester
- ✓ Determination of the residual moisture content after lyophilization (BDS 1109-89) - the method is modified, with the use of an electronic balance "Sartorius" with infrared heating of the samples.
- ✓ Determination of active acidity /pH/ - potentiometric with pH - meter "CONSORT", model C830.
- ✓ Determination of titratable acidity - (BDS 1111-80).
- ✓ Determination of total protein content - Kjeldahl method (BDS 6231-73).
- ✓ Determining the fat content - automatically using the "Soxtec" apparatus (BDS 1671-89).
- ✓ Determination of total ash – (BDS 6154-74)
- ✓ Energy value of 100g of product - kJ /kcal/ - calculation based on the chemical composition.

2.3 Biochemical methods

2.3.1 Determination of enzyme activity - the proteolytic activity of the enzymes papain, bromelain and chymosin was determined and calculated according to Anson's method.

2.3.2 Determination of degree of hydrolysis - the method is based on the reaction of amino acids with Ninhydrin hydrate ($C_9H_6O_4 \cdot xH_2O$) at pH 5.0 and temperature 100°C for a standard period of time.

2.3.3 Determination of antioxidant activity - the antioxidant capacity of whey and milk hydrolysates was determined according to the method of Brand-Williams et al. (1995).

2.3.4 Determination of Amino Acid Composition of Bioactive Products - The identification and quantification of amino acids in the final products

was performed by ultra-high performance liquid chromatography (HPLC) using an Agilent 1260 Infinity Quaternary LC, DAD.

2.3.5 Macroelemental and microelemental composition - the analysis was carried out on an atomic emission spectrophotometer "AES-ICP" in the Central Laboratory for Chemical Testing and Control at the Food Agency - Sofia.

2.4 Microbiological methods

2.4.1 Cultivation (recovery and activation) of strains of probiotic bacteria to conduct the experimental part.

2.4.2 Determination of total number of viable cells (CFU/ml) *Lactiplantibacillus plantarum*, *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus*, cfu/ml - BDS ISO 7889: 2005.

Steps of counting the survival of probiotic bacteria:

✓ Immediately after the completion of the fermentation process when cultured in a medium with fresh cow's milk and whey and the hydrolyzates obtained from them with the enzymes bromelain and papain.

✓ Reporting the survival of the probiotic lactic acid microflora after lyophilization using two cryoprotectants.

✓ Evaluation of the survival of lactic acid probiotic bacteria during storage at three different temperature conditions - room temperature, refrigerated conditions - 4°C and in a freezer at -18°C.

2.4.3 Microbiological studies of raw materials and products - (BDS 1670-82).

2.4.3.1 Total number of mesophilic aerobic and facultative anaerobic microorganisms - CFU/ml, (BDS EN ISO 4833-1,2)

2.4.3.2 Lactic acid bacteria, CFU/ml, (BDS ISO 15214)

2.4.3.3 Spores of microscopic moulds and yeasts, CFU/ml, (BDS ISO 6611:2006)

2.4.3.4 *Escherichia coli* in 0.1 g of the product (ISO 16649 -2:2004)

2.4.3.5 Coagulase-positive staphylococci in 1.0 g of the product (BDS EN ISO 6888-1)

2.5 Differential thermal analysis

Measurements were performed with a DSC 204F1 Phoenix (Netzsch Gerätebau GmbH, Germany). Heat flux and temperature were calibrated with an indium standard (melting temperature $T_m=156.6^{\circ}\text{C}$, melting enthalpy $H_m=28.5\text{ J/g}$) for all values of heating or cooling rates used. Initially, the samples were cooled to -60°C at a rate of 5 K/min , then heated to 200°C at a rate of 10 K/min .

2.6 Technological methods

On the basis of all performed analyzes and reported results, a technological scheme was developed for the experimental production of two bioactive products, including milk or whey hydrolyzate, a probiotic strain that showed the highest values of survival during fermentation and after the lyophilization process, prebiotic components, fruits and natural sweeteners.

2.6.1 Lyophilization

Sublimation drying was carried out in a semi-industrial sublimation installation "LYOBETA 6PL" of the TELSTAR company.

2.7 Statistical processing of the results

Mean values and standard deviation were calculated from experimental data for 3 replicates. The t-test function was used for statistical reliability of the difference between individual values. The results were processed using MS Office Excel 2007 software.

V. RESULTS AND DISCUSSION

1. Selection of proteolytic enzymes of different origins and determination of their enzyme activity in order to obtain protein hydrolysates as a source of biologically active peptides.

To conduct a series of experiments on the hydrolysis of the protein components in the starting substrates, three commercial proteases were used - papain, bromelain and chymosin. Their proteolytic activity was determined by Anson's method, under conditions and pH of the medium, specific for the given enzyme. The results obtained are shown in Table 1.

Table 1. Catalytic activity of the enzyme preparations used

Enzyme	Activity
Papain	67550,00 U/g
Bromelain	256475,00 U/g
Chymosin	79,64 U/ml

2. Selection of a suitable protein substrate. Preliminary studies on the physicochemical and organoleptic parameters of fresh cow's milk and cow's milk whey.

2.1 Organoleptic evaluation

Fresh cow's milk is a homogeneous liquid, without external impurities and the presence of agglomerates of fat lumps, with a white color with a slightly creamy tint. Whey is a milk serum with a watery consistency with a predominant pale yellow color, unattractive taste and aroma, which is obtained as a waste product of cheese production.

2.2 Physicochemical evaluation

Figure 1 graphically shows a comparative analysis of the physicochemical parameters between cow's milk and its whey.

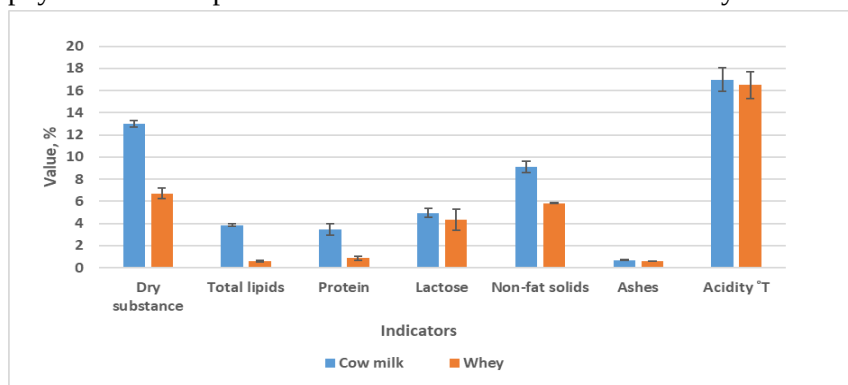


Figure 1. Comparative physicochemical analysis between cow's milk and cow's milk whey

Comparative physicochemical analysis shows that the dry matter content of cow's milk is almost twice as high as that of whey. The content of proteins in whey (α -lactalbumin and β -lactoglobulin) is lower

compared to milk, because during the production of cheese the main milk protein - casein, coagulates under the action of rennet enzymes, retaining most of the milk fat and part of mineral salts and lactose. For this reason, it is also low in fat. The titratable acidity varies between 16-17°T in both starting substrates.

2.3 Biochemical assessment

The fatty acid profile of fresh cow's milk and whey was determined experimentally. The data are presented in table 2.

Table 2. Content of essential groups of fatty acids in cow's fresh milk and whey g/100g fat (x±sd)

Fatty acid groups, %	Cow's milk	Whey
Σ saturated fatty acids	66,12±0,26	52,76±0,32
Σ monounsaturated fatty acids	27,72±0,45	31,46±0,25
Σ polyunsaturated fatty acids	5,76±0,22	15,38±0,42
Σ C-18:1trans	5,94±0,15	
Σ CLA	2,56±0,04	
Σ ω-3	1,13±0,02	
Σ ω-6	2,85±0,28	
ω-6/ ω-3	2,58	

A high content of vaccenic acid and CLA was found, but the amount of essential ω-6 and ω-3 fatty acids was low compared to other components. The ratio of ω-6/ω-3 fatty acids in cow's milk has values lower than 5.0, which characterizes it as milk with a low risk factor for human health.

The content of saturated fatty acids in whey is high - almost 53% (g/100 g fat). The amount of unsaturated fatty acids is slightly over 30%. Many authors believe that unsaturated fatty acids increase the biological value of milk fat in milk and products derived from it.

2.4 Antioxidant capacity

The antioxidant capacity of the starting products - fresh cow's milk and whey - was measured by the DPPH method with Trolox standard. The results are presented as Trolox equivalents antioxidant capacity (TE) mg /100 ml product in table 3.

Table 3. Antioxidant activity of fresh cow's milk and whey ($\bar{x} \pm \text{sd}$)

Raw material	Antioxidant capacity, mg TE/100 ml
Cow fresh milk	2,21 $\pm$ 0,03
Cow's milk whey	1,67 $\pm$ 0,02

2.5 Microbiological status of the starting raw materials - cow's milk and whey

Cow's fresh milk and whey are pasteurized for 10 minutes at 90-95°C before use for further experiments. Regarding the total number of microorganisms, the starting raw materials meet the requirements of BDS EN ISO 4833-1, 2. The presence of microscopic moulds and yeasts was not detected.

3. Carrying out experiments on the hydrolysis of the protein components of the substrate

The efficiency of the hydrolysis process was evaluated for each substrate by modifying the following parameters:

- enzyme concentration (0.1; 0.5 and 1 mg/ml for papain and bromelain and 1; 5 and 10 $\mu\text{l/ml}$ for chymosin)
- the reaction time on the starting protein substrates – 10, 20, 30 and 60 min.

The variant hydrolysates and abbreviations of the resulting samples are shown in tables 4 and 5.

Table 4. Variants of cow's milk hydrolysates

Table 5. Variants hydrolysates from whey

Enzyme	Enzyme concentration	Hydrolysis time (min)	Sample variant
Papain	0,1 mg/ml	10	MP1
		20	MP2
		30	MP3
		60	MP4
	0,5 mg/ml	10	MP5
		20	MP6
		30	MP7
		60	MP8
	1 mg/ml	10	MP9
		20	MP10
		30	MP11
		60	MP12
Bromelain	0,1 mg/ml	10	MB1
		20	MB2
		30	MB3
		60	MB4
	0,5 mg/ml	10	MB5
		20	MB6
		30	MB7
		60	MB8
	1mg/ml	10	MB9
		20	MB10
		30	MB11
		60	MB12
Chymosin	1 µl/ml	10	MCh1
		20	MCh2
		30	MCh3
		60	MCh4
	5 µl /ml	10	MCh5
		20	MCh6
		30	MCh7
		60	MCh8
	10 µl /ml	10	MCh9
		20	MCh10
		30	MCh11
		60	MCh12

Enzyme	Enzyme concentration	Hydrolysis time (min)	Sample variant
Papain	0,1 mg/ml	10	WP1
		20	WP2
		30	WP3
		60	WP4
	0,5 mg/ml	10	WP5
		20	WP6
		30	WP7
		60	WP8
	1 mg/ml	10	WP9
		20	WP10
		30	WP11
		60	WP12
Bromelain	0,1 mg/ml	10	WB1
		20	WB2
		30	WB3
		60	WB4
	0,5 mg/ml	10	WB5
		20	WB6
		30	WB7
		60	WB8
	1mg/ml	10	WB9
		20	WB10
		30	WB11
		60	WB12
Chymosin	1 µl/ml	10	WCh1
		20	WCh2
		30	WCh3
		60	WCh4
	5 µl /ml	10	WCh5
		20	WCh6
		30	WCh7
		60	WCh8
	10 µl /ml	10	WCh9
		20	WCh10
		30	WCh11
		60	WCh12

3.1 Biochemical studies of the obtained hydrolysates

Degree of hydrolysis

The degree of hydrolysis /DH/ was determined, and the result was expressed as a percentage of hydrolyzed peptide bonds. The results are presented in tables 6 and 7.

Table 6. Degree of hydrolysis of fresh milk samples ($x \pm sd$)

Sample	DH, %	Sample	DH, %	Sample	DH, %
MP1	2,56±0,02	MB1	6,46±0,12	MCh1	4,66±0,06
MP2	2,88±0,03	MB2	7,94±0,14	MCh2	4,70±0,07
MP3	3,15±0,05	MB3	9,00±0,19	MCh3	5,58±0,09
MP4	3,39±0,04	MB4	10,16±0,24	MCh4	6,35±0,08
MP5	8,11±0,12	MB5	30,46±0,36	MCh5	8,50±0,09
MP6	8,43±0,16	MB6	30,77±0,34	MCh6	8,93±0,1
MP7	10,18±0,14	MB7	34,24±0,38	MCh7	9,53±0,12
MP8	10,86±0,17	MB8	34,42±0,4	MCh8	9,80±0,11
MP9	24,05±0,31	MB9	37,93±0,41	MCh9	8,29±0,10
MP10	24,50±0,29	MB10	39,51±0,46	MCh10	8,49±0,12
MP11	24,74±0,30	MB11	42,82±0,52	MCh11	9,37±0,14
MP12	28,76±0,41	MB12	46,14±0,57	MCh12	12,18±0,18

Table 7. Degree of hydrolysis of whey samples ($x \pm sd$)

Sample	DH, %	Sample	DH, %	Sample	DH, %
WP1	33,22±0,52	WB1	28,88±0,32	WCh1	13,18±1,51
WP2	33,81±0,51	WB2	29,19±0,38	WCh2	15,62±1,59
WP3	35,16±0,54	WB3	30,24±0,46	WCh3	15,62±1,58
WP4	41,63±0,65	WB4	31,91±0,48	WCh4	19,77±1,61
WP5	60,43±1,52	WB5	47,02±0,71	WCh5	29,03±1,73
WP6	60,78±1,55	WB6	49,77±0,70	WCh6	32,66±1,85
WP7	62,02±1,68	WB7	54,81±0,68	WCh7	32,82±2,04
WP8	63,22±1,72	WB8	58,18±0,74	WCh8	34,84±2,12
WP9	60,39±1,54	WB9	54,54±0,67	WCh9	40,31±1,98
WP10	73,80±1,72	WB10	56,05±0,72	WCh10	45,78±2,37
WP11	74,57±1,78	WB11	60,66±0,85	WCh11	47,36±2,56
WP12	75,62±1,83	WB12	74,45±0,92	WCh12	47,36±2,95

The lowest degree of hydrolysis was reported for fresh milk sample MP1 (10 min treatment with 0.1 mg/ml papain), and the highest percentage was obtained from high concentration bromelain in

hydrolyzate MB12 (1 mg/ml, 60-th processing minute). For the serum samples, the hydrolysis percentage for sample WCh1 (chymosin 1 μ l/ml at 10 min reaction time) was only 13.18%. The highest percentage was achieved with WP12 hydrolyzate using papain at 1 mg/ml.

3.2 Electrophoretic analysis of the obtained protein hydrolysates

Polyacrylamide SDS electrophoresis was performed to determine the change in the state of the protein substances in the cow's milk and whey variants (figures 2 and 3). β -Lactoglobulin (β -Lg), α -Lactalbumin (α -La), α -casein and serum albumin (BSA) and standard protein markers are given for comparison.

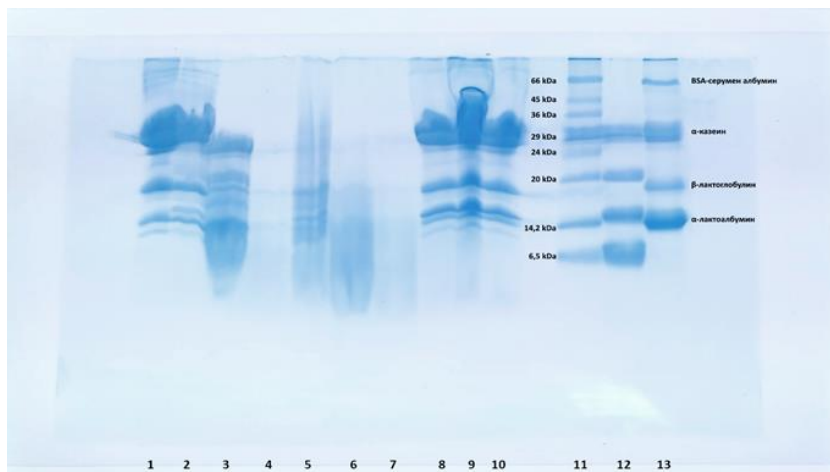


Figure 2. SDS-PAGE of cow's milk protein hydrolysates

1- Fresh milk, 2- MP4, 3- MP8, 4- MP12, 5- MB4, 6- MB8, 7- MB12, 8- MCh4, 9 -MCh8, 10 -MCh12, 11- Protein marker (SigmaMarker low range), 12- Protein marker (protein test mixture 5- SERVA), 13- BSA (serum albumin), α -casein, β -Lactoglobulin, α -Lactalbumin.

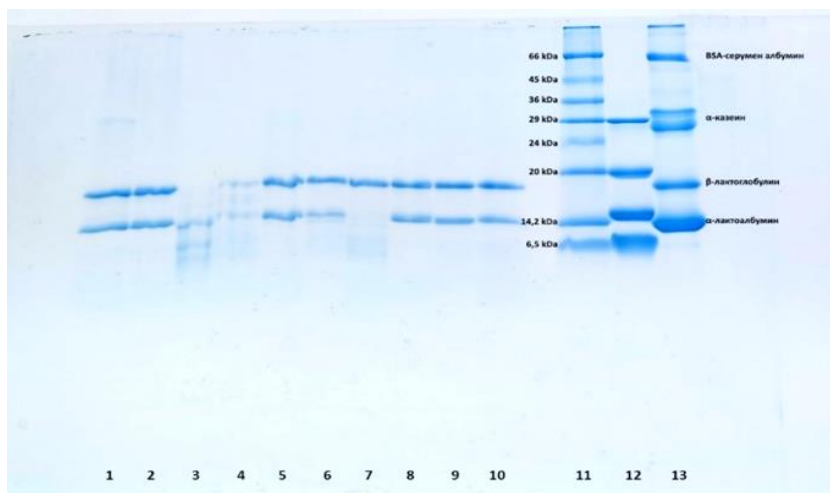


Figure 3. SDS-PAGE of whey protein hydrolysates

1- Whey, 2 - WP4, 3- WP8, 4- WP12, 5- WB4, 6- WB8, 7- WB12, 8- WCh4, 9- WCh8, 10- WCh12, 11- Protein marker (SigmaMarker low range), 12- Protein marker (protein test mixture 5- SERVA), 13- BSA (serum albumin), α -casein, β -Lactoglobulin, α -Lactalbumin

In the hydrolysates obtained after treatment with the enzymes papain and bromelain, it can be seen that the obtained products have undergone proteolytic degradation.

The main protein components of milk are completely degraded after treatment with papain and bromelain at a concentration of 1mg/m.

Electropherograms of papain- and bromelain-treated whey variants showed significant changes in the amount of α -Lactalbumin and β -Lactoglobulin fractions.

No significant difference was reported in the protein profile of the chymosin-treated variants.

3.3 Antioxidant capacity

The antioxidant activity of the obtained hydrolysates from cow's fresh milk and whey are presented in tables 8 and 9.

Table 8. Antioxidant capacity of milk hydrolysates obtained with the enzymes papain, bromelain and chymosin ($\bar{x} \pm \text{sd}$).

Enzyme	Enzyme concentration	Hydrolysis time (min)	Sample variant	Antioxidant activity (mg TE/100 ml)
Papain	0,1 mg/ml	10	MP1	4,38 $\pm$ 0,03
		20	MP2	4,58 $\pm$ 0,05
		30	MP3	5,94$\pm$0,04
		60	MP4	4,86 $\pm$ 0,29
	0,5mg/ml	10	MP5	5,88 $\pm$ 0,32
		20	MP6	7,57 $\pm$ 0,29
		30	MP7	9,31$\pm$0,36
		60	MP8	8,40 $\pm$ 0,06
	1mg/ml	10	MP9	5,26 $\pm$ 0,03
		20	MP10	5,67 $\pm$ 0,05
		30	MP11	5,90$\pm$0,12
		60	MP12	5,58 $\pm$ 0,17
Bromelain	0,1 mg/ml	10	MB1	9,37 $\pm$ 0,23
		20	MB2	9,74 $\pm$ 0,09
		30	MB3	11,32$\pm$0,41
		60	MB4	10,57 $\pm$ 0,37
	0,5mg/ml	10	MB5	7,56 $\pm$ 0,26
		20	MB6	7,72 $\pm$ 0,11
		30	MB7	9,36$\pm$0,15
		60	MB8	8,76 $\pm$ 0,09
	1mg/ml	10	MB9	6,97 $\pm$ 0,07
		20	MB10	7,20 $\pm$ 0,13
		30	MB11	7,31$\pm$0,18
		60	MB12	6,93 $\pm$ 0,08
Chymosin	1 μ l/ml	10	MCh1	3,26 $\pm$ 0,02
		20	MCh2	3,38 $\pm$ 0,04
		30	MCh3	3,61$\pm$0,03
		60	MCh4	3,54 $\pm$ 0,07
	5 μ l /ml	10	MCh5	3,43 $\pm$ 0,08
		20	MCh6	3,73 $\pm$ 0,11
		30	MCh7	4,07 $\pm$ 0,12
		60	MCh8	4,26$\pm$0,16
	10 μ l /ml	10	MCh9	4,03 $\pm$ 0,13
		20	MCh10	4,40 $\pm$ 0,20
		30	MCh11	4,61 $\pm$ 0,23
		60	MCh12	4,90$\pm$0,22

The results show that in all investigated protein hydrolysates from milk, a higher antioxidant activity was found compared to the starting substrate (2.21mg TE/100 ml). The highest antioxidant activity was reported for the milk variant hydrolyzed with 0.1 mg/ml bromelain for 30 min.

The group of whey samples hydrolyzed with the enzyme papain exhibited higher antioxidant activity than the hydrolysates obtained with the enzymes bromelain and chymosin. The highest value was found with 0.5 mg/ml papain for 30 min.

The enzyme chymosin has a weak impact on the hydrolysis process and the antioxidant activity in this group of samples is the least pronounced.

Table 9. Antioxidant capacity of whey hydrolysates with papain, bromelain and chymosin enzymes ($\bar{x} \pm \text{sd}$).

Enzyme	Enzyme concentration	Hydrolysis time (min)	Sample variant	Antioxidant activity (mg TE/100 ml)
Papain	0,1 mg/ml	10	WP1	2,30 $\pm$ 0,03
		20	WP2	2,55 $\pm$ 0,06
		30	WP3	3,07$\pm$0,05
		60	WP4	2,91 $\pm$ 0,01
	0,5mg/ml	10	WP5	5,62 $\pm$ 0,11
		20	WP6	6,80 $\pm$ 0,12
		30	WP7	7,83$\pm$0,21
		60	WP8	6,19 $\pm$ 0,19
	1mg/ml	10	WP9	5,36 $\pm$ 0,16
		20	WP10	5,46 $\pm$ 0,09
		30	WP11	6,15$\pm$0,18
		60	WP12	5,71 $\pm$ 0,21
Bromelain	0,1 mg/ml	10	WB1	3,12 $\pm$ 0,04
		20	WB2	3,46 $\pm$ 0,05
		30	WB3	4,06$\pm$0,16
		60	WB4	3,80 $\pm$ 0,08

	0,5mg/ml	10	WB5	4,53±0,22
		20	WB6	4,78±0,25
		30	WB7	4,99±0,32
		60	WB8	4,76±0,33
	1mg/ml	10	WB9	2,32±0,02
		20	WB10	2,39±0,07
		30	WB11	3,08±0,25
		60	WB12	2,90±0,05
Chymosin	1 µl/ml	10	WCh1	2,23±0,08
		20	WCh2	2,31±0,09
		30	WCh3	2,55±0,12
		60	WCh4	2,82±0,17
	5 µl /ml	10	WCh5	2,38±0,15
		20	WCh6	2,54±0,26
		30	WCh7	3,05±0,22
		60	WCh8	3,19±0,24
	10 µl /ml	10	WCh9	3,36±0,29
		20	WCh10	2,30±0,03
		30	WCh11	2,55±0,06
		60	WCh12	3,07±0,05

The group of whey samples hydrolyzed with the enzyme papain exhibited higher antioxidant activity than the hydrolysates obtained with the enzymes bromelain and chymosin. The highest value was found with 0.5 mg/ml papain for 30 min. The enzyme chymosin has little effect on the hydrolysis process and the antioxidant activity in this group of samples is the least expressed. All variant hydrolysates exhibited higher antioxidant activity than the native substrate (1.67 mg TE/100 ml).

Based on the obtained results, in the following studies and activities, milk and whey hydrolysates were used, showing the highest antioxidant capacity, namely:

- Hydrolysates obtained by hydrolysis of fresh cow's milk with the enzyme bromelain in concentrations of 0.1mg/ml, 0.5mg/ml and 1.0mg/ml and during 30 min
- Hydrolysates obtained by hydrolysis of milk whey with papain enzyme in concentrations of 0.1mg/ml, 0.5mg/ml and 1.0mg/ml and during 30 min

4. Fermentation with selected strains of lactic acid bacteria and the preparation of functional products with new physiological effects.

4.1 Recovery and activation of bacterial cultures

For the fermentation experiments, three probiotic strains of lactic acid bacteria with proven health benefits *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 (A), *Lactobacillus gasseri* NBIMCC 2450 (B) and *Lacticaseibacillus rhamnosus* NBIMCC 1013 (B) (figure 4).

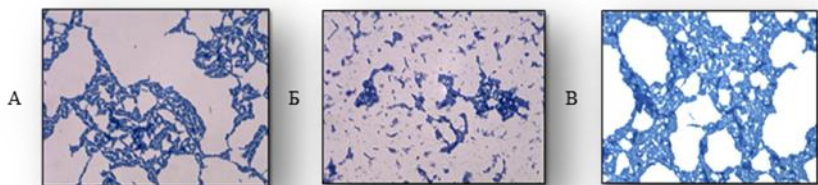


Figure 4. Photomicrograph of probiotic strains

MRS broth (de man, Rogosa, Sharpe, Oxoid) previously sterilized was used for their recovery and activation. Activated probiotic strains in an amount of 5-10% are cultivated in pre-sterilized MRS broth, statically at a temperature of 37°C for 16-18 hours. After completion of the cultivation process, the technological status of the cultures was evaluated by determining the total number of viable cells (CFU/ml) and by microscopic analysis to study the morphology of the cells.

4.2 Determination of technological parameters for fermentation

Technological schemes of the fermentation process were created for each strain separately.

Because a strain of *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 was isolated from sauerkraut, which implies a wide range of cultivation temperature variation, and three temperature regimes - 32°C, 37°C and 40°C were set and experimented in the technological scheme for the cultivation of the strain in order to determine the optimal temperature for our goals (figure 5).

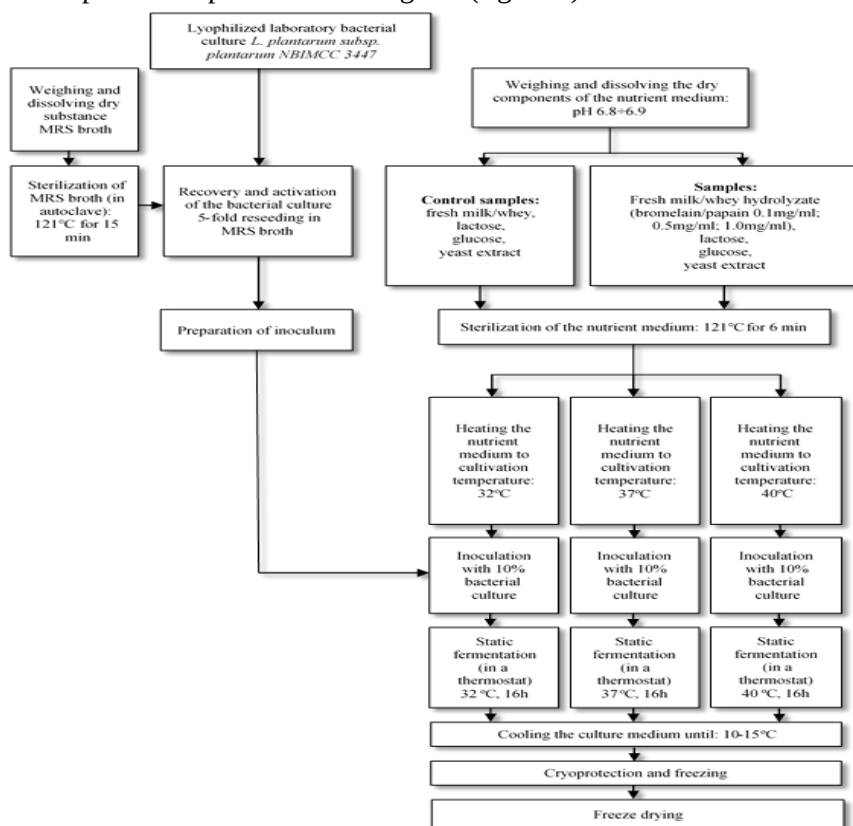


Figure 5. Technological scheme for fermentation of strain *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447

Strain *Lactobacillus gasseri* NBIMCC 2450 was isolated from faeces, and *Lacticaseibacillus rhamnosus* NBIMCC 1013 was isolated from fermented milk products and the certificate from NBIMCC indicated the optimal temperature for cultivation - 37°C in the technological scheme only one temperature regime was applied (figure 6).

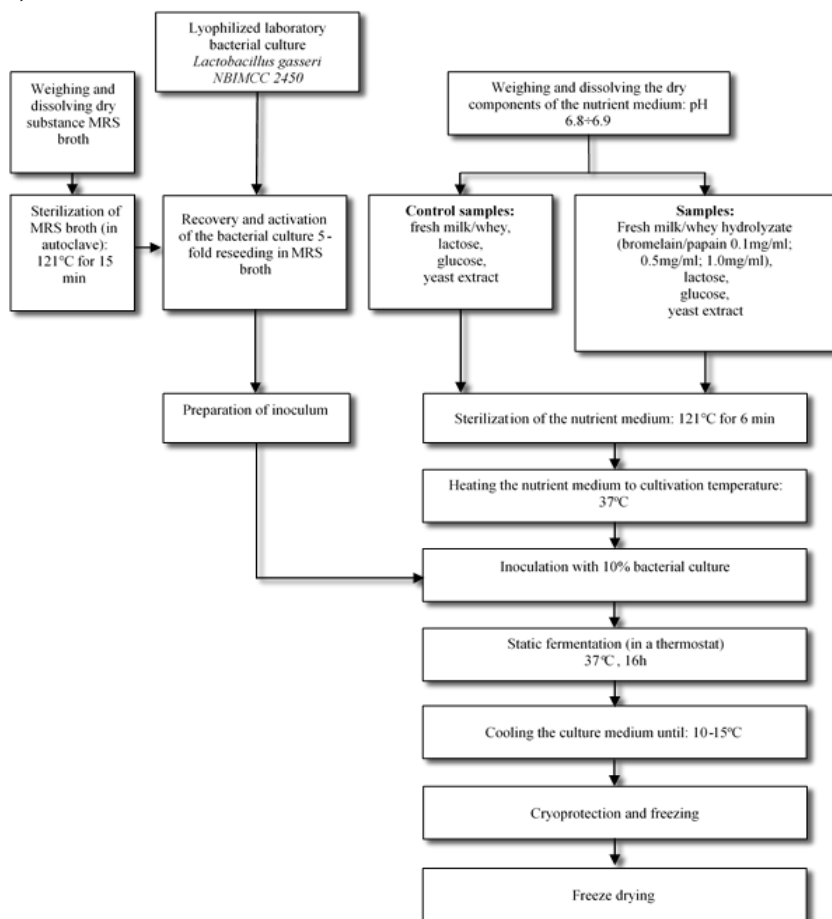


Figure 6. Technological scheme for fermentation of *Lactobacillus gasseri* strain NBIMCC 2450

The main stages in the cultivation of the probiotic strains are: recovery and activation of the bacterial cultures; preparation of inoculum and inoculation in culture medium; static fermentation; Cooling of the cultural environment; cryoprotection and freezing and freeze drying.

The data from the reported active acidity of the medium at the beginning and at the end of the fermentation process with the participation of the probiotic strains are presented in figures 7 and 8.

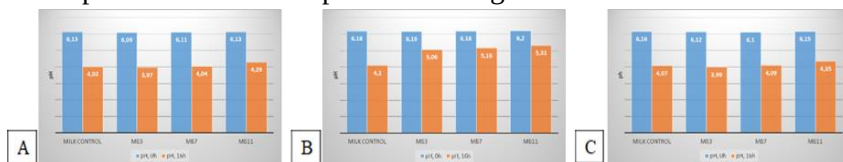


Figure 7. Change in the active acidity of the fermentation medium with the participation of A – *L. plantarum*, B – *L. gasseri* and C – *L. rhamnosus* during their cultivation in milk and fresh milk hydrolysates with bromelain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

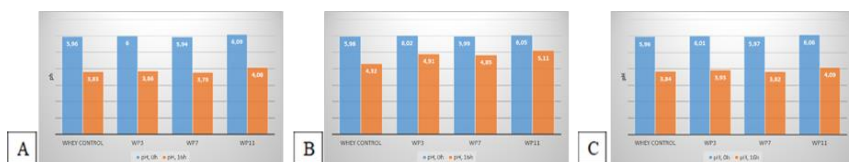


Figure 8. Change in the active acidity of the fermentation medium with the participation of A – *L. plantarum*, B – *L. gasseri* and C – *L. rhamnosus* during their cultivation in whey and whey hydrolysates with papain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

A criterion for the completion of the fermentation process is reaching a pH lower than 5

The results show that the active acidity in the fermentation process gradually decreases and reaches this value at the end of the fermentation process in *L. plantarum* and *L. rhamnosus*. There is a delay in the acid-forming activity of *Lactobacillus gasseri* strain NBIMCC 2450 (16-hour pH is above 5.00) compared to the other two strains.

Viable cells of the probiotic bacteria were counted on a solid nutrient medium - MRS agar by deep seeding (figure 9).



Figure 9. Deep culture in MRS agar of A - *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447, Б - *Lactobacillus gasseri* NBIMCC 2450, B - *Lacticaseibacillus rhamnosus* NBIMCC 1013

The number of viable cells of the three studied probiotic bacteria, counted in the process of cultivation in milk and whey and in their protein hydrolysates at different temperature regimes are indicated in Tables 10, 11, 12 and 13.

Table 10. Number of viable cells *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 when cultured in milk and fresh milk hydrolysates with bromelain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Survival after fermentation					
	32°C		37°C		40°C	
	CFU/ml	log	CFU/ml	log	CFU/ml	log
Milk control	$9,7 \times 10^8$	8,98	$1,2 \times 10^9$	9,08	$4,2 \times 10^8$	8,62
MB3	$1,3 \times 10^9$	9,11	$1,6 \times 10^{10}$	10,20	$8,5 \times 10^8$	8,93
MB7	$8,1 \times 10^8$	8,91	$1,9 \times 10^9$	9,28	$3,9 \times 10^8$	8,59
MB11	$1,7 \times 10^8$	8,23	$9,7 \times 10^8$	8,99	$8,7 \times 10^7$	7,93

Table 11. Number of viable cells of *Lactobacillus gasseri* NBIMCC 2450 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 when cultivated in milk and fresh milk hydrolysates with bromelain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Survival after fermentation, 37°C			
	<i>L. gasseri</i>		<i>L. rhamnosus</i>	
	CFU/ml	log	CFU/ml	log
Milk control	$4,0 \times 10^8$	8,60	$1,1 \times 10^9$	9,04
MB3	$1,5 \times 10^9$	9,18	$8,2 \times 10^9$	9,91
MB7	$1,8 \times 10^9$	9,25	$2,6 \times 10^9$	9,41
MB11	$8,5 \times 10^8$	8,92	$9,3 \times 10^8$	8,97

The presented results show that the cultivation process of all three probiotic strains was successful in hydrolyzed milk medium.

For the hydrolysates cultured with the *Lactiplantibacillus plantarum* strain, higher survival values were reported at a temperature of 37°C. The highest of 1.6×10^{10} CFU/ml (log 10.20) was reported for sample MB3 (0.1mg/ml).

For *Lacticaseibacillus rhamnosus* NBIMCC 1013, the highest survival of 8.2×10^9 CFU/ml (log 9.91) was reported with the same variant hydrolyzate (0.1mg/ml enzyme).

Lower survival was observed with *Lactobacillus gasseri* NBIMCC 2450 1.8×10^9 CFU/ml (log 9.25).

Table 12. Number of viable cells *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 when cultured in whey medium and whey hydrolysates with papain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Survival after fermentation					
	32°C		37°C		40°C	
	CFU/ml	log	CFU/ml	log	CFU/ml	log
Whey control	$8,4 \times 10^8$	8,92	$1,4 \times 10^9$	9,15	$3,4 \times 10^8$	8,53
WP3	$8,6 \times 10^8$	8,93	$1,6 \times 10^9$	9,20	$4,2 \times 10^8$	8,62
WP7	$9,5 \times 10^8$	8,98	$3,0 \times 10^9$	9,48	$5,5 \times 10^8$	8,74
WP11	$5,1 \times 10^7$	7,71	$3,1 \times 10^8$	8,50	$1,1 \times 10^7$	7,04

Table 13. Number of viable cells of *Lactobacillus gasseri* NBIMCC 2450 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 when cultured in whey and whey hydrolysates with papain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Survival after fermentation, 37°C			
	<i>L. gasseri</i>		<i>L. rhamnosus</i>	
	CFU/ml	log	CFU/ml	log
Whey control	4×10^8	9,04	$3,9 \times 10^8$	8,59
WP3	$1,3 \times 10^9$	9,11	$1,1 \times 10^9$	9,04
WP7	$1,6 \times 10^9$	9,20	$2,1 \times 10^9$	9,32
WP11	$2,3 \times 10^8$	8,36	$6,9 \times 10^8$	8,84

Similar to the fermentation in milk hydrolysates, we observed the highest fermentative capacity of strain *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 at a temperature of 37°C.

The highest cell viability was observed in the samples treated with the papain enzyme at a concentration of 0.5 mg/ml.

From the results obtained and the reported lower survival of *Lactobacillus gasseri* NBIMCC 2450, the next fermentation trials will continue with *Lactiplantibacillus plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013.

4.3 Selection and application of cryoprotectant

The effect of two cryoprotectants on probiotic bacteria after lyophilization was investigated.

I - 3% sucrose, 2% pectin and 95% dH₂O.

II - 0.1% Locust bean gum solution (E410)

The amount of cryoprotective medium is 10% of the amount of the culture liquid pre-sterilized in an autoclave for 15 min at 121°C.

4.4 Freezing and freeze-drying of probiotic cultures after fermentation

The process was carried out in a semi-industrial sublimation installation "LYOBETA 6PL" of the TELSTAR company. The variants are pre-frozen at a temperature of -30 to -35°C in a refrigeration plant. The lyophilization moves to a second phase - sublimation (water removal at -22°/-25°C under deep vacuum) and finally the process ends with a third phase - desorption (further drying) by heating at positive temperatures up to +28 to +30°C.

4.5 Survival of lactic acid microflora after lyophilization.

Survival of probiotic bacteria after lyophilization was monitored using two cryoprotectants. The results of the study for the number of viable cells of *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC

3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 with fresh milk hydrolysates are presented in table 14 and 15.

Table 14. Survival after lyophilization of *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 when cultured with fresh milk hydrolysates with bromelain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Before freeze-drying, log	After freeze-drying			
		1 variant of cryoprotectant		1 variant of cryoprotectant	
		log	% survival	log	% survival
Milk control	9,08	8,99	81,3	8,97	77,5
MB3	10,20	10,13	84,9	10,18	94,0
MB7	9,28	9,16	76,0	9,20	84,2
MB11	8,99	8,85	73,5	8,89	79,8

Table 15. Survival after lyophilization of *Lacticaseibacillus rhamnosus* NBIMCC 1013 when cultured with fresh milk hydrolysates with bromelain enzyme concentrations of 0.1; 0.5; 1.0 mg/ml.

Samples	Before freeze-drying, log	After freeze-drying			
		1 variant of cryoprotectant		1 variant of cryoprotectant	
		log	% survival	log	% survival
Milk control	9,04	8,95	80,5	8,93	78,0
MB3	9,91	9,83	82,3	9,88	91,5
MB7	9,41	9,27	71,5	9,33	83,0
MB11	8,97	8,80	68,0	8,86	77,6

In all samples with included hydrolyzate, higher survival of probiotic strains was reported using cryoprotectant 2 - locust bean gum. We observe a tendency to decrease the number of viable cells with increasing concentration of the enzyme to obtain a hydrolyzate. From the obtained results, it can be seen that Locust bean exhibits better cryoprotective properties in the freezing process of the hydrolyzate

samples, preserving the integrity of the cells that successfully survive the lyophilisation process.

4.6 Antioxidant activity of selected hydrolysates included in the culture medium of the two probiotic strains *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013.

The results for antioxidant activity of the culture medium with and without milk or whey hydrolyzate after completion of the fermentation process with *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 are shown in Table 16 and 17.

Table 16. Antioxidant activity of milk culture medium and its hydrolysates after fermentation.

A sample	TEAA mg/100 ml	A sample	TEAA mg/100 ml
<i>L. plantarum</i> fermentation in milk medium	13,94	<i>L. rhamnosus</i> fermentation in milk medium	12,82
<i>L. plantarum</i> fermentation in MB 3 medium	21,28	<i>L. rhamnosus</i> fermentation in MB 3 medium	20,49
<i>L. plantarum</i> fermentation in MB 7 medium	16,95	<i>L. rhamnosus</i> fermentation in MB 7 medium	16,30
<i>L. plantarum</i> fermentation in MB 11 medium	12,87	<i>L. rhamnosus</i> fermentation in MB 11 medium	12,43

The culture liquid of the probiotic strains in a medium with non-hydrolyzed milk showed a lower antioxidant activity compared to the samples where the bacteria underwent fermentation in a medium with included milk hydrolysates. During the hydrolysis of milk with the enzyme bromelain, the breakdown of milk into low-molecular peptides is observed, which are easily absorbed by probiotic bacteria, as a result of which the antioxidant value of the sample increases after fermentation.

In all fermentation media with included hydrolyzate, an increase in antioxidant activity was observed for both probiotic bacteria, the highest being in the medium with the lowest amount of the enzyme.

Table 17. Antioxidant activity of whey culture medium and its hydrolysates after fermentation.

A sample	TEAA mg/100 ml	A sample	TEAA mg/100 ml
<i>L. plantarum</i> fermentation in whey medium	9,53	<i>L. rhamnosus</i> fermentation in whey medium	8,92
<i>L. plantarum</i> fermentation in medium with WP 3	10,37	<i>L. rhamnosus</i> fermentation in medium with WP 3	9,94
<i>L. plantarum</i> fermentation in medium with WP 7	16,70	<i>L. rhamnosus</i> fermentation in medium with WP 7	17,08
<i>L. plantarum</i> fermentation in medium with WP 11	11,78	<i>L. rhamnosus</i> fermentation in medium with WP 11	10,59

In all media with added whey hydrolyzate, we observed an increased antioxidant capacity compared to the control fermentation media. No significant difference was reported in the antioxidant activity between the two probiotic bacteria, as in *L. plantarum* and *L. rhamnosus* it was highest in the culture medium with hydrolyzate obtained with 0.5mg/ml enzyme.

Therefore, the whey hydrolysates added to the fermentation culture medium of the two probiotic strains can be used as natural biofunctional ingredients to improve the antioxidant properties of new functional products.

4.7 Electrophoretic analysis

Electrophoretic analyses of the hydrolysates variants, which showed the highest antioxidant activity and were selected for carrying out fermentation with strains of *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013. SDS-PAGE electrophoresis of milk and whey samples and their

hydrolysates, respectively, before and after fermentation with the determined strains are shown in Figure 10 and 11.

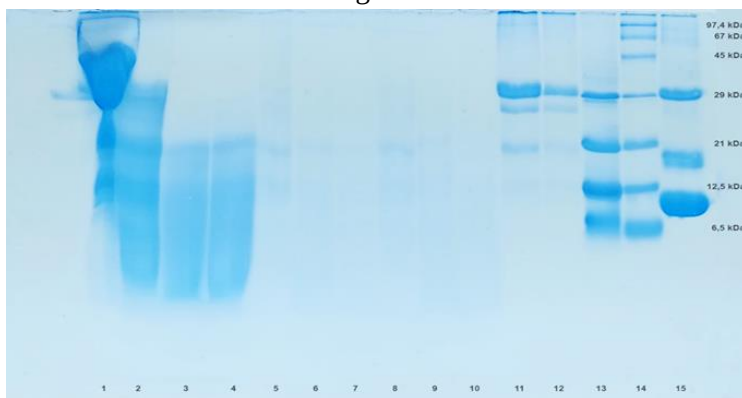


Figure 10. SDS-PAGE of milk protein hydrolysates before and after fermentation with probiotic strains.

1- Milk, 2- MB3, 3- MB7, 4- MB11, 5- MB3+*L. plantarum*, 6- MB7+*L. plantarum*, 7- MB11+*L. plantarum*, 8- MB3+*L. rhamnosus*, 9- MB7+ *L. rhamnosus*, 10- MB11+ *L. rhamnosus*, 11- Milk +*L. plantarum*, 12- Milk+ *L. rhamnosus*, 13- Protein marker (protein test mixture 5 - SERVA 6.5 - 29.0 kDa), 14- Protein marker (protein test mixture 6 - SERVA 6.5 - 97.4 kDa), 15- mixture of standard proteins (BSA, α -casein, β -casein, α -lactalbumin, β -lactoglobulin).

In the hydrolysates obtained after enzymatic treatment with bromelain, significant proteolytic degradation is clearly visible. In the variant treated with the lowest concentration of bromelain - 0.1mg/ml, degradation to lower molecular peptides (from 6.5 to 24 kDa) was observed. In the samples treated with bromelain at concentrations of 0.5mg/ml and 1mg/ml, the lines are faintly distinct, which gives us reason to assume that a large part of the proteins have been degraded to amino acids.

In all variants fermented with *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 practically no lines corresponding to milk proteins were observed. In all probability, after the fermentation of the hydrolysates

(especially those obtained with high concentrations of the enzyme) we have a complete degradation of the protein fractions to amino acids.

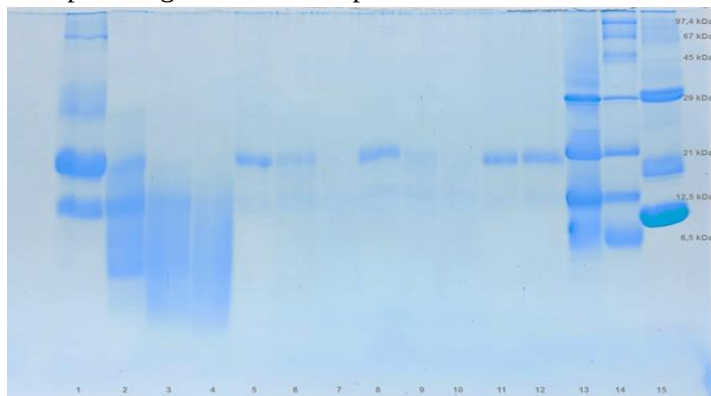


Figure 11. SDS-PAGE of whey protein hydrolysates before and after fermentation with probiotic strains.

1- Whey, 2- WP3, 3- WP7, 4- WP11, 5- WP3+*L. plantarum*, 6- WP7+ *L. plantarum*, 7- WP11+*L. plantarum*, 8- WP3+*L. rhamnosus*, 9-WP7+*L. rhamnosus*, 10- WP11+*L. rhamnosus*, 11- Whey + *L. plantarum*, 12- Whey + *L. rhamnosus*, 13- Protein marker (protein test mixture 5 - SERVA 6.5 - 29.0 kDa), 14- Protein marker (protein test mixture 6 - SERVA 6.5 - 97.4 kDa), 15- BSA - serum albumin, α -casein, β -casein, α -lactalbumin, β -lactoglobulin.

The serum electrophorogram (lane 1) shows two intense bands corresponding to the main protein components - α -lactalbumin and β -lactoglobulin. A fainter band corresponding to serum albumin and traces of casein fractions are also noted. In the variants treated with a different concentration of papain enzyme (WP3, WP7 and WP11), a tendency of hydrolytic degradation of the main whey protein fractions was observed, which increased with the concentration of the enzyme.

In the samples fermented with the two probiotic strains, we can note that where a more complete degradation of the protein components was achieved, the lines are of minimal intensity. The intensity of the protein fractions during fermentation of the hydrolysis products obtained after treatment with 0.1 mg/ml papain decreased to a lesser extent.

5. Formulation of a recipe composition and preparation of a technological scheme for the production of bioactive products from hydrolyzed protein

From the conducted experiments and the results obtained from them for inclusion in the composition of the functional products, the following enzymatic hydrolysis were determined:

- Lyophilized hydrolyzate of fresh milk with probiotic strains *L. plantarum* and *L. rhamnosus*, obtained by enzymatic treatment with bromelain at a concentration of 0.1 mg/ml for 30 minutes (MB3)
- Lyophilized whey hydrolyzate with probiotic strains *L. plantarum* and *L. rhamnosus*, obtained by enzymatic treatment with papain at a concentration of 0.5 mg/ml for 30 minutes (WP7)

Table 18 presents the recipe composition of the developed products.

Table 18. Product 1 and 2 with enzymatic hydrolysates of milk and whey fermented with probiotic strains

Composition	Quantity in 100g/ g	Composition
Lyophilized fresh milk hydrolyzate with probiotic strains <i>Lactiplantibacillus plantarum</i> subsp. <i>plantarum</i> NBIMCC 3447 and <i>Lacticaseibacillus rhamnosus</i> NBIMCC 1013	60	Lyophilized whey hydrolyzate with probiotic strains <i>Lactiplantibacillus plantarum</i> subsp. <i>plantarum</i> NBIMCC 3447 and <i>Lacticaseibacillus rhamnosus</i> NBIMCC 1013
Freeze-dried mango fruit	10	Lyophilized raspberry fruit
Fructooligosaccharides	10	Fructooligosaccharides
Xylitol	9,4	Xylitol
Mannitol	5	Mannitol
Fucose	3	Fucose
Microcrystalline cellulose	2	Microcrystalline cellulose
Locust bean gum	0,6	Locust bean gum

6. Study of the quality characteristics of the obtained products - organoleptic, physicochemical, biochemical and microbiological indicators, with a view to determining their health benefits.

6.1 Organoleptic assessment

A 9-point Hedonic scale was used to study the organoleptic indicators - appearance, consistency, color, taste and aroma. The results are presented in table 19.

Table 19. Organoleptic evaluation of lyophilized synbiotic foods

Product	Appearance	Consistency	Aroma	Taste	Overall evaluation
1. Freeze-dried product based on fresh cow's milk hydrolyzate with 10% added fruit - freeze-dried mango	Lyophilic product with a yellow color	Fine, powdery	Pleasant, composition specific	Pleasant, lactic acid, with a taste of mango fruit	8.9
evaluation	9.0	9.0	88	8.9	
2. Lyophilized product based on whey hydrolyzate with 10% added fruit - lyophilized raspberry	Lyophilic product with a pale pink color	Fine, powdery	Pleasant, composition specific	Pleasant, lactic acid, with a hint of raspberry	8.9
evaluation	9.0	9.0	89	8.7	



Figure 12. Lyophilized product 1



Figure 13. Lyophilized product 2

6.2 Physicochemical indicators

The main physicochemical parameters of the lyophilized product 1 (based on milk hydrolyzate) and product 2 (based on whey hydrolyzate) are presented in table 20.

Table 20. Physicochemical indicators of lyophilized products ($\bar{x} \pm s_d$)

Indicators	Values by analysis		Research methods and standards
	Product 1 based on milk hydrolyzate	Product 2 based on whey hydrolyzate	
Water content, %	2,12 $\pm$ 0,02	3,32 $\pm$ 0,01	wet balance „Sartorius”
Dry substance, %	97,88 $\pm$ 0,04	96,68 $\pm$ 0,01	
Total protein, %	21,37 $\pm$ 0,01	6,01 $\pm$ 0,03	Kjeldahl method (BDS 6231)
Fibers, %	11,87 $\pm$ 0,03	15,26 $\pm$ 0,03	
Total lipids, %	12,22 $\pm$ 0,07	5,80 $\pm$ 0,02	apparatus “Soxtex” (БДЦ 1671 -89),
General ash, %	2,32 $\pm$ 0,10	3,11 $\pm$ 0,03	weight method BDS 6191-74
Carbohydrates (Inc. reducing sugars), %	35,40 $\pm$ 0,01	51,8 $\pm$ 0,01	BDS 9373-80
Polyols, %	14,7 $\pm$ 0,01	14,7 $\pm$ 0,01	by product composition
Active acidity - pH	3,87 $\pm$ 0,02	3,92 $\pm$ 0,03	potentiometric, pH-meter “Hanna”
Titrateable acidity, °T	71,5 $\pm$ 0,04	72,5 $\pm$ 0,01	BDS 1111- 80
Vitamin C, %	3,45 $\pm$ 0,2	4,26 $\pm$ 0,04	titrimetrically
Energy value / 100 g product, (kcal/ kJ /)	396,07/1683,31	349,24/1484,27	by chemical composition

The water content is below 5%, which is an important indicator of successful lyophilisation.

The total protein content is relatively low, since the starting raw materials themselves are not high in protein and they were additionally subjected to enzymatic hydrolysis.

Its quantitative values of total fat are relatively low - 12.22% for the milk-based product and 5.80% for the product with included whey hydrolyzate.

Carbohydrates, in this amount reducing sugars and fibers have increased values, which is due to the added additional ingredients in the recipe formulas of the two products.

6.3 Biochemical indicators

The analyzed functional foods have a high content of potassium from the macro elements. And of the trace elements, an increased content of manganese, iron and zinc is reported (table 21).

Table 21. Micro and micro element composition of lyophilized fresh cow's milk and whey and lyophilized products from their hydrolysates

Research A sample	Macro elements, g/kg				Micro elements, mg/kg			
	Ca	P	Mg	K	Mn	Fe	Cu	Zn
Functional product based on milk hydrolyzate	1,28	1,51	0,24	6,42	21,80	8,45	0,76	9,87
Functional product based on whey hydrolyzate	0,76	1,85	0,19	5,90	13,89	8,36	0,78	8,36

Table 22. Fatty acid groups product 1 (g/100g fat)

Fatty acid groups	Control milk	Milk hydrolyzate with <i>L. plantarum</i>	Milk hydrolyzate with <i>L. rhamnosus</i>	Functional product based on milk hydrolyzate
SFA	66,39	68,08	65,48	66,96
MUFA	29,22	23,75	30,12	29,16
PUFA	4,43	6,40	4,55	4,14
$\Sigma n-3$	0,75	1,44	0,72	0,80
$\Sigma n-6$	3,52	4,75	3,63	3,01
$\Sigma n-6/\Sigma n-3$	4,69	3,31	5,05	3,75

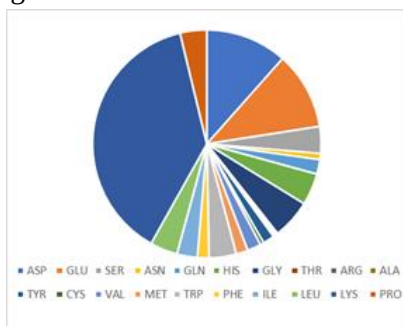
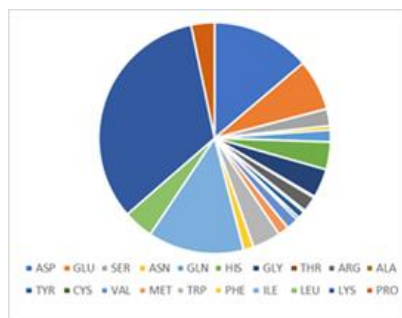
Table 23. Fatty acid groups product 2 (g/100g fat)

Fatty acid groups	Control whey	Whey hydrolyzate with <i>L. plantarum</i>	Whey hydrolyzate with <i>L. rhamnosus</i>	Functional product based on whey hydrolyzate
SFA	57,47	59,19	58,51	58,59
MUFA	35,59	35,01	35,20	35,55
PUFA	6,59	6,05	6,07	6,26
$\Sigma n-3$	0,90	0,87	0,86	0,89
$\Sigma n-6$	4,73	4,23	4,45	4,63
$\Sigma n-6/\Sigma n-3$	5,24	4,89	5,17	5,20

From the fatty acid analysis, it is reported that the amounts of the main fatty acid groups unsaturated, polyunsaturated and saturated fatty acids remain unchanged in the final products compared to the starting raw materials.

6.4 Amino acid profile

The amino acid profiles of the new functional products were determined by HPLC liquid chromatography. The results are presented in figures 14 and 15.

**Figure 14.** Amino acid profile of product 1**Figure 15.** Amino acid profile of product 2

The starting raw materials - milk and whey contain a more pronounced amount of lysine (14.97 $\mu\text{M/ml}$), proline (5.96 $\mu\text{M/ml}$), aspartic acid, glutamic acid and arginine.

After carrying out enzymatic hydrolysis, the number of free amino acids increases and some of the essential amino acids for humans are released - valine, tryptophan, phenylalanine, isoleucine and leucine. The fermentation process carried out with the probiotic strains *L. plantarum* and *L. rhamnosus* leads to the additional release and accumulation of amino acids, such as asparagine, glycine, alanine, tryptophan, phenylalanine and lysine. In both the milk and whey variants, we see a complete amino acid spectrum.

6.5 Microbiological determination of the number of viable cells of the probiotic bacteria and microbial seeding of the new products

The survival of *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 in the final products over a period of 1 year at three storage temperature regimes (table 24).

Table 24. Viability of probiotic strains in the new functional products during storage (CFU/ml).

Term, days	Survivability, CFU/ml					
	Product 1			Product 2		
	A room T, °C	4°C	-18°C	A room T, °C	4°C	-18°C
1 day		2,5x10 ⁹			2,0x10 ⁹	
30 day	6,4x10 ⁷	2,2x10 ⁹	2,5x10 ⁹	5,6x10 ⁷	6,1x10 ⁸	1,0x10 ⁹
60 day	1,5x10 ⁷	1,2x10 ⁹	2,2x10 ⁹	1,0x10 ⁷	2,5x10 ⁸	7,6x10 ⁸
3 month / 90 day	1,8x10 ⁶	8,7x10 ⁸	1,4x10 ⁹	1,5x10 ⁶	1,2x10 ⁸	6,8x10 ⁸
120 day	1,2x10 ⁵	3,2x10 ⁸	9,3x10 ⁸	1,0x10 ⁵	1,0x10 ⁷	2,3x10 ⁸
150 day		8,5x10 ⁷	5,8x10 ⁸		2,2x10 ⁶	7,9x10 ⁷
6 month / 180 day		1,2x10 ⁶	2,8x10 ⁸		1,2x10 ⁵	2,2x10 ⁷
210 day		2,3x10 ⁵	7,1x10 ⁷			7,9x10 ⁶

240 day			$2,4 \times 10^7$			1×10^6
9 month / 270 day			$9,4 \times 10^6$			$4,4 \times 10^5$
300 day			$2,6 \times 10^6$			
330 day			$8,5 \times 10^5$			
12 month / 360ден						

From the data presented, it can be seen that the survival of the probiotic cells in the products stored at room temperature drops sharply, and in both products after the third month the cells are below 1.0×10^6 .

After this period, the products lose the name "probiotic product" by definition, because the number of viable cells in them is less than 10^6 degrees. Storing the products under refrigerated conditions preserves the viability of the probiotic cells until the end of the 6th month in product 1 and up to the 5th month in product 2.

Storage at -18°C maintains high survival of the strains in product 1 - up to the tenth month, and for product 2 it is up to the eighth month.

Table 25 shows the indicators of microbial contamination of the new products after the lyophilization process.

Table 25. Values of microbial insemination.

Indicators	Product 1	Product 2
Total number of mesophilic aerobic and facultative anaerobic microorganisms, CFU/g	$3,6 \times 10^2$	$2,2 \times 10^2$
Spores of microscopic moulds and yeasts, CFU/g	$2,1 \times 10^1$	$1,8 \times 10^1$
<i>Escherichia coli</i> в 0.1 g of the product	of are established	of are established
Coagulase-positive staphylococci in 1.0 g of the product	of are established	of are established

The survival of pathogenic microflora after lyophilization is

influenced by the residual moisture content of the products, temperature parameters and storage conditions.

The studies of the developed products were carried out according to standard microbiological methods. The indicators of microbial insemiation are in accordance with the norms defined by the standard for foods containing lactic acid bacteria and prove the absence of pathogenic microflora.

VI. MAIN CONCLUSIONS

- The influence of the technological parameters - type, concentration and time of action of the proteolytic enzymes - papain, bromelain and chymosin on the degree of hydrolysis of fresh cow's milk and whey was determined and the antioxidant activity of the obtained hydrolysates was determined.
- Degradation of the main protein components of the starting substrates to low-molecular peptides after hydrolysis has been electrophoretically proven.
- The degree of hydrolysis was determined as a percentage of hydrolyzed peptide bonds to quantify the process. The lowest degree of hydrolysis was reported for fresh milk MP1 (10 minutes of treatment with 0.1 mg/ml papain), and the highest percentage was obtained from bromelain with a high concentration in hydrolyzate SP12, the 60th minute of treatment. For the serum samples, the hydrolysis percentage for sample CX1 (chymosin 1 μ l/ml at 10 min reaction time) was only 13.18%. The highest percentage was achieved with SP12 hydrolyzate using papain at 1mg/ml.
- The antioxidant capacity values of the hydrolyzed products, which show higher activity compared to the original substrate, were determined. The highest activity of 11.32 mg TE/100 ml was found in variant MB3 (milk hydrolysed with bromelain at a concentration of

0.1 mg/ml for 30 minutes) In the variants treated with a higher concentration of enzyme (1 mg/ml) the value of antioxidant capacity decreases. The variants hydrolyzed with the papain enzyme show their strongest antioxidant capacity at 30 minutes from the start of hydrolysis.

- Fermentation experiments were performed with selected probiotic strains of lactic acid bacteria: *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447, *Lactobacillus gasseri* NBIMCC 2450 and *Lacticaseibacillus rhamnosus* NBIMCC 1013, which show that the most favorable for the reproduction of probiotic bacteria are the media variants MB3 - hydrolyzed milk with bromelain with an enzyme concentration of 0.1mg/ml and WP7 - hydrolyzed whey with papain with an enzyme concentration of 0.5mg/ml.
- We found the highest number of viable cells in *Lactiplantibacillus plantarum* NBIMCC 3447 at a cultivation temperature of 37°C - log 10.20 in milk hydrolyzate and log 9.48 - in whey, *Lacticaseibacillus rhamnosus* NBIMCC 1013 - log 9.91 in milk hydrolyzate and log 9.32 – in whey and *Lactobacillus gasseri* NBIMCC 2450 - log 9.25 in milk hydrolyzate and log 9.20 - in whey.
- The optimal parameters of the freeze-drying process were determined, and it was found that the use of cryoprotectants increased the survival of bacterial cells of *Lactiplantibacillus plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 in milk and whey samples after freezing and after lyophilization.
- The higher cryoprotective effect of locust bean gum (cryoprotectant 2) compared to the combination of sucrose and pectin (cryoprotectant 1) on bacterial cell survival was demonstrated.
- A technology has been created for the production of biologically active products based on hydrolyzates of fresh milk and whey, fermented with the selected strains of lactic acid bacteria.
- The formulated multicomponent composition of the developed functional products includes enzymatically derived protein

hydrolysates enriched with proven health-promoting probiotic strains *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 and physiologically active substances from different origins.

- The summarized results of the conducted organoleptic, physicochemical and biochemical studies prove that the new biologically active products have a balanced composition and high biological value.

VII. CONTRIBUTIONS

Scientific contributions

- It has been experimentally proven that enzymatic hydrolysis of fresh cow's milk and whey leads to an increase in their antioxidant activity.
- Fermentation of selected milk and whey hydrolysates with the probiotic bacteria *L. plantarum* and *L. rhamnosus* was found to further increase their antioxidant activity.
- The high cryoprotective effect of locust bean gum (cryoprotector 2) on the survival of bacterial cells in fermented milk and whey hydrolysates after freezing and lyophilization has been proven.

Scientific - applied contributions

- The influence of the technological parameters - type, time of action and concentration of the proteolytic enzymes papain, bromelain and chymosin on the degree of hydrolysis of two protein substrates - fresh cow's milk and whey - was determined.
- The methods for enzymatic hydrolysis of casein and whey proteins in fresh cow's milk and whey have been optimized in order to obtain

bioactive hydrolysates and peptides.

- The effect of the addition of hydrolyzed protein substrates in the culture medium on the duration of the fermentation process and the survival of probiotic strains of lactic acid bacteria - *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447; *Lactobacillus gasseri* NBIMCC 2450 and *Lacticaseibacillus rhamnosus* NBIMCC 1013.
- It has been proven that the most favorable alternative breeding media for the strains of lactic acid bacteria: *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447, *Lactobacillus gasseri* NBIMCC 2450 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 are MB3 – hydrolyzed milk with bromelain with an enzyme concentration of 0.1mg/ml and WP7 – hydrolyzed whey with papain with an enzyme concentration of 0.5mg/ml.
- The main technological parameters of the freeze-drying process of the strains of lactic acid bacteria *Lactiplantibacillus plantarum* subsp. *plantarum* NBIMCC 3447 and *Lacticaseibacillus rhamnosus* NBIMCC 1013 in the milk and whey hydrolysates.

Applied Contributions

- The possibility of using fresh cow's milk and whey (a waste product from cheese production) as protein substrates for carrying out enzymatic hydrolysis and turning them into products with high added value has been proven.
- Formulations were formulated and technology was developed for the production of new functional foods based on milk hydrolysates.

DISSERTATION RELATED PUBLICATIONS

1. **Loginovska, K.**, 2021. Receiving of Protein Hydrolysates from Cow's Milk by Enzymatic Treatment, Journal of Mountain Agriculture on the Balkans, 24 (4), 10-22
2. **Loginovska, K.**, A. Valchkov, M. Doneva, P. Metodieva, I. Nacheva, 2023. Technology for Obtaining a Lyophilized Product Based on Milk Hydrolyzate, 2023, Journal of Mountain Agriculture on the Balkans, 26 (5), 47-65
3. Doneva, M., **K. Loginovska**, S. Dyankova, I. Nacheva, P. Metodieva, N. Ninova-Nikolova, 2024. Application of whey protein hydrolysates as a fermentation medium with strains of lactic acid bacteria, Journal of Chemical Technology and Metallurgy, 59, 1, 15-22