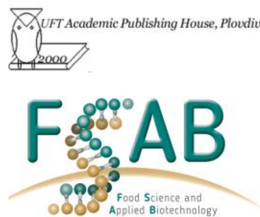

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Research Article

Products of metabolism and processing of lactic acid bacteria and bifidobacteria as functional ingredients

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Abstract

Lactic acid bacteria (LAB) and bifidobacteria (BB) are unique microorganisms that have a lot of biological and physiological effects. Structural components of LAB and BB – peptidoglycans, compounds of the muramylpeptide series, teichoic acids – have powerful immunological properties. Metabolites of LAB and BB – organic acids, hydrogen peroxide, bacteriocins, etc. – provide antagonistic activity, have an indirect impact on the immune system, reducing the antigenic load caused by pathogenic microorganisms. The expediency of peptidoglycans degradation of LAB and BB cell walls is substantiated. Low molecular weight products of the degradation can easily be absorbed and enter into biochemical processes, accelerating the expected functional-physiological effect. To obtain low-molecular products of peptidoglycans degradation, a combination of LAB and BB was used. The combination of LAB and BB is the sum of the test cultures of *Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Bifidobacterium bifidum, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Streptococcus thermophilus*. Destruction of peptidoglycans of bacterial cell walls was carried out using a combination disintegrating factors. The efficiency of destruction was determined by the accumulation of low molecular weight peptides (with molecular weight up to 1500 Da), amino acids and soluble protein in the disintegrate. It has been established that the highest accumulation of low molecular weight degradation products occurs when using autolysis followed by enzymatic hydrolysis during 180 min with the ratio of the enzyme: substrate 1:100. At the same time ≈ 53% of protein substances pass from insoluble to soluble state. The molecular weight of the obtained products is determined by the gel chromatography method. The qualitative and quantitative content of organic acids, amino acids and vitamins of group B in the hydrolysis products composition was investigated. It was shown that the obtained product possesses high biological effect in the experiment on animals.

Practical applications. The expediency of cell walls destruction of the bacterial composition for the purpose of obtaining of low molecular weight immunological compounds has been proved. The conditions of degradation of LAB and BB cells are investigated and products of their destruction are characterized. It is proved that as a result of enzymatic hydrolysis of autolysate of bacterial biomass on selected parameters, low molecular weight peptides with a molecular weight in the range of 294-650Da form, which corresponds to the molecular weight of the muramyldipeptide – a powerful immunological compound. The qualitative and quantitative content of organic acids, amino acids and vitamins of group B in the hydrolysate composition is investigated. Conducted biomedical researches confirm the safety and efficacy of the resulting product. It is proved that the received preparation contains a large number of biologically active substances and can be used as a functional and physiological food ingredient for the nutritional support of the population with suppressed state of immunity. The use of products of processing and metabolism of LAB and BB biomass as food immunological ingredients or dietary supplements is promising.

Keywords: muramyldipeptide, cell wall, lactic acid bacteria, bifidobacteria, autolysis, enzymatic hydrolysis, pancreatic, low molecular weight peptides

Abbreviations: LAB-lactic acid bacteria; BB-bifidobacteria; GRAS-generally recognized as safe; PG-peptidoglycans; MDP-muramyldipeptide; BM-biomass; LMWP-low molecular weight peptides; GMDP-glucosaminylmuramyl dipeptide; PAMP-pathogen-associated molecular patterns

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Introduction

An extremely pressing problem of the present day is an increase of infectious diseases cases among the population. The state of the population health of many countries in the world is significantly depressed under the influence of anthropogenic and social factors. The creation and implementation of effective preventive measures is necessary for the overcoming this global problem. At the same time, the nutritional support of population is very important, namely, the introduction of functional food in the diet containing the functional-physiological immunological ingredients that can increase the body's resistance to various diseases. It is promising and expedient to use, as immunological food ingredients, products of metabolism and processing of lactic acid bacteria (LAB) and bifidobacteria (BB) (Glushanova 2003; Molohova 2011). It is well known that the LAB and BB are an integral part of the functioning of the immune system of macroorganisms. Products of their vital functions – organic acids, hydrogen peroxide, bacteriocins and others – exhibit antagonistic activity, have indirectly impact on the immune system, reducing the antigenic load caused by pathogenic microorganisms (Cherno 2016; Stojanova 2012).

It is widely known, that most of the *Lactobacillus* and *Bifidobacterium* species show no pathogenicity or acute oral toxicity (Jia 2011; Denkova 2014). LAB and BB have the "GRAS" status (Generally Recognized as Safe) and considerable cultivation experience has been accumulated to obtain eubiotic preparations.

The structural components of the LAB and BB cell walls – peptidoglycans (PG), muramyldipeptide (MDP), teichoic acids – are objects for the recognition of the innate immunity system (Chapot-Chartier 2014). It is known, that peptides of the MDP series with a molecular weight of up to 1500 Da have high immunological activity (Gavrilin et al., 2007). Fragmented PG is transported inside the cells and is recognized by the cytoplasmic receptors (Nod 1 and Nod 2 proteins) (Harris 2008; Fournier 2005; Kawai et al. 2010). Once activated, these molecules trigger intracellular signalling pathways that lead to the activation of transcriptional responses culminating in the expression of a subset of inflammatory genes (Moreira 2012).

Thus, the signals for the start of the adaptive immune response are not living bacteria, but their fragments or products of vital functions, which reach the cells of the immune system, passing through the intestinal epithelium. In this regard, the direct partial destruction of bacterial cells is expedient in order to obtain biologically active substances, which are easier to digest and enter into biochemical processes, accelerating the expected immunological effect (Kapustian 2015).

The destruction of microorganisms cell walls is carried out using physical, chemical or combined techniques of influence (Telishevskaya 2000; Shaphaev 2015)

As a rule, physical disintegration of microbial cells leads to irreversible violation of their anatomical integrity (Zhou et al. 2010). In order to produce the low molecular weight glycopeptide products of regular structure, chemical and enzymatic methods of degradation are usually used (Kapustian 2017). The enzymatic methods of the peptidoglycan hydrolysis are milder compared with chemical ones. Typically, enzymatic hydrolysis is carried out at physiological pH values of the medium and temperature corresponding to the maximum of the enzymes activity. Enzymes are specific catalysts, which, unlike acids and alkalis, act only on strictly defined groups of compounds and bonds. For the destruction of the bacterial cell walls peptidoglycans, it is advisable to use proteolytic enzymes that can break down peptide bonds in its structure.

Autolytic processes can lead to partial disintegration of the cell too (Humann et al. 2009). Most bacteria synthesize a group of enzymes known as autolysins that are capable of hydrolysing the peptidoglycan of their own cell wall. Autolysins are localized on the outer surface of the membrane, but in the logarithmic phase of growth, they are associated with the cell wall, and are released when the cells die.

The purpose of the work is obtaining and characterization of the functional-physiological immunotropic ingredients on the basis of products

of metabolism and processing of LAB and BB composition.

Materials and Methods

Materials. Composition of LAB and BB represents a sum of test cultures: *Lactobacillus acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus, Bifidobacterium bifidum, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Streptococcus thermophilus*, from the collection of Scientific and Production Enterprise "Ariadna" (Odessa) with a concentration $5 \cdot 10^9$ cfu.cm^3. This bacterial composition has a commercial name "Bacterial starter cultures Symbinorm". The dry matter content of the bacterial composition was 7.12 ± 0.02%, total protein – 27.25 ± 0.04%. Enzymatic degradation biomass (BM) cell was performed by pancreatin treatment with a proteolytic activity of 370 UN (Ternopharm, Ternopil).

Analysis

Destruction of cells of bacterial composition. BM after exponential growth phase was maintained during 48 hours at 37°C for the autolysis processes. After that, the biomass with the culture liquid was heated to a temperature of 90°C and kept for 15 min, then the mixture was cooled and enzymatic hydrolysis was carried out. The constant parameters of hydrolysis were temperature 37°C and pH=7.4. The ratio of the enzyme to the substrate (dry matter content of BM) was varied in the range from 1:20 to 1:300 and the duration of the incubation of the reaction mixture was varied in the range 10-300 min. In the obtained hydrolysates, the content of free amino acids was monitored by the method of formalin titration (Semak et al. 2007), soluble protein – by the Benedict method (Semak et al. 2007), low molecular weight peptides (LMWP) – by Benedict's method after precipitation of high molecular proteins by 10% solution of trichloroacetic acid (Gavrilin et al. 2007). Enzymatic hydrolysis was stopped by heating at the temperature 100°C, the mixture was cooled, centrifuged for 10 min at 8000 min^{-1}, decanted, further lyophilic drying of supernatant containing low molecular weight soluble biological active substances carried out. As a control, enzymatic hydrolysis was carried out for the bacterial composition that was not incubated after exponential growth phase during 48 hours at 37°C. The molecular weight of the protein nature compounds of hydrolysate was determined by gel chromatography on column with sephadex G-15. Samples were prepared using ion exchange chromatography with cation exchanger KU-2 (column sizes: H=30cm, D=1.8cm). Received preparation contained amino acids and low molecular weight peptides and deprived of acids, neutral carbohydrates and salts. The column with sephadex G-15 (H=28cm, D=2.8cm, V=112cm^3) was calibrated with markers of known molecular weight, namely: I – GMDP (MW 650 Da), II – aspartame (L-Aspartil-L-phenylalanine, MW 294 Da), III – glycine (MW 75 Da). Qualitative and quantitative content of organic acids in the bacterial hydrolysate was determined by the method of capillary electrophoresis (device Capel 105/105M). To 0.5g of the preparation 50cm^3 of distilled water heated to 70°C were added. The mixture was stirred on a laboratory shaker for 10 min. After that, 1 cm^3 of filtrate was taken out, centrifuged and the determination of quantitative and qualitative content of organic acids was carried out. Detection was performed at wavelengths of 190 nm (Kotsyumbas et al. 2013). Qualitative and quantitative content of amino acids in the bacterial hydrolysate was determined by the method of capillary electrophoresis (device Capel 105/105M). To 0.1g of the drug was added 10cm^3 of hydrochloric acid, sealed and mixed. Hydrolysis was carried out at a temperature of 110°C for 16 hours. After completion of the hydrolysis, the mixture was cooled to room temperature and filtered. In glass vats, 0.05cm^3 of hydrolysate was collected and evaporated, after which 0.15cm^3 of sodium carbonate solution and 0.3cm^3 of phenyl isothiocyanate solution were added, thoroughly mixed, left for 35 minutes at room temperature and dried. The dry residue was dissolved in 0.5cm^3 of bidistilled water and used for investigation. The detection of amino acids was carried out at a wavelength of 254nm (Kotsyumbas et al. 2013). Qualitative and quantitative content of the vitamins

group B in the bacterial hydrolysate was determined by the method of capillary electrophoresis (device Capel 105/105M). To 0.2g of the preparation 5cm^3 of the working solution prepared in accordance with the guidelines were added. The mixture was heated for 5 min at 100°C, cooled, filtered. After that vitamins at wavelengths of 200 and 267nm were detected (Kotsyumbas et al. 2013). The acute toxicity of the resulting hydrolysate was determined in two stages: indicative and expanded experiments. In the indicative experiment, the preparation was administered intragastrically at doses of 50, 500 and 5000mg.kg^{-1} body weight. For each dose, three white rats were used. In the expanded trial, the resulting drug was administered intragastrically at doses of 5000 and 10000mg.kg^{-1} body weight. Animals of control groups were administered physiological solution. Animals were observed for 14 days. The degree of toxicity of the drug was assessed by changing the general condition of the animals, behavioural reactions, reactions to external stimuli, mortality, changes in body mass dynamics of animals, and mass coefficients of internal organs. The effective dose of the resulting hydrolysate was determined on white non-breeding rats aged 2-3 months, with a body weight of 160-180g, which were kept on a standard vivarium diet. On the principle of analogues five groups of animals were formed. There were 20 rats in each group. Animals of the control group were administered physiological solution. Animals of the first group received a dose of 5μg·kg^{-1}, the second group – 10μg·kg^{-1}, the third – 12.5μg·kg^{-1}, and the fourth – 25μg·kg^{-1}. The preparation was administered with a gastric probe for 14 days on an empty stomach. On the seventh and fourteenth days of the administration of the drug, as well as after discontinuation of the 21st and 28th day of the experiment, the animals were decapitated under anaesthesia and blood samples were taken for research. The concentration of haemoglobin, the number of red blood cells and leukocytes, the concentration of total protein and its fractions were determined in the blood.

Results and Discussion

Destruction of cells of LAB and BB composition. In order to establish the destruction parameters of the cell walls of the LAB and BB composition, in which the maximum accumulation of LMWP with a molecular weight <1500Da occurs, a series of experiments were carried out, in which the concentration of pancreatin in the reaction mixture and the time of enzymatic hydrolysis was varied. The research results are shown in Fig. 1a,b and 2.

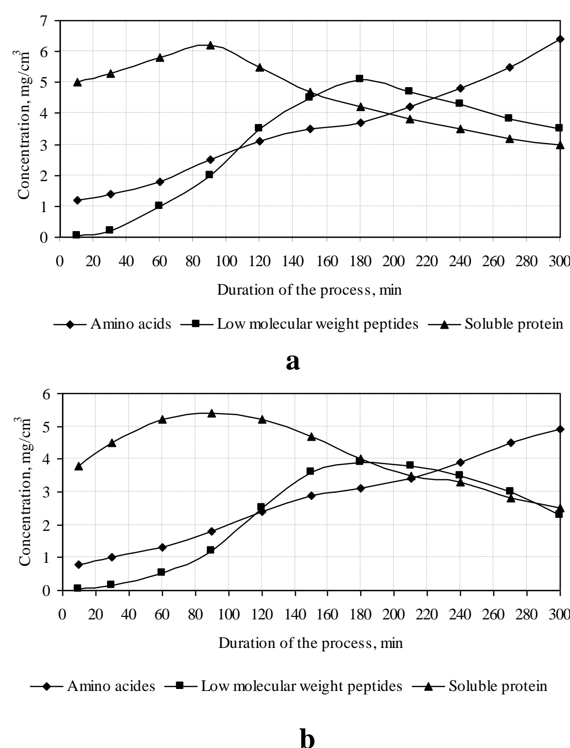

Figure 1. Dependence of the degradation products accumulation on the duration of the process of enzymatic hydrolysis of the bacterial composition (with a ratio of enzyme: substrate 1:100)
a) using autolysis; b) without autolysis

At the first stage it was necessary to determine the influence of the duration of enzymatic destruction of the cell walls of the bacterial composition on the accumulation of hydrolysis products in the reaction medium. The ratio of enzyme: substrate was chosen arbitrarily 1:100. Analysing the results of the studies presented in the graphical dependence of Fig. 1a, we can state that the accumulation of

amino acids in the hydrolysate during 10 to 300 min is almost linear. Accumulation of soluble protein in the hydrolysate during the first 90 min increases from 5 to 6.2mg.cm^{-3}, after which its amount gradually decreases. Some increase in the amount of soluble protein under the action of the proteolytic enzyme can be explained by the beginning of destruction of the cell walls of the bacterial composition and the release of intracellular protein compounds. A graphic representation of the LMWP accumulation in the hydrolysate is parabolic with a maximum at its peak, which corresponds to a LMWP concentration 5.1mg.cm^{-3}. As the process progresses, the amount of LMWP in the hydrolysate decreases, which is apparently due to the destruction of peptide bonds in their structure under the influence of pancreatin. As for the control samples, the regularity of the accumulation of the hydrolysis products in the reaction medium is similar to the hydrolysis of the bacterial composition using autolysis. The efficiency of hydrolysis of control samples, which is expressed in the amount of hydrolysis products, is inferior to the experimental samples. The maximum accumulation of soluble protein in the hydrolysate of the control sample is 5.4mg.cm^{-3}. This is 13% less compared to the test sample. The maximum accumulation of LMWP in the hydrolysate of the control sample is 3.9mg.cm^{-3}, which is 24% less compared to the test sample. At the next stage of the study it was necessary to determine the influence of the enzyme:substrate ratio on the accumulation of target LMWP in the hydrolysate reaction medium.

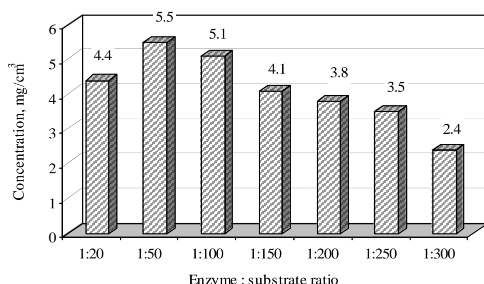

Figure 2. Dependence of the accumulation of LMWP in hydrolysate medium on the enzyme: substrate ratio (using autolysis; duration of enzymatic hydrolysis180 min)

The enzymatic hydrolysis was carried out according to the parameters shown in Fig. 1a, at which the maximum accumulation of LMWP was observed. Thus, the duration of the process was 180 min. The diagram depicted in Fig. 2 demonstrates that the maximum LMWP accumulation in the BM hydrolysate occurs at the ratio of the enzyme: substrate 1:50 and is 5.5mg.cm^{-3}. With a decrease of the pancreatin concentration in the reaction mixture by 2 times (the ratio of enzyme: substrate 1: 100), the amount of LMWP decreases insignificant and is 5.1mg.cm^{-3}. The molecular weight of the low molecular compounds of the protein nature of the product of BM destruction was investigated by the gel chromatography method (Fig. 3a). There are two pronounced peaks on the 36th, 50thcm^{3} elute on the gel chromatographic curve of the low molecular weight fraction of the hydrolysate (Fig. 3b). Peaks correspond to the markers with molecular weights in the range 294 - 650 Da.

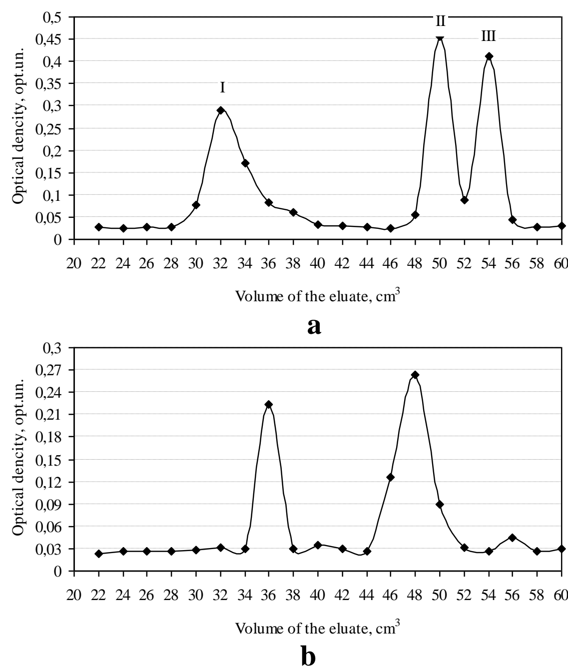

Figure 3. Molecular weight distribution of low molecular compounds of protein nature:
a) Gel chromatography with markers: I – GMDP (MW 650 Da), II – aspartame (L-Aspartil-L-phenylalanine, MW 294 Da), III – glycine (MW 75 Da);
b) Gel chromatography of LMWP

Determination of the qualitative and quantitative organic acids content. It is established that a number of organic acids were in the composition of BM hydrolysate of lactic acid bacteria. Quantitative content of the following organic acids was deter-mined. There are oxalic acid ($1.6 mg.cm^3$), lemon acid ($22.1 mg.cm^{-3}$), acetic acid ($575.8 mg.cm^{-3}$), dairy acid ($236.3 mg.cm^{-3}$), benzoic acid ($1.5 mg. cm^{-3}$), sorbic acid ($5.3 mg.cm^3$).

Content of amino acids and vitamins of group B. In order to predict the physiological and functional properties of bacterial hydrolysate as perspective nutritional ingredients, the qualitative and quantitative content of amino acids and vitamins of group B in their composition has been investigated (Tables 1 and 2).

Table 1. The content of essential amino acids in the hydrolysate of bacterial composition

Amino acids	Concentration in hydrolysate, $mg \cdot g^{-1}$	Daily need, mg	Providing daily need, %
Lysine	4.62	55	8.36
Phenylalanine and tyrosine	2.54	60	4.16
Leucine and isoleucine	7.51	110	6.81
Methionine	0.36	35	0.85
Valine	3.92	50	7.80
Threonine	3.93	40	9.75

The results of the studies allow us to ascertain the presence of seven essential amino acids in the hydrolysate. Methionine is the limiting amino acid, its content in the hydrolysate is $0.3 mg.g^{-1}$, which provides only 0.85% of the daily human need. The highest content is characterized by the amino acid lysine, its concentration in the hydrolysate is $4.6 mg.g^{-1}$, which provides 8.36% of the daily human need. The results of studies on the presence and content of vitamins of group B prove that the composition of the obtained product contains a sufficiently high number of vitamins in this group, in particular, riboflavin (22.62%). The role of B vitamins in the life of an organism is difficult to overestimate, because they perform a number of extremely important functions.

The acute toxicity determination (unpublished data). Due to the fact that the mid-lethal dose (DL50) at the intra-gastro-intestinal and subcutaneous methods of administration was not possible to establish, the drug was administered at maximum doses. As a result of the conducted research, it was founded that there were no deaths and diseases of laboratory rats at single-dose of 5000 and $10000 mg.kg^{-1}$ for gastric administration and for subcutaneous administration at doses of 1500, 3000 and $5000 mg.kg^{-1}$, clinical symptoms of poisoning and deviations in the behaviour of animals were not founded too. Besides, changes in weight ratios of the mass of internal organs are not established.

Table 2. The content of vitamins in the hydrolysate of bacterial composition

Vitamins	Concentration in hydrolysate, $mg.g^{-1}$	Daily need, mg	Providing daily need, %
B_1(thiamine chloride)	0.08	1.50	5.80
B_2 (riboflavin)	0.40	1.80	22.61
B_3 (calcium pantothenate)	0.63	5.00	1.27
B_5 (nicotinic acid)	0.67	12.00	0.50
B_6 (pyridoxine hydrochloride)	0.13	2.00	6.90
B_9 (folic acid)	0.11	0.40	26.50

The effective dose determination (unpublished data). Positive changes in haematological parameters of blood have been observed in the application of the preparation, in particular, in laboratory animals in experimental groups, an increase of haemoglobin concentration, the number of leukocytes and erythrocytes was founded, compared with the animals of the control group. At the concentration determining of the total protein and its fractions in blood serum, the increase in total protein level, the number of albumin and the concentration of γ-globulins in the animals of all experimental groups was determined, that indicated activation of the liver's protein synthesis function. A pronounced pharmacological effect was observed among all experimental groups of animals receiving a preparation at a dose of

12.5mg.kg^{-1} body weight. LAB and BB are gram-positive bacteria containing up to 70% peptidoglycan. This biopolymer is main barrier to bacterial cells destruction, since it is multi-layer and contains many specific bonds. In this regard, a combined method of disintegration of the bacterial composition cell walls was used in this study. This method consists in enzymatic hydrolysis of biomass, which was previously subjected to autolysis. It is widely known, that the LAB and BB are producers of bacteriocins, which can lead to autolysis and degradation of their own cells. This is rather not desirable process for the production of eubiotic preparations, but for targeted cell lysis in order to obtain immunological fragments of the peptidoglycan cell wall of bacteria this is very justified. Autolysis processes certainly do not provide complete destruction of cellular structures, but they can lead to disruption of integrity, cell perforation, partial destruction of the cell. This will significantly intensify enzymatic hydrolysis. The conducted researches have confirmed this judgment. The content of degradation products in the hydrolysate of the bacterial composition that was subjected to autolysis is much higher. And this means that the autolysines of the LAB and BB composition contributed to the primary destruction of cells. This, in turn, increased the efficiency of the complex enzyme preparation of pancreatin, since the outer cell protective membrane was obviously violated. This allowed to increase the probability of interaction of the active centers of the proteolytic component of pancreatin with peptide bonds in the peptidoglycan structure. The study of enzymatic degradation of the autolysate of the BM has shown that the most rational hydrolysis parameters are the enzyme: substrate 1: 100 ratio and the incubation time of 180 min. At the same time, in the mixture, the highest content of LMWP are accumulated (5.1m.cm^{-3}), which possesses high biological activity and immunological properties (Fig. 1). An increase of the enzyme concentration in the reaction mixture does not lead to a significant increase in the amount of LMWP in the hydrolysate. Thus, with the ratio of the enzyme: substrate 1: 100 and incubation duration 180 min, the concentration of peptides reaches 5.5mg.cm^{-3} (Fig. 2). Therefore, from an economic point of view, it is more expedient to use the ratio of enzyme: substrate 1: 100. The efficiency of bacterial cell destruction can be clearly demonstrated by pointing to changes in the ratio of soluble and insoluble protein substances in the reaction mixture before and after enzymatic hydrolysis. Insoluble protein is a peptidoglycan protein of cell walls of bacteria, which is transformed during enzymatic hydrolysis. Thus, in the enzymatic degradation of autolysate of the bacterial composition containing 27.25% of the total protein from which 6.25% is soluble, about 53% of protein substances (the sum of proteins, peptides, amino acids) are transformed from the insoluble in the soluble state. After achieving the main objective of the study, that is, destroying the cells of the bacterial composition and obtaining low molecular weight hydrolysis products, it was necessary to establish a molecular weight distribution of LMWP in order to detect peptides corresponding to the molecular weight of the MDP. It is now established that the MDP has all the necessary properties for the pathogen-associated molecular patterns (Pathogen-Associated Molecular Patterns, PAMPs), expressed in the stimulation of innate immunity and the ability to form a protection against microbial pathogens in vertebrates (Traub 2006). Exactly MDP initiates a signal of cascade reactions that leads to the synthesis of pro-inflammatory cytokines by immunocompetent cells and activation of immunological defence mechanisms of the organism (Matsui et al. 2014). The results of the studies allow us to ascertain that group of peptides with a molecular weight in the range corresponding to the molecular mass of the MDP are presented in the obtained hydrolysate (Fig. 3). The study of the qualitative and quantitative composition of organic acids has proved their presence in a sufficiently high amount. This allows us to predict, that the composition of organic acids in the resulting preparation will exhibit antagonistic properties against pathogens. The presence of essential components in the obtained preparation as essential amino acids and vitamins B significantly increase its biological activity. This can attributed the obtained preparation to the category of multifunctional. Investigation of the

toxicity of this preparation in experiments on animals allows to classify it to 4 class of chemicals on the level of danger (low toxicity substances).

Conclusions

The efficiency of combining the processes of autolysis and enzymatic hydrolysis of the LAB and BB composition with the aim of destroying their cell walls was proved. It was found that the enzymatic degradation of biomass, which was not subjected to autolysis, the concentration of hydrolysis products is lower. In particular, the content of target peptides is lower by 24%, amino acids by 23%. The highest accumulation of low molecular weight degradation products occurs after autolysis with the following enzymatic hydrolysis during 180 min with the ratio of the enzyme: substrate 1: 100. At that parameters of enzymatic hydrolysis, the maximum accumulation of LMWP with immunological activity takes place. It is noted that approximately 53% bacterial peptidoglycan of cell walls transforms into soluble products of the hydrolysate during enzymatic hydrolysis. The presence in the hydrolysate obtained from the BM of the LAB composition LMWP, of amino acids, organic acids, vitamins allows to attribute this product to the category of immunological polyfunctional ingredient, the effectiveness of which is confirmed by medical biological studies with the definition of the parameters of acute toxicity and effective dose.

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