Вивчали протеолітичну, α-галактозидазну і β-глюкозидазну активності консорціумів Lactobacillus acidophilus 317/402 з Bifidobacterium longum-Я3 і Bifidobacterium adolescentis-С52 в процесі ферментації соєвого молока. Встановили, що досліджувані культури мають активні ферментні апарати і допомагають мінімізувати чинники, що обмежують використання соєвого молока для виробництва функціональних продуктів харчування – присутність олігосахаридів, що не перетравлюються, і бобового смаку. За протеолітичною активністю, яка становила 30 mU через 6 годин ферментації, лідирував консорціум Lactobacillus acidophilus 317/402 з Bifidobacterium adolescentis-С52. Найбільшу α-галактозидазну і β-глюкозидазну активності – 98 U/mg і 81 U/mg відповідно – проявляв консорціум Lactobacillus acidophilus 317/402 з Bifidobacterium longum-Я3. При цьому кількість даідзину, гліцитину і геністину знижувалася відповідно на 93 %, 75 % і 99,6 %, а кількість відповідних агліконів зростала на 278 %, 153 % і 338 %. Тоді як зазначені ферментні активності Lactobacillus acidophilus 317/402 з Bifidobacterium adolescentis-С52 не перевищували 78 і 75 U/mg відповідно, та процеси біотрансформації ізофлавонів йшли менш інтенсивно. Показано, що в соєвому молоці формуються певні симбіотичні взаємини між обраними штамами біфідобактерій та ацидофільною паличкою, що дає можливість отримувати високі титри пробіотичних культур в готовому продукті, причому з перевагою біфідобактерій. Через 9 годин ферментації середня кількість клітин біфідобактерій і лактобацил по обох консорціумах становила (0,9–2)·108 КУО/см3 и (0,8–4)·109 КУО/см3. Доведено підвищення пробіотичної та естрагенної активності ферментованих напоїв на основі сої при зниженні кількості галактоолігосахаридів в середньому на 50–70 %

Ключові слова: ферментація, соєве молоко, лактобацили, біфідобактерії, пробіотики, олігосахариди, ізофлавони, α-галактозидаза, β-глюкозидаза

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BIOTECHNOLOGICAL ASPECTS OF OBTAINING FERMENTED SOYBEAN PRODUCTS WITH INCREASED PHYTOESTROGENIC ACTIVITY

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1. Introduction

In recent years, increased attention has been paid to public health, improving the quality of life, promoting healthy lifestyles, as well as well-balanced diets. In turn, this stimulates the development of the concept of population health and prevention of aging of the body by incorporating functional foods into the diet, including those containing probiotic cultures [1–3].

A study of soybean composition shows that they are a rich source of biologically active substances, many of which are functional, thereby making soy processing products an important part of functional foods and nutraceuticals [4, 5]. In addition, soy products contain a small amount of lipids, especially saturated, they lack cholesterol, they have low energy value, that is, they can be used in dietary nutrition. An alternative solution is to use such products for people with lactase deficiency and intolerance to milk protein. Phytocomponents of soybeans could make it possible to receive products with additional therapeutic and preventive properties.

Soy products attract special attention not only as sources of critical nutrients but also as active carriers of probiotic microorganisms, prebiotics, and synbiotics. The fermentation of soy extracts (soy milk) with Lactobacillus and other beneficial microorganisms makes it possible not only to improve their nutrient component but also to strengthen it, enriching with biologically active compounds.

The mechanism of therapeutic and preventive action of probiotics is multifaceted and is predetermined not only by the high content of viable cells, the accumulation of their extracellular metabolites, which enhances the probiotic effect, but also other positive effects on the health of consumers. Today we already know about the ability of probiotic

microorganisms to reduce low-density lipoprotein (LDL) cholesterol and symptoms of lactose intolerance, to increase the bioavailability of mineral elements (in particular, calcium, magnesium, zinc) and isoflavones. In addition, they can improve protein absorption, gut health, support the immune system, demonstrate antioxidant, anti-carcinogenic, and antihypertensive activity, etc. Clinical studies show that they promote the reproduction of useful microbiota while the metabolites produced in parallel reduce the risk of digestive and cardiovascular diseases, as well as some cancers [2, 5–8].

New experimental data highlight the potentially positive role of probiotics in multifactorial diseases, including those caused by an unbalanced diet and abnormalities in healthy lifestyles. Factors such as stress, overtime work, smoking, combined with high-calorie and low-nutrient diets can lead to weakening the body's natural defenses and to disturbances of homeostatic mechanisms. This contributes to the development of metabolic syndrome, which is a combination of risk factors for cardiovascular diseases, such as abdominal obesity, increased blood pressure, insulin resistance, chronic pro-inflammatory processes of low gradations, and prothrombotic changes [9].

Therefore, it is a relevant task, based on the interest of consumers, to study the creation of new functional fermented soy products, to study the biochemical processes occurring during fermentation by probiotic microorganisms, to investigate the composition and properties of finished products.

2. Literature review and problem statement

To date, many technologies have been developed to produce soy products, in particular, fermented [10]. However, soy-based products are generally consumed by the Asian population, and their introduction to the European market is limited by several factors – the bean taste and intestinal dysfunction associated with the presence of indigestible oligosaccharides. This problem can be solved by using soy extract, fermented by lactic acid bacteria, which synthesize a wide range of enzymes, including α-galactosidase [11, 12]. At the same time, it is important to monitor the strains of lactic acid cultures for the ability to develop on soy milk, giving the finished product the necessary organoleptic characteristics, to accumulate a sufficient amount of lactic acid and synthesize α-galactosidase.

In the last few decades, many studies have been conducted around the world on soy milk fermentation using various strains of *Lactobacillus*, which are known to actively synthesize α-galactosidase. The fermentation processes of soy milk derived from different varieties of soybeans by the cultures of *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus cremoris, and Lactobacillus casei* were investigated. It was established that all cultures reached 10^8 CFU/cm^3 after 24 hours of fermentation at 30 °C, which is enough for a probiotic effect. In addition, all cultures have shown good results in reducing the number of soy galactooligosaccharides, but only *L. reuteri, L. brevis, and L. plantarum* have caused a sufficient decrease in pH and an increase in viscosity characteristic of the fermented product [5, 13]. Scientists have investigated different strains of *Lactobacillus* for soy milk fermentation and obtained similar results. The cultures developed well on soybean milk and accumulated the biomass necessary for the probiotic effect, reducing the number of soy oligosaccharides by 30–90 % as a result of 24 hours of fermentation but did not produce the desired result in terms of the organoleptic indicators of the finished product [14, 15]. In addition, the duration of the fermentation process over 24 hours is significantly different from the classical technologies for the production of fermented products, the fermentation process of which is limited to 9–12 hours on average.

At present, the genus *Bifidobacterium* has become particularly popular in the manufacturing of probiotic products. Some strains of *Bifidobacterium* are well known as probiotics, which have a wide range of health-beneficial effects and play an important role in the microbial ecology of the human gut. Sucrose, raffinoses, stachyose, proteins, vitamins, and microelements contained in soy milk provide favorable conditions for the development of *Bifidobacterium*, so many authors use them to produce fermented soy products of the probiotic direction. Note that there is very little information in the literature about the properties of the *Bifidobacterium* α-galactosidase enzyme [16]. Typically, *Bifidobacterium* recycle glucose through the so-called "Bifidus pathway", which leads to high levels of acetic acid, which negatively affects the taste-flavor of the finished product. This is a major drawback of the use of these bacteria in the fermentation system. There is no doubt that the molar ratio of milk and acetic acids varies from species to species, even from strain to strain, and depends on fermentation conditions. Most often for soy milk fermentation, *Bifidobacterium* are used in a consortium with lactic acid bacteria. As a result, products are obtained with the number of lactic- and *Bifidobacterium* cells sufficient for the probiotic effect, with a reduced content of soy oligosaccharides; however, with not always satisfactory organoleptic indicators [17].

Some scientists in the process of soy milk fermentation, both with monocultures and the consortia of probiotic microorganisms, focus on the study of their proteolytic properties, the accumulation of biologically active peptides, and the increase in antioxidase activity [12, 18]. In addition, in the case of lactic acid bacteria, the authors report that an increase in the number of nitrogen sources leads to an increase in the concentration of lactic acid, hence the taste of the product should be better [19].

Isoflavones belong to phytoestrogens, have a stabilizing effect on the hormonal status of the human body, making up for the lack of sex hormones or contributing to their less production by competitive binding to cell receptors. They also prevent hormone-dependent breast and prostate cancer, prevent the onset of menopause symptoms, and premature aging, especially in post-menopausal women. In addition, they regulate the blood glucose content, prevent blood from entering cancer cells, thereby stopping their growth. They show chemoprotective properties, contribute to the preservation of bone tissue, reduce total cholesterol and LDL cholesterol, prevent the occurrence of cardiovascular diseases, etc. [20, 21]. Therefore, studies of the estrogenic activity of fermented soy products have become particularly popular in recent years. They study the bioconversion of isoflavones in the process of soy milk fermentation by lactic acid bacteria. They analyze the synthesis and activity of β-glucosidase of the studied cultures and, respectively, the hydrolysis of glucose conjugates of soybean isoflavones with the formation of their aglycone forms. Aglycones are biologically more accessible and absorbed by the human body faster and in large quantities [22–24].

Thus, it has been shown that the fermentation of soy extract by lactic acid bacteria contributes to the hydrolysis of soy oligosaccharides, due to the synthesis of the enzyme α-galactosidase, that is, eliminates bowel dysfunction when using such products. It also helps overcome the taste of beans. In addition, fermentation increases the digestibility of nutrients, in particular proteins and minerals, improves the bioavailability of isoflavones, activates the immune system, lowers cholesterol, inhibits the development of pathogens in the gut, etc. Thus, the fermented soy extract is a carrier of the probiotic, antioxidant, protea, β-glucosidase, and α-galactosidase activities [5, 25].

However, previous studies have lacked a comprehensive approach to examining all the metabolic changes that occur in the soy milk fermentation by probiotic microorganisms. Such an approach would minimize the limits of soy consumption, while increasing the bioavailability and, therefore, assimilation of all biologically active components of the finished product. All this suggests that research into the conversion of the soy milk, fermented by probiotic microorganisms, into a multi-component biologically active functional food system, which has a comprehensive positive impact on human health, is appropriate.

3. The aim and objectives of the study

The aim of this study is to obtain a soy fermented functional drink with increased probiotic and estrogenic activity.

To accomplish the aim, the following tasks have been set:

– to study the fermentation process of soy milk by the consortia of probiotic cultures;

– to determine the percentage of soy oligosaccharides utilization by the *Lactobacillus* and *Bifidobacterium* during soybean milk fermentation;

– to study the dynamics of the proteolytic activity of the consortia of probiotic cultures in the soy milk fermentation;

– to study the bioconversion of isoflavones in the process of soy milk fermentation by the cultures of *Lactobacillus* and *Bifidobacterium*.

4. Materials and methods to study the profile of the soy milk fermentation process by probiotic cultures

4. 1. The examined materials used in the study and the methodology of the experiment

This work was performed using the following cultures from the Museum of the Department of Biochemistry, Microbiology, and Nutrition Physiology at the Odesa National Academy of Food Technology: *Bifidobacterium longum-Ya3, Bifidobacterium adolescentis-C52, Lactobacillus acidophilus 317/402*.

Soy milk was obtained from soy, which met the requirements of DSTU 4964:2008 "Soya. Specifications.", according to the technology that involves the following operations: coy peeling, moisture-thermal treatment for 5 minutes at 120 °C, in 0.5 % solution Na_2CO_3 at hydro module 1:6, removal of Na_2CO_3 solution, disintegration, water extraction at hydro module 1:5, filtration. Additional ingredients (salt, sugar, vanilla) are recommended to give the soy extract more pleasant taste indicators. The milk is then pasteurized at 90 °C °C for 15 minutes (a regime justified by the high protein content in soy milk), cooled to a temperature of 2–6 °C, and stored no more than 36 hours [26].

This technology makes it possible to obtain a sweet, pleasant-to-taste drink, of saturated white-cream color, with a slight pleasant smell. Moisture-thermal treatment implies the removal of foreign substances from soybeans that give foreign flavors and odors (for example, lipoxygenase), as well as anti-nutrient factors such as phytates, trypsin and lectin inhibitors. During water extraction, the milk is entered with mainly water-soluble components of soybeans, as well as soy polysaccharides, partially, poorly soluble in water, and other components.

Restoration of *Bifidobacterium* and *Lactobacillus acidophilus* cultures, stored in the museum of the Department of Biochemistry, Microbiology, and Nutrition Physiology in a lyophilized form, was carried out as follows. The lyophilized culture was converted into a suspension using the appropriate sterile liquid medium – MRS for *Lactobacillus acidophilus* and corn-lactose medium with neomycin for *Bifidobacterium*. After careful mixing, the suspension was introduced into the environment of the same composition and incubated for 1–3 days at a temperature of (37±1) °C. Next, we identified and tested the purity of the obtained sourdough cultures, determining their morphological, cultural, biochemical properties. In addition, we determined the concentration of cells in sourdough and calculated its required quantity, based on the calculation that the initial concentration of probiotic microorganisms in the mixture is at least 10^6 CFU/cm^3. The process of soy milk fermentation was carried out at a temperature of 37 °C.

Accounting for the titer of cultures was carried out by sieving ten-fold dilutions of the product in special elective media for *Bifidobacterium* and *Lactobacillus* – corn-lactose medium with neomycin and MRS, respectively. After the thermosetting of cultures at a temperature of (37±1) °C for 48 hours, the characteristic colonies were counted. In addition to the cultural attributes of the received colonies, we studied the morphology of the microorganisms that formed them.

Changes in active acidity were controlled in the soy milk fermentation process every 3 hours using a pH-meter. The activity of acid formation of the samples studied was determined by a titrimetric method also every 3 hours. We titrated 0.1 M NaOH in the presence of phenolphthalein as an indicator. The titrated acidity was calculated from the following formula:

$$^{\circ}T = A \times K \times 10, \quad (1)$$

where A is the number of milliliters of 0.1 M NaOH, used for the titration of 10 cm^3 of cultural fluid; K is the titration coefficient for 0.1 M of hydroxide sodium solution; $^{\circ}T$ is the conditional value expressed in Turner's degrees.

4. 2. Methods of experimental study of the enzymatic activity of probiotic cultures and the product's biochemical indicators

In unfermented soy milk, as well as after fermentation, carbohydrates were separated by highly effective liquid chromatography (HELC). The eluent consisted of 75 % acetonitrile and 25 % distilled water and was maintained at a flow rate of 1 ml/min isocratically. The volume of injection of 20 ml was used for both samples and standards. The time of holding the standards for raffinose, stachyose, and sucrose was 11.4, 19.1, and 7.8 minutes, respectively. Standard solutions of raffinose (2.53 g/100 ml), stachyose (2.03 g/100 ml), and sucrose (2.53 g/100 ml) were used to make a standard

calibration curve. The concentration of oligosaccharides was determined based on a standard curve and expressed in milligrams of sugar per 100 ml of soy milk [27].

The extraction of sugars for this purpose was carried out using a method described earlier [28] with some modifications: to remove the protein, 3 ml of each sample was centrifuged at 14,000×*g* for 30 minutes, followed by filtration using a membrane filter of 0.20 μm.

The amount of lactic acid was determined by the titrimetric method, followed by a recalculation [29].

The proteolytic activity of sourdough cultures on soybean milk was controlled based on the dynamics of amine nitrogen during the fermentation process, which was determined by the colorimetric method, as well as by the accumulation of free groups of NH_3, which were measured by the method of o-phthalic dialdehyde. To determine the free groups of NH_3, 100 μl of the sample of fermented soy milk was added to 750 μl of o-phthalic dialdehyde, it was aged for 2 minutes at room temperature, and then we measured the optical density of the solution with a spectrophotometer at 340 nm. The proteolytic activity was defined as the absorption of free amino groups, measured at 340 nm. The relative degree of proteolysis was determined as the difference between the proteolytic activity in fermented soy milk and the activity of unfermented milk [30, 31].

To determine the α-galactosidase and β-glucosidase activity, the samples were selected every three hours for 12 hours of soy milk fermentation by probiotic cultures. They were then centrifuged at 4,000×*g* (4 °C) for 30 minutes to produce extracellular enzyme extracts, which were analyzed for the activity of both enzymes in accordance with the method described in [28]. To this end, 60 μl of extracellular enzyme extract was mixed with 120 μl of 5 mM of the corresponding specific substrates pNPαGal (p-nitrophenyl-α-D-galactopyranoside) or pNPβGlu (p-nitrophenyl-β-D-glucopyranoside) and incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 120 μl of cold 0.2 M sodium carbonate. The amount of the released p-nitrophenol was measured using a spectrophotometer at 405 or 420 nm, respectively, using a calibration linear method, obtained with n-nitrophenol under the same conditions. We adopted, as a unit of enzyme activity, the amount of enzyme, which is released by one micromole of p-nitrophenol from the substrate ρNPG per milliliter per minute under the appropriate conditions. Specific activity was expressed in units (*U*) of the activity of both enzymes per milligram of protein [14, 32].

Isoflavones were extracted by water solutions of polar solvents. The polar solvents used were 70 % water solution of ethanol or 80 % water solution of methanol. The duration of extraction varies from 2 to 24 hours and depends on the temperature, which can range from room to 80 °C. However, as some authors claim, high extraction temperatures are undesirable because they increase the likelihood of hydrolysis of the bonds of complex carbohydrate esters, which, in turn, can lead to a significant change in the output of isomeric composition of isoflavones [32, 33].

The isoflavones were quantified at the gas-liquid chromatographer "Knauer" (Germany) with the SGX–C_{18} (3×250 mm) column made by Separon (Czechoslovakia), which has a return phase and a moving phase – water and a polar solvent (methanol) in the gradient of concentration 30–90 %, respectively, as well as the UV detector. The registration of output components was conducted at 220 nm [32, 33].

5. Research results

5. 1. Examining the profile of the soy milk fermentation process by the *Lactobacillus* and *Bifidobacterium* consortia

In the process of fermentation, changes in the active, titrated acidity, and the number of cells of probiotic microorganisms (Fig. 1) were controlled.

The results of our study show a radical change in the competitive relationships between the lactic and *Bifidobacterium* at joint cultivation on soybean milk in comparison with the development of a given consortium on cow's milk. It is known that *Lactobacillus acidophilus,* when growing on cow's milk, manifests itself as a strong and fast acid-forming agent, leading the process of fermentation along the homo-fermenting path. At the same time, it accumulates 90 % of lactic acid; milk is fermented in 3–5 hours, the threshold acidity, in this case, can reach 200–250 °T. For *Bifidobacterium*, milk is not a natural habitat; they develop very slowly in it. The main reasons for this are dissolved oxygen, the ability to absorb casein only after its partial hydrolysis, low phosphatase activity, etc. In addition, *Bifidobacterium* do not develop at the pH of a medium below 4.5, so do not compete with Lactobacillus acidophilus when co-cultivated in cow's milk.

The rate of growth of titrated acidity and the reduction of active one in different phases of the fermentation process in cow's and soy milk is different. When cultivating symbiotic sourdough on cow's milk, the adaptation phase lasts about 6 hours, then the acidity builds up, which is intensified towards the end of the fermentation process. In a given case, the fermentation process involves mainly *Lactobacillus*, *Bifidobacterium* develop only at the initial stage, and then their growth slows down due to unfavorable medium conditions: increased acidity, the lack of growth factors, the lack of energy material.

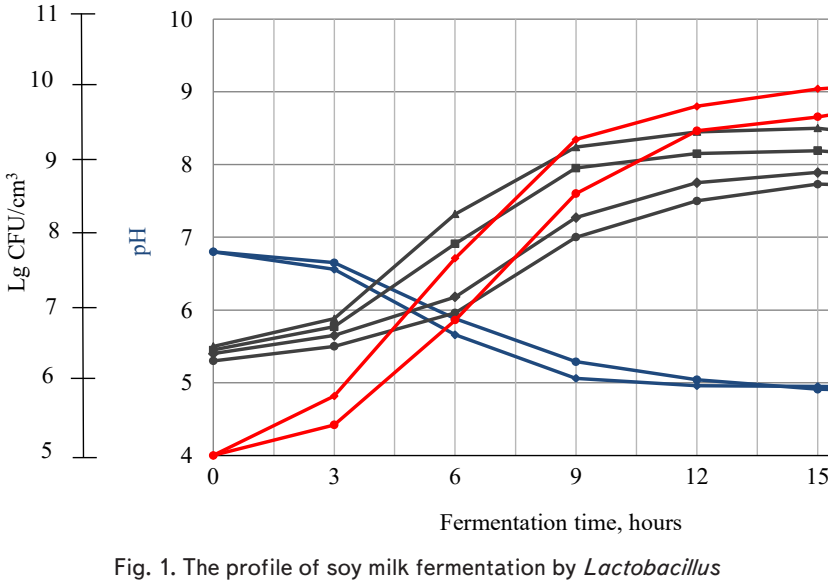

Fig. 1. The profile of soy milk fermentation by *Lactobacillus acidophilus 317 / 402* together with *Bifidobacterium* at 37 ° C

When developing on soy milk, the adaptation of probiotic cultures takes 3.0–3.5 hours, the entire fermentation process lasts 8–12 hours, depending on the clot's organoleptic indicators (acidity, density, viscosity, etc.).

Thus, after 12 hours of fermentation, pH is on average 5.0–5.2, the maximum titrated acidity is 53–58 degrees °T. Such indicators indicate a softer and gentler taste of the finished product and the possibility of prolonging the shelf life of fermented soy products while maintaining their probiotic properties (Fig. 1).

It is shown that certain symbiotic relationships between selected strains of *Bifidobacterium* and acidophilus bacilli are formed in soybean milk, which makes it possible to obtain the high titers of probiotic cultures in the finished product, and with the predominance of *Bifidobacterium*. Thus, when using the combination of *Lactobacillus acidophilus 317/402* with *Bifidobacterium longum-Ya3*, after 9 hours of fermentation, the number of cells was, respectively, $(8–10)\cdot10^7$ CFU/cm^3 and $(7–9)\cdot10^8$ CFU/cm^3, for the case of combining *Lactobacillus acidophilus 317/402* with *Bifidobacterium adolescentis-C52* – $(1–3)\cdot10^8$ CFU/cm^3 and $(2–5)\cdot10^9$ CFU/cm^3 (Fig. 1) With further cultivation, the number of cells of the probiotic cultures slightly increased and entered a stationary phase.

Table 1

Utilization of soy oligosaccharides by the cultures of *Lactobacillus* and *Bifidobacterium*

Culture used	Before fermentation	After fermentation	Decrease percentage
Stachyose, % per dry matter			
L. acidophilus	4.3±0.2	4.0±0.2	7
B. adolescentis		2.06±0.1	52
B. longum		1.89±0.09	56
L. acidophilus+B. adolescentis		2.02±0.1	53
Raffinose, % per dry matter			
L. acidophilus	1.5±0.08	1.28±0.06	15
B. adolescentis		0.69±0.03	54
B. longum		0.41±0.02	73
L. acidophilus+B. adolescentis		0.44±0.02	71
Sucrose, % per dry matter			
L. acidophilus	4.5±0.2	1.98±0.1	56
B. adolescentis		4.14±0.21	8
B. longum		4.05±0.2	10
L. acidophilus+B. adolescentis		1.85±0.09	59

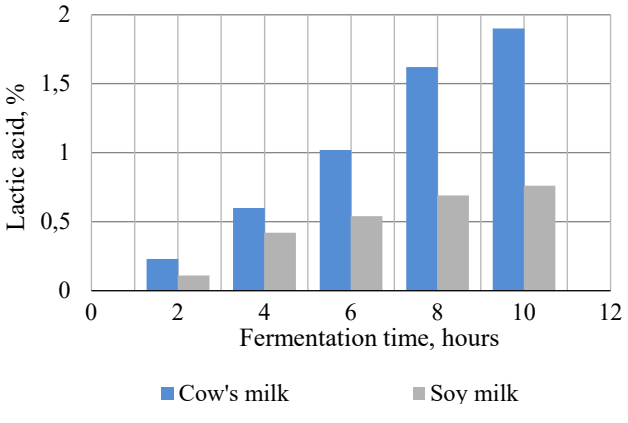

Fig. 2. The accumulation of lactic acid by sourdough microorganisms in the process of fermenting cow's and soy milk

5. 2. Determining the utilization of soy oligosaccharides by the *Lactobacillus* and *Bifidobacterium* cultures in the process of soy milk fermentation

One of the prerequisites for the development of lactic acid bacteria is the presence of a source of easily utilized energy – carbohydrates. Soy milk contains *about 3 % of* carbohydrates. The carbohydrate composition of soy milk is represented mainly by galactooligosaccharides – raffinose (1.5 %), stachyose (4.3 %), and sucrose (4.5 % recalculated for the dry matter).

This carbohydrate composition is a natural medium for the development of *Bifidobacterium* as they can synthesize, in significant quantities, α-galactosidase, hydrolyzing soy oligosaccharides, but not sufficient for *Lactobacillus acidophilus*. Therefore, of interest is to study the utilization of sugars and the accumulation of lactic acid in the process of soy milk fermentation. Table 1 gives data on the utilization of soy oligosaccharides, stachyose, raffinose, and sucrose, by the cultures of *Lactobacillus* and *Bifidobacterium*, after 10 hours of fermentation. It should be noted that the calculation of oligosaccharides in the fermented products was performed on the basis of recalculating for 14 % of dry matter.

In the process of cultivating probiotic cultures, the main products of metabolism are organic acids, especially lactic and acetic. The average values of the accumulation of lactic acid by sourdough microorganisms in the fermentation process of soy milk in comparison with cow's milk are shown in Fig. 2.

5. 3. Studying the dynamics of the proteolytic activity of the consortia of probiotic cultures in the soy milk fermentation

When making dairy products, one of the most important criteria in the selection of sourdough cultures is their proteolytic activity. This is one of the most important properties of lactic acid bacteria, characterizing their ability to break down milk proteins with the formation of simpler nitrogen-containing compounds – peptones, peptides, free amino acids, etc. Most lactic acid bacteria are characterized by a high need for growth factors such as peptides and amino acids, and the raw material does not contain enough of them to ensure the desired growth of sourdough cultures. Consequently, lactic acid and *Bifidobacterium* have a complex system of proteinases and peptidases that make it possible for them to use milk or soy protein as a source of amino acids and nitrogen. Different types of lactic acid microorganisms have different proteolytic activity, and even within one species, there are strains that differ from each other in this attribute. *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* are the leading ones among lactic acid bacteria in this attribute [34, 35]. There is also evidence of a high enough proteolytic activity of *Bifidobacterium*, the exception being the genus *B. bifidum*, which almost does not hydrolyze the protein [36, 37].

The proteolytic activity of sourdough cultures on soy milk, in addition to the ability to release free NH_3 groups, was also controlled based on the dynamics of the amount of non-protein nitrogen during fermentation. Before fermentation, soybean milk contained about 4.5 mg/ml of non-protein nitrogen. After 3–4 hours of fermentation, its amount decreased by an average of 35–40 %, and then the change in the amount of amine nitrogen in the mixture depended on the strain of *Bifidobacterium* included in the sourdough (Fig. 3).

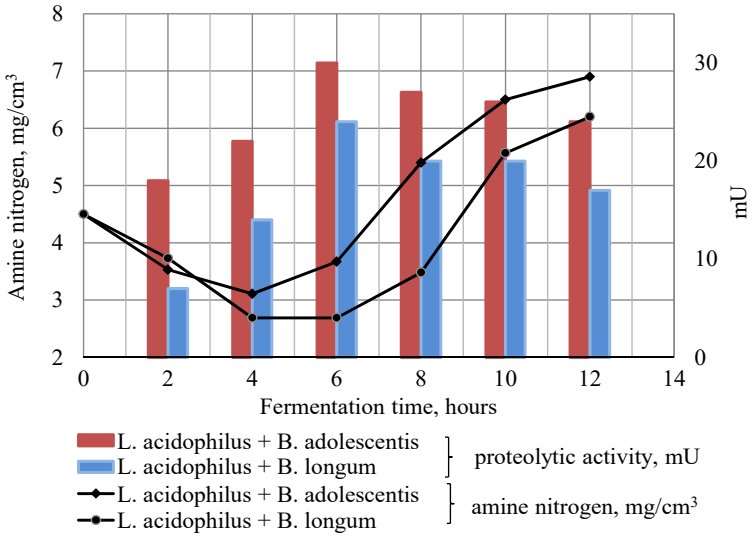

Fig. 3. The dynamics of proteolytic activity and the amount of amino nitrogen during soy milk fermentation by the culture *Lactobacillus acidophilus 317 / 402* in combination with *Bifidobacterium*

5. 4. Studying the quantitative and qualitative bioconversion of isoflavones in the process of soy milk fermentation by the cultures of *Lactobacillus* and *Bifidobacterium*

Isoflavones are part of the diphenol compounds, they are called phytoestrogens as they are structurally and functionally similar to estradiol, human estrogen, but much less potent. Due to this similarity, there is a large body of research on the preventive effects that soy isoflavones have on many types of hormone-dependent diseases [38]. Only twelve forms of isoflavones, which are found in four chemical forms, were isolated from soybeans and their processing products. They include three loose forms called aglycones (genistein, daidzein, and glycitein), and 3 conjugated forms with each aglycon called glucosides. Conjugated forms have an additional glucose component, which may not contain other groups (β-glucosides, namely genistein, daidzein, and glycitein) or may be associated with either the acetyl group (6"-O-acetylglucosides) or with a malonyl group (6"-O-malonyl glucosides). Thus, in soybeans and unfermented soy products, isoflavones are mainly in the form of biologically inactive glycoside (or glucoside) conjugates, which make up 80 to 95 % of their total content. Biologically active, estrogen-like, isoflavone compounds are the aglycone configurations of genistein, daidzein, and glycitein, which are absorbed by the human body faster and in higher amounts than their respective glucosides (Fig. 4).

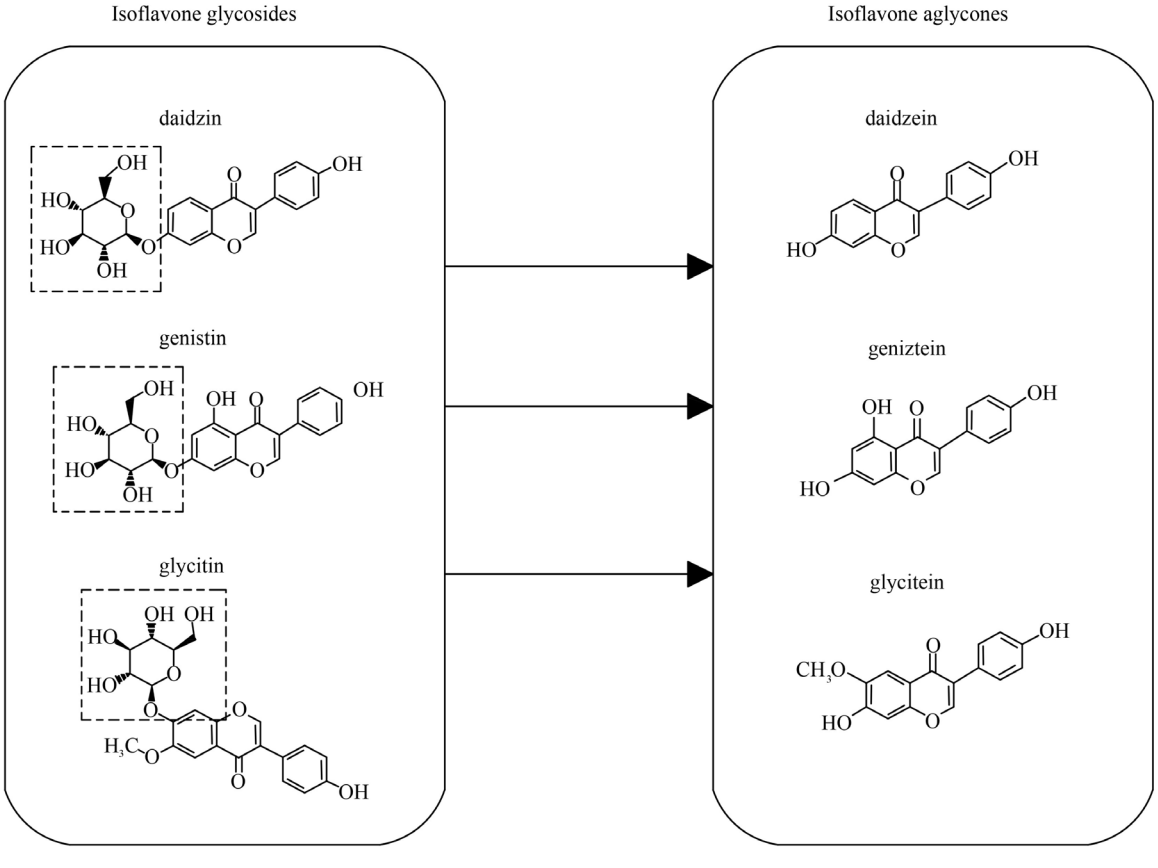

Fig. 4. The scheme of isoflavone glycosides bioconversion reaction

It is assumed that the representatives of the intestinal microbiota play an important role in the metabolism and bioavailability of isoflavones, as they, due to the synthesis of β-glucosidase, lead to the hydrolysis of glucosides components, releasing a bioavailable and biologically active form of aglycone [42]. The methods of enzymatic transformation using microbial glucosidases have great potential for the production of biologically active aglycones through the hydrolysis of sugar fragments of various glycosides in food products. It is known that the genus *Bifidobacterium* and *Lactobacillus* contain many glycosyl hydrolases, such as amylase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, α-L-arabinofuranosidase, and β-fructofuranosidase. These enzymes play a major role in the intestinal hydrolysis of various oligosaccharides, polysaccharides, and glycoconjugate phytochemicals, including isoflavone glucosides, whose transformation into bioactive aglycon forms requires β-glucosidase [24, 31, 40].

Table 2

Quantitative and qualitative bioconversion of isoflavones in the process of soy milk fermentation by the cultures of *Lactobacillus* and *Bifidobacterium*

Sample	Fermentation time, h	Amount of isoflavones, μg/100 g dry sample					
		Daidzein	Glycitein	Genistein	**Daidzein**	Glycitein	Genistein
Soy milk	0	1,333±65	291±14	1,568±77	335±15	157±7	5,08±24
Soy milk, fermented by *L. acidophilus 317/402* with *B. longum-Ya3*	9	123±6	133±6	15±0.7	1,242±61	354±17	2,163±107
	12	91±4	72±3	6±0.3	1,267±62	398±19	2,224±110
Soy milk, fermented by *L. acidophilus 317/402* with *B. adolescentis-C52*	9	198±9	190±9	48±2	1,051±51	233±11	1,905±94
	12	127±6	178±8	26±1	1,204±59	287±13	2,058±101

When studying the dynamics of quantitative and qualitative changes in the composition of soy glucosides and aglycones, a correlation with the dynamics of β-glucosidase activity in the process of fermentation of soybean milk by the selected cultures (Table 2) has been established.

6. Discussion of results of studying the enzymatic activity of *Lactobacillus* and *Bifidobacterium* in the process of soy milk fermentation

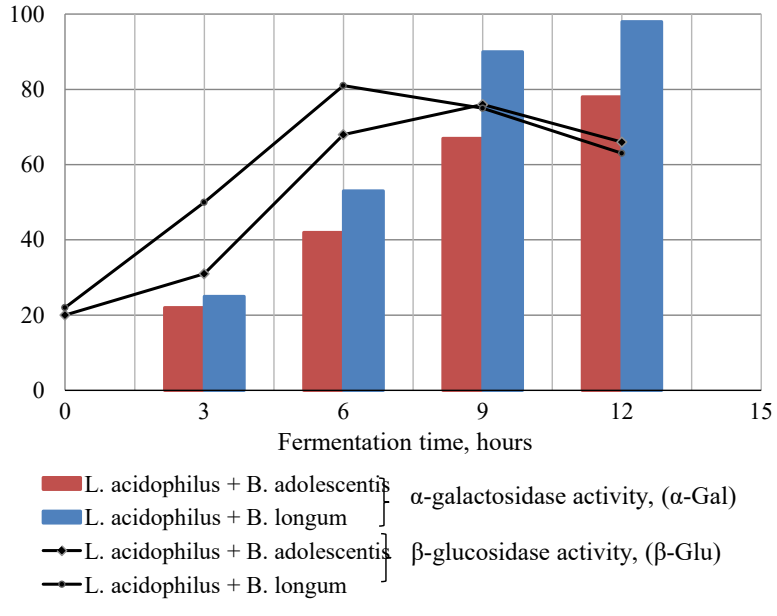

Fig. 5. The dynamics of β-glucosidase and α-galactosidase activity of probiotic cultures at joint cultivation in soybean milk at 37 °C

The β-glucosidase activity of the consortia of *Lactobacillus* and *Bifidobacterium* was determined during the fermentation of soy milk at 37 °C every 3 hours (Fig. 5).

Of particular interest were the changes in the number of isoflavones and aglycones in the process of soy milk fermentation by the selected cultures. The results are given in Table 2. The number of isoflavones and aglycones in the original soybean milk and after 9 and 12 hours of fermentation by sourdough cultures was determined. The duration of fermentation is chosen on the basis that the necessary fermentation time is 9 to 12 hours to obtain the finished drink, depending on the desired consistency.

In soy milk, the process of adaptation of symbiotic sourdough is shorter and is 3.0–3.5 hours. The active growth phase of sourdough microorganisms occurs earlier and lasts 6–7 hours, then the rate slows down in contrast to the cultivation on cow's milk (Fig. 1). At the same time, *Bifidobacterium* are mainly involved in the fermentation process. The rate of their growth in the logarithmic phase is much higher compared to the growth rate of *Lactobacillus*. This can be explained by the presence of various growth stimulants and prebiotics in soybean milk, which activates the growth and development of bifidoflora (Fig. 1).

This ratio of *Lactobacillus* and *Bifidobacterium* in the development in soy milk can be explained as follows. *Lactobacillus acidophilus* is known to ferment amygdalin, cellobioses, fructose, glucose, galactose, lactose, maltose, salicin, sucrose, and trehalose. Some strains ferment glycogen (usually weakly), as well as melibioses and/or raffinose. It is also known that lactic acid bacteria can change their classic saccharolytic pathway depending on the sugars present in the medium. They show considerable proteolytic and lipolytic activities. Their growth requires the presence of acetate or mevalonic acid, riboflavin, calcium pantothenate, niacin, and folic acid, do not need the additions of thiamine, pyridoxal, thymidine, and vitamin B_{12} (cyanocobalamin) [43, 44].

The culture B. longum is isolated from the feces of an adult and infants. The range of hydrolyzed sugars includes lactose, galactose, sucrose, fructose, maltose, stachyose, raffinose, ribose, xylose, and other sugars. This indicates the presence of a wide range of saccharolytic enzymes in a given culture, including α- and β- galactosidase. For its development, it requires the presence in the environment

of cultivation of such free amino acids as cysteine, proline, serine, alanine, as well as nicotine and folic acids. It has good proteolytic activity [37]. Thus, soy milk has enough easily utilized energy sources for the development of B. longum and, therefore, in cases of combining this culture with Lactobacillus acidophilus, it develops more actively.

The culture *B. adolescentis* is isolated from adult feces, it has the most active saccharolytic properties among all types of *Bifidobacterium*, can absorb a wide range of carbohydrates and even polysaccharides. It requires for its development the presence in the nutrient environment of riboflavin, pyridoxine, thiamine, calcium pantothenate, nicotinic acid, alanine, lysine, serine, proline, glutamic and aspartic acids. At the same time, it has a high proteolytic activity [35, 37]. It was established that this culture develops best in soy milk while suppressing the growth of Lactobacillus acidophilus.

We studied the carbohydrate composition of soy milk before and after fermentation (Table 1). It was noted that *L. acidophilus* almost did not utilize stachyose; we reduced the amount of raffinose by 15 % and sucrose – by 56 %. This was indicative of the lack of synthesis of α-galactosidase by this culture.

Both species of *Bifidobacterium* showed a high enough α-galactosidase activity. Fermenting the soy milk with *B. adolescentis* for 24 hours led to the complete hydrolysis of oligosaccharides. The highest α-galactosidase activity was shown by *B. longum* – over 10 hours of fermentation, at achieving the pH of 5.2, there was a decrease in the amount of raffinose and stachyose by 73 % and 56 %, respectively. *B. adolescentis* were slightly worse at the utilization of oligosaccharides, reducing their amount by 54 % and 52 %, respectively. In addition, the results of the utilization of soy galactooligosaccharides were confirmed by studying the dynamics of α-galactosidase activity of probiotic cultures at joint cultivation on soybean milk, shown in Fig. 5. At both combinations of *L. acidophilus* with *B. adolescentis and L. acidophilus* with *B. longum,* their α-galactosidase activity increased rapidly over 12 hours of cultivation. At the same time, after the passage of the lag phase, the phase of adaptation, the enzymatic activity of *L. acidophilus* with *B. longum* exceeded the activity of *L. acidophilus* with *B. adolescentis* by 20 % or more (Fig. 5).

In addition, *Bifidobacterium* can utilize -1,2-glycoside bonds in the sucrose molecule. As shown by data in Table 1, when *B. adolescentis* was cultivated, the amount of sucrose decreased by 8 %, and at the cultivation of *B. longum* by 10 %.

Thus, it should be noted that when soy milk is fermented by sourdoughs containing *Bifidobacterium*, it significantly reduces the number of galactooligosaccharides, that is, the possibility of discomfort in the intestines, bloating, flatulence for consumers is significantly reduced. At the same time, soy galactooligosaccharides are prebiotics that stimulate the growth and development of both *Bifidobacterium* sourdough and the beneficial intestinal microbiota.

In our study, we noted a significant difference in the accumulation of lactic acid by sourdough microorganisms in the fermentation of cow and soy milk at a slight difference in their acidity. This can be explained by the composition of sourdoughs, the lack of easily utilized sugars in soy milk, as well as the way in which *Lactobacillus* and *Bifidobacterium* conduct the process of sugar conversion (Fig. 2). *L. acidophilus* is an active acid-forming agent, conducts the fermentation process by the glycolytic pathway, in which the main metabolite is lactic acid. *Bifidobacterium* ferment sucrose, glucose, fructose, stachyose, maltose, galactose, lactose by a bifid bridge with the formation of mainly acetic and lactic acids in the molar ratio of 3:2, they can also form acetate and formate [35]. And since the joint cultivation on soy milk is dominated by *Bifidobacterium*, the rapid increase in acidity is accompanied by a slight accumulation of lactic acid.

According to our data, the proteolytic activity of the examined cultures in the soy milk fermentation depends on the strain and duration of fermentation. However, both combinations of probiotic cultures reached maximum proteolytic activity after 6 hours of soy milk fermentation, which later began to decrease slightly. And after 12 hours of fermentation, it was 80 % of the maximum value for *Lactobacillus acidophilus 317/402* and *Bifidobacterium adolescentis-C52* and 70 % – for *Lactobacillus acidophilus 317/402* with *Bifidobacterium longum-Ya3*. This is confirmed by the dynamics of the amount of amine nitrogen (Fig. 3).

Over 4 to 6 hours of soy milk fermentation by the cultures *Lactobacillus acidophilus 317/402* with *Bifidobacterium longum-Ya3*, the amount of amino nitrogen does not change. And then, at fermentation hour 8, it starts to increase; by the end of the process, it is about 125 % of the original value. When using symbiotic sourdough, which includes *Lactobacillus acidophilus 317/402 and Bifidobacterium adolescentis-C52*, the amount of amine nitrogen begins to increase within 5 hours from the onset of the fermentation process. After 7 hours, the amount of amine nitrogen equals the original value, and after 12 hours is more than 150 % of the original amount (Fig. 3). This distinguishes *B. adolescentis* among other *Bifidobacterium* strains studied as the strain with the greatest proteolytic activity. Thus, the high proteolytic activity of *Bifidobacterium*, in addition to their ability to metabolize soy oligosaccharides, explains their active growth and development on soy milk.

It can be stated that the correlation between the culture growth and their proteolytic activity shows the positive effect of the soy protein proteolysis process on the accumulation of biomass by *Lactobacillus acidophilus 317/402* and *Bifidobacterium adolescentis-C52*.

Our study has shown that the -glucosidase activity of both consortia increased rapidly from the first hours of fermentation (Fig. 5). In this case, we observed the most intense activity at the joint cultivation on soybean milk of *Lactobacillus acidophilus 317/402* with *Bifidobacterium longum-Ya3*, it reached its maximum after 6 hours and amounted to 81 U/mg of soy protein. Next, the -glucosidase activity slowly decreased and, after 12 hours of fermentation, was 63 U/mg of soy protein. The -glucosidase activity of the consortium of *Lactobacillus acidophilus 317/402* with *Bifidobacterium adolescentis-C52* increased more slowly and reached its maximum after 9 hours of cultivation on soybean milk (75 U/mg of soy protein). Then it also began to decrease and, on hour 12, was 66 U/mg of soy protein. Thus, one can see that the increase and decrease in the -glucosidase activity of both consortia during the fermentation of soybean milk corresponded to the exponential and stationary phases of development, respectively (Fig. 5).

We analyzed changes in the number of isoflavone glucosides and aglycones in the process of soy milk fermentation by the selected cultures (Table 2). It was revealed that the decrease in the content of isoflavone glucosides and the increase in the number of related aglycones coincided with an increase in the -glucosidase activity in the fermentation process of soybean milk by certain cultures. Over 12 hours

of soy milk fermentation by *L. acidophilus 317/402* with *B. longum-Ya3*, the amount of daidzein, glycitein, and genistein decreased by 93 %, 75 %, and 99.6 %, respectively. At the same time, the number of relevant aglycones increased by 278 %, 153 %, and 338 %. When fermenting soy milk by *L. acidophilus 317/402* with *B. adolescentis-C52*, the process of biotransformation of soy isoflavone glucosides into aglycones was less intense. This can be explained by the fact that the maximum of β-glucosidase activity is reached by these cultures only at hour 9 of the fermentation, while *L. acidophilus 317/402* with *B. longum-Ya3* reach it after 6 hours. In addition, the β-glucosidase activity of *L. acidophilus 317/402* with *B. longum-Ya3* is 8 % higher than that of *L. acidophilus 317/402* with *B. adolescentis-C52*. However, within 12 hours of soy milk fermentation by *L. acidophilus 317/402* with *B. adolescentis-C52*, the number of transformed glucosides into the corresponding aglycons was closer to the results of the biotransformation of their opponents. Thus, 90 %, 39 %, and 98 %, respectively, of daidzin, glycitin, and genistin were destroyed; with 259 %, 83 %, and 305 %, respectively, of daidzein, glycitein, and genistein formed.

The main purpose of the current study was to obtain a soy fermented functional drink, which, first, would be acceptable to consumers in terms of its organoleptic indicators and contain a minimum amount of soy galactooligosaccharides, which could lead to discomfort in the intestines. Second, we wanted to get a product with increased probiotic and estrogenic activity. The solution to the problem was approached comprehensively, so we confined ourselves to 9–12 hours of soy milk fermentation, taking into consideration the costs of production capacity (the process of fermenting sour-milk drinks takes, on average, 6 to 12 hours). The duration of the fermentation process depends on the desired consumer properties of the finished product (acidity, density, viscosity, etc.). Further continuation of the fermentation process beyond 12 hours was unacceptable as the product obtained was not satisfactory in terms of its organoleptic indicators.

However, the current study could be advanced by prolonging the fermentation process of soy milk and by investigating the enzymatic activity of the selected cultures, aiming to obtain poly- or mono-component dietary supplements with enhanced probiotic and estrogenic activity.

7. Conclusions

1. It was established that, when developing on soy milk, the adaptation of probiotic cultures takes 3.0–3.5 hours, the entire fermentation process lasts 8–12 hours depending on the clot's organoleptic indicators (acidity, density, viscosity, etc.). Thus, after 12 hours of fermentation, pH is, on average, 5.0–5.2, the titrated acidity is 53–58 °T. Such indicators testify to a softer and gentler taste of the finished product and to the possibility of prolonging the shelf life of fermented soy products while maintaining their probiotic properties. It has been shown that certain symbiotic relationships between the selected strains of *Bifidobacterium* and *Lactobacillus acidophilus* are formed in soybean milk, which makes it possible to obtain the high titers of probiotic cultures in the finished product, and with the predominance of *Bifidobacterium*. Thus, after 9 hours of fermentation, the average number of *Lactobacillus* and *Bifidobacterium* cells for both consortia was $(0.9–2)\cdot10^8$ CFU/cm^3 and $(0.8–4)\cdot10^9$ CFU/cm^3, respectively. With further cultivation, the number of cells of probiotic cultures increased slightly and entered a stationary phase.

2. We studied the carbohydrate composition of soy milk before and after fermentation. It was noted that *L. acidophilus* almost did not utilize stachyose, reduced the amount of raffinose by 15 %, and by 56 % – sucrose. This was indicative of the insufficient synthesis of α-galactosidase by a given culture.

Both species of *Bifidobacterium* showed a high enough α-galactosidase activity. *B. longum* over 10 hours of fermentation, at achieving the pH of 5.2, led to a decrease in the amount of raffinose and stachyose, by 73 % and 56 %, respectively. The oligosaccharides were slightly worse utilized by *B. adolescentis*, reducing their amount by 54 % and 52 %, respectively. The results of the utilization of soy galactooligosaccharides were confirmed by studying the dynamics of the α-galactosidase activity of probiotic cultures when co-cultured on soy milk. The α-galactosidase activity of both combinations of *L. acidophilus* with *B. adolescentis* and *L. acidophilus* with *B. longum* rapidly increased throughout all 12 hours of cultivation. Moreover, after passing through the adaptation phase, the enzymatic activity of *L. acidophilus* with *B. longum* exceeded the activity of *L. acidophilus* with *B. adolescentis* by 20 % or more. The maximum values of the α-galactosidase activity of both consortia were 98 U/mg and 78 U/mg, respectively.

Thus, it was established that the soy milk fermentation by sourdoughs containing *Bifidobacterium* significantly reduces the amount of galactooligosaccharides, that is, the possibility of discomfort in the intestines, bloating, flatulence for consumers is significantly reduced.

3. We have studied the dynamics of the proteolytic activity of the consortia of *Lactobacillus* and *Bifidobacterium*. A correlation between cultures' growth and their proteolytic activity has been established, which shows the positive effect of the soy protein proteolysis process on the accumulation of biomass by probiotic cultures. Both consortia reached the maximal proteolytic activity (30 and 24 mU) after 6 hours of soy milk fermentation, which later began to decline slightly. The consortium of *Lactobacillus acidophilus 317/402* with *Bifidobacterium adolescentis-C52* was the leader in proteolytic activity. After 12 hours of fermentation, the proteolytic activity was 80 % of the maximum value for *Lactobacillus acidophilus 317/402* and *Bifidobacterium adolescentis-C52* and 70 % – for *Lactobacillus acidophilus 317/402* with *Bifidobacterium longum-Ya3*. The dynamics of amine nitrogen in the soy milk fermentation process was also determined; the results indicate that *B. adolescentis-C52* is the strain with the greatest proteolytic activity. With the use of symbiotic sourdough, which includes *Lactobacillus acidophilus 317/402* and *Bifidobacterium adolescentis-C52*, the amount of amine nitrogen begins to increase within 5 hours of the onset of the fermentation process. After 7 hours, the amount of amine nitrogen equals the original value and, after 12 hours, it is more than 150 % of the original amount. Thus, the high proteolytic activity of Bifidobacterium, in addition to their ability to metabolize soy oligosaccharides, explains their active growth and development on soy milk.

4. The bioconversion of isoflavones in the process of fermentation of soybean milk by the cultures of *Lactobacillus* and *Bifidobacterium* was studied in parallel with the dynamics of the β-glucosidase activity. The results showed that the decrease in the content of isoflavone glucosides and the increase in the number of related aglycones coincided with

an increase in the β-glucosidase activity in the fermentation of soybean milk by certain cultures. Over 12 hours of soy milk fermentation by *L. acidophilus 317/402* withc *B. longum-Ya3*, the amount of daidzin, glycitin, and genistin decreased by 93 %, 75 %, and 99.6 %, respectively. At the same time, the amount of related aglycones increased by 278 %, 153 %, and 338 %. When fermenting soy milk by *L. acidophilus 317/402* c *B. adolescentis-C52*, the process of the biotransformation of isoflavone glucosides of soy into aglycones was less intense. This can be explained by the fact that the maximum of the β-glucosidase activity (75 U/mg) is reached by these cultures only on hour 9 of the fermentation, while *L. acidophilus 317/402* with *B. longum-Ya3* reach the maximum value of activity (81 U/mg) after 6 hours. In addition, the β-glucosidase activity of *L. acidophilus 317/402* with *B. longum-Ya3* is 8 % higher than that by *L. acidophilus 317/402* with *B. adolescentis-C52*. However, within 12 hours of soy milk fermentation by *L. acidophilus 317/402* with *B. adolescentis-C52*, the number of transformed glucosides into the corresponding aglycones was closer to the results of the biotransformation of their opponents. Thus, 90 %, 39 %, and 98 %, respectively, of daidzin, glycitin, and genistin were destroyed; with 259 %, 83 %, and 305 %, respectively, of daidzein, glycitein, and genistein formed. Further, the β-glucosidase activity of both consortia decreased. Thus, it is clear that the increase and decrease in the β-glucosidase activity of both consortia during the fermentation of soybean milk corresponded to the exponential and stationary phases of development, respectively. These results make it possible to plan the process of soy milk fermentation so that the microorganisms of sourdough, in addition to their probiotic properties, have time to execute the assigned biotechnological tasks.

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