IMPACT OF BETALACTOGLOBULIN HYDROLYSATE ON STRUCTURAL AND MECHANICAL PROPERTIES OF ALLERGENIC POTENCY-RESTRICTED YOGURT

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Abstract: Creation and development of low-allergenic food products in sour-milk drink groups is the urgent trend in dairy industry development. By the results of patent search, one of challenging methods to reduce the dairy product allergenicity has been identified. To produce such products, the method involves using whey protein hydrolysates, in particular, β-lactoglobulin hydrolysate obtained by using enzyme preparations Flavorpro 750MDP and Promod 439L. The research aimed to study normalized milk mixtures fermented with β-lactoglobulin hydrolysate with the resulting changes in the chemical composition, as well as to select starter microorganisms that provide the required stability and structure of the milk coagulum by producing exopolysaccharides. The researches were carried out at the base of several laboratories as follow: The Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences, Christian Hansen LLC, the Voronezh State University of Engineering Technologies and the Federal Research Centre of Nutrition and Biotechnology. This work is based on standard and conventional research methods. Advanced tools and information technologies were used to assess the properties of raw materials, semi-finished products and food products. By the results of studies completed, it was found unreasonable to replace over 20% of whole milk with β-lactoglobulin hydrolysate during normalization, since such replacement resulted in an increase in the mixture acidity and prevented the normal growth and development of Streptococcus thermophilus, the basic exopolysaccharide producer. At the same time, the length of fermentation increased up to 5–6 h as the mass fraction of β-lactoglobulin hydrolysate increased. The viscosity of the resulting coagulum increased due to the reaction of exopolysaccharides with the protein gel mesh and fixation thereof on the surface of protein matrix. The residual antigenicity of the finished product decreased to 48.5% relative to that of sour-milk drinks produced as per traditional technology.

Keywords: Low-allergy yogurt, β-lactoglobulin hydrolysate, starter microorganisms, exopolysaccharides, rheological behavior

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INTRODUCTION

Currently, the sour-milk production, particularly production of beverages, is reported to develop at the rapid rates as compared to other dairy products [1], and to employ greater development challenges. This benefits from price affordability of this food category. In addition, the increase in output and consumption of sour-milk drinks is due to the widespread promotion of healthy food among the population.

These products are reported in the high content of essential amino acids (7–11 times higher than in fresh milk), magnesium salts, phosphorus, calcium, vitamins A, D, E that are involved in the human metabolism [2]. Organic acids and carbon dioxide have a strong stimulating effect on digestive glands, thereby improving the process of digestion and intestinal uptake. Sour-milk products enrich the gastrointestinal tract with lactic acid and other bacteria that can considerably increase the human immune activity, and some of them can also "settle down" in the intestinal tract [3]. These microorganisms are antagonists of pathogenic and opportunistic pathogenic microflora as well as of putrefactive bacteria in the human intestine. The calcium of fermented dairy products is easier digested since it transforms to a soluble state in the acid environment and it partially releases from protein molecules due to protein hydrolysis by starter microorganisms [4].

Currently, the Russian market of fermented drinks offers quite a wide range of products. However, of special importance are the development and integration of technologies to produce hypoallergic and

low-allergenic dairy products with the known reduced residual antigenicity that ensure immunological tolerance.

In our country, the product line of hypoallergic and low-allergenic dairy products is limited to imported dry infant milk mixes [5]. In this sector, the integration of import substitution technologies is of great scientific and practical importance.

As per the data by the International Union of Immunological Societies, the known food-borne allergens in dairy products are the following: caseins, immunoglobulins, β-lactoglobulins, α-lactoalbumins, bovine serum albumin [6]. Hereat, as per the database by IUIS, BiPep, Allergen Online, AllerMatch, β-lactoglobulin is the most allergenic fraction [7, 8]. This is because the β-lactoglobulin is not hydrolyzed during ingestion affected by the pepsin (gastric juice enzyme) unlike other milk proteins. When proteins interact with cells of specific immunity in the human body, IgE and IgG are synthesized to different antigenic determinants of these proteins.

Various technologies are reported to ensure low-allergenic properties of dairy products as follow: isolation of protein substances using specific sorbents, physical effect on protein molecules, protein proteolysis affected by enzyme preparations [9]. It results in conformational transitions in protein molecules causing changes in the structure and digestibility of denatured proteins, as well as the destruction or access restriction to antigenic epitopes [10, 11].

The most challenging approach to reduce the allergenicity of dairy products is the biocatalytic conversion of milk proteins focused on obtaining hydrolysates thereof with specified molecular mass distribution and residual antigenicity. The specificity of proteolytic enzymes with respect to the type of peptide bond is one of the main properties of such systems that allow obtaining food products of various level of protein hydrolysis. The use of enzymatic hydrolysates as a protein component of specialized products is aimed at reducing or eliminating the milk protein allergenicity and concurrent eliminating the risk of the body sensitization. In addition, hydrolysed proteins are reported to have certain physiological effect on the human body resulting from the accelerated indigestion process of short-chain peptides in the intestinal tract as compared with native proteins and amino acids [12].

To obtain the yogurt with the reduced residual antigenicity, we proposed to use β-lactoglobulin hydrolysate developed using enzymatic preparations Flavorpro 750MDP and Promod 439L [13]. It is a source of functional ingredients as follow: protein (3.18%, including the pure protein 1.17%), lactose (4.5%), calcium (84 mg%), phosphor (157 mg%), magnesium (10 mg%), potassium (102 mg%), retinol (0.03 mg%), tocopherol (0.03 mg%), Vitamin C (1.17 mg%), pyridoxine (0.07 mg%), riboflavin (0.14 mg%), thiamine (0.035 mg%), vitamin PP (0.05 mg%) [14]. As components of the β-lactoglobulin hydrolysate, calcium and phosphor are at the ratio best effective for indigestion, where the balanced content of essential aminoacids ensures its high biological value and digestibility. The biofunctional properties studied *in vitro* and *in vivo* proved that resulting from the cheese whey bioconversion, the hydrolysate obtained is described to have the antioxidant, hypolipidemic and hypocholesteremic action.

The specific whey flavor of the hydrolysate may be eliminated by the lactic acid fermentation since lactic acid microorganisms are known for their ability to use certain amino acids and peptides as a source of nitrogen for their metabolic needs. This accelerates the fermentation process and avoids the potential bitter taste of the finished product.

The proportioning of the normalized formula and the β-lactoglobulin hydrolysate was of great importance when developing the formulation since hydrolysis products (peptides, amino acids) are known for their bitterness. Challenging was to ensure a decrease in antigenicity and acceptable organoleptic properties of the sour-milk drink obtained. To this end, the feasibility to introduce various mass fractions of β-lactoglobulin hydrolysate to normalized mixtures was studied (Table 1). The change in the ratio of casein to whey proteins in the normalized formula resulting from β-lactoglobulin hydrolysate available in the formulation may cause a decrease in the ability of the mixture to acid gelation due to different spatial structure of molecules [15].

Monomeric casein components are associated in submicelles resulting from the hydrophobic and weak electrostatic interaction, and also due to the formation of phosphate-calcium bridges. The high content of proline uniformly distributed over the surface of casein micelles prevents the formation of the compact globular structure. The decrease in the surface potential results in reduction of the thickness of micelles hydrated shell and the strength of their intermicellar repulsion. When the isoelectric point is attained, the micelles are associated with gel formation or casein coagulation.

Table 1. Ratio of formulation ingredients in normalized formulas studied

Sample	Milk mix / β-lactoglobulin hydrolysate, %	Mass fraction of β-lactoglobulin hydrolysate, %	Weight ratio, % of total protein, %	Mass fraction of pure protein, %	Ratio casein/serum proteins/peptides + free amino acids	Mass fraction of lactose, %
Control	100/0	0.33	3.2	3.17	88/10/2	4.70
1	90 : 10	0.29	3.2	3.06	80/11/9	4.72
2	80 : 20	0.25	3.2	2.93	73/12/15	4.74
3	70 : 30	0.22	3.2	2.72	65/13/22	4.76

Serum proteins are known for the compact structure of spherical or ellipsoidal shape. Intramolecular disulfide bridges stabilize the folds of the polypeptide chain. Hydrophobic side chains are inside the molecules where polar and electrically charged groups are found on the surface that forms the hydrated shell with the help of hydrogen bonds. Electric charges are uniformly distributed over the surface with low total value thereof. These molecules form the hydrophilic sol that does not precipitate at the isoelectric point, and coagulates when the larger amount of electrolyte is added. Due to that, caseins coagulate at the isoelectric point unlike serum proteins that remain in the serum as dissolved [16].

For sour-milk drinks, the strength of the gel obtained is the quality parameter. It depends on the structure of aggregates formed, including on the strength and location of casein fibers, dimensions of cavities in the gel mesh. Gels with larger cells of the spatial structure are more delicate, too sensitive to physical impact where the presence of large capillaries facilitates the retention of water by these gels. The excess content of denatured β-lactoglobulin bound to κ-casein prevents the heavier association of casein particles during the gel structure formation.

Since β-lactoglobulin hydrolysate added in the course of normalization process results in considerable changes in the ratio of casein/whey proteins/peptides and free amino acids, as well as in an increase in the lactose mass fraction in the mixture, the research aimed at studying the fermentation of normalized mixtures with β-lactoglobulin hydrolysate in view of the ability of starter microorganisms to synthesize exopolysaccharides (EPS) and to participate in the formation of structural and mechanical properties of the finished product. EPSs are known for the certain physiological effect. The availability of these substances increases the time for the consumed product to be ingested in the gastrointestinal tract that may be useful for the short-term colonization of probiotic bacteria.

OBJECTS AND METHODS OF STUDY

Research objects are the normalized formulas with β-lactoglobulin hydrolysate obtained by using enzyme preparations Flavorpro 750MDP and Promod 439L (STO 00426012-005-2014) [11] and the yogurt based on them.

Pilot studies were conducted at the base of The Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences, Christian Hansen LLC, the Voronezh State University of Engineering Technologies and the Federal Research Centre ofNutrition and Biotechnology (Russian Federation).

Research methods are standard and common for research practice, as well as modified, improved and specific implemented by using the advanced equipment and information technologies to assess properties of raw materials, semi-finished products and food products.

To isolate EPSs from the resulting sour-milk coagulum, pronase and sodium azide were used. The enzymatic reaction was blocked by boiling followed by centrifugation to separate germ cells and proteins. EPS was coagulated by adding ethanol and sodium chloride solution at the concentration of 5 mol/dm^3 to the supernatant. Prior to the test, the EPS was dissolved in the distilled water. The EPS concentration in the resulting solution was determined by the colorimetric phenol-acid method [17]. As a standard substance, glucose was used.

The structural and mechanical properties of yogurts were evaluated using the Brookfield RVDV-II + Pro rotary viscometer (USA). The mechanical stress was determined as the function of shear rate. The mechanical shear was established by the time of increase or decrease in the shear rate. The study results were evaluated by the deflection curve used to calculate the apparent (effective) viscosity at different shear rates, as well as hysteresis (cyclic zone between the upper and lower curve).

The impact of the mass fraction of β-lactoglobulin hydrolysate added and the type of starter culture used on the viscosity of the finished product was evaluated upon the fermentation of the normalized mixture for 5–6 hours at the temperature of (40 ± 2)°C [18, 19].

The experimental study results have been processed using the method of mathematical statistics as the data of various experiments were evaluated in triple sequence. Calculations, plotting and description thereof were supported by the Brookfield Rheocalc32 software and Microsoft Office 13 Application for Windows 8. The MathCad 16.0 application software package was used for graphical interpretations and data processing. The results obtained are known for high reproducibility, mutual consistency of test values and the correct statistical processing.

The study results were validated under factory conditions at the PJSC Dairy Plant "Voronezhsky" (Voronezh, Russian Federation).

RESULTS AND DISCUSSION

In compliance with the technical regulations of the Customs Union "On safety of milk and dairy products" TR TS 033/2013, a mixture of strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* is used to produce the product line of yogurt [20].

As the strains are added to the normalized milk, they rapidly multiply since the mixture acidity furthers their growth. At the initial stage, they use free amino acids of milk to synthesize proteins and enzymes in the course of their activity the amount of which decreases during the first hours of culturing. Further on, the proteolytic decomposition of milk proteins occurs when exoenzymes of bacterial cells are generated. Concurrently, to obtain the right volume of energy, lactose is fermented by lactic-acid bacterium to form the lactic acid [21]. As they multiply, the lactic acid intensely accumulates, the medium pH decreases and at pH = (4.6–4.7) the milk proceeds to gel formation.

The lactic acid formation determines the process of protein coagulum formation to define the consistency of the finished product. It adds some pleasant and sourish flavor to the yogurt, and its content depends on the composition of the normalized formula, the

composition of the bacterial starter and process parameters. It is known that when mixed cultures of lactic microorganisms are used, the rate of acid formation is higher than separate strains are used [21]. This is due to the fact that resulting from the symbiosis, the cultures known for weak proteolytic activity (for example, *Str. Thermophilus*) are stimulated by cultures with higher activity (*Lbm. Bulgaricus*) that ensures an increase in the overall degree of protein hydrolysis. Therefore, the possibility to use the formula of various starter cultures has been studied to produce the high quality yogurt. When selecting strains of microorganisms the following factors were considered:
– maximum dynamics of acid formation during fermentation in the medium with high content of free amino acids;
– high capacity of starter cultures to synthesize exopolysaccharides that compact the product consistency by binding unbound moisture and slowing the serum separation.

EPSs are the carbohydrates of high molecular weight consisting of repeated residues of monosaccharides. The concentration of EPS found in fermented milk products usually ranges from 50 to 600 mg/dm^3 depending on the culture and fermentation conditions. The amount of EPS is also dependent on the structure of the growth medium, especially the presence of carbon and nitrogen sources [22]. The ability to produce EPSs is common among some lactic bacteria, in particular, in some strains of *S. thermophilus*, *Lb. Bulgaricus* and *Lactococcus lactis subsp. cremoris* that are widely used in production of sour-milk drinks.

The EPS bio-synthesis may be divided in two stages: The first stage is the central carbohydrate metabolism when precursors of nucleotide sugars are formed. On the second stage, nucleotide sugars are combined to form the oligosaccharide repeating unit that polymerizes and is released. These stages are encoded in genes in the EPS gene cluster, for example, in the chromosome of *S. thermophilus*. The production of EPS is unstable, they may break down when cultivating microorganisms. This may result in the loss of plasmids in their cells as a result of mobile genetic element emergence or DNA erasure, as well as of DNA rearrangement [22].

The milk gel containing EPS is the mixture of bio-polymer proteins and EPS along with other components. The properties of this mixture depend on whether the bio-polymers are segregational at certain concentration (thermodynamically incompatible, resulting in aggregation of molecules of the similar type or in phase separation), compatible (coexisting in solution) or associative (capable of complex aggregation) [22, 23]. In this case, their charge is important since it is known that direct interaction can occur between negatively charged EPS and the protein structure only. This makes it possible to obtain the protein coagulum of high gloss and viscosity and of low degree of strength and syneresis.

The reason of syneresis may be the casein particle restructuring in the gel structure and the degree of dissolution of the colloidal calcium phosphate during the coagulum formation. The protein structure compression, as well as the internal pressure of the coagulum and protein sedimentation during storage may also cause the syneresis. This phenomenon results in the reduction in the shelf life of fermented milk products, so it is undesirable for the process cycle.

It is established that the maximum volume of EPSs was produced by cultures fermented by F-DVS YF-LX700 (Fig. 1). This sample of yogurt was known for its high viscosity, low syneresis and higher resistance to stirring. The reduction in serum separation in the finished product is due to resulting EPSs that further bind the protein mesh encasing the serum by its strands. The syneresis prevention is directly proportional to the EPS concentration (the higher is the EPS content, the better is the encapsulation).

All the samples studied may be classified as pseudo-plastic rheological objects described by the Ostwald equation and are known for their coagulation-condensation structure [24]. Their response to the applied deformation varied depending on the shear rate. The presented rheological curves describe the properties of structured systems that characterizes intermolecular interaction between the EPS and the protein coagulum in all fermented drinks. Hereat, the plastic flow of milk gels and disproportionate dependence between the shear rate and internal stress in samples were noted. The transition of tested samples to the state of spreadable flow upon application of the constant load resulted in stabilization of viscosity values of mixtures corresponding to the horizontal section of curves. The tested food systems had stable structures. As the shear rate increased, the viscosity of samples reduced to be explained by the breakdown of the protein coagulum structure. The maximum values of critical shear stress were reported in drinks produced by using starters F-DVS YF-LX700 and F-DVS YoFlex Harmony 1.0 (Fig. 2).

It was found that an increase in the mass fraction of β-lactoglobulin hydrolysate in the normalized mixture resulted in the decrease in the critical shear stress of yogurt samples (Fig. 3). An inverse relationship between the viscosity and stability of the resulting milk gel was reported. The concept of stability includes the values of elastic and viscous modulus, depends on the length of load application and is the qualitative parameter of organoleptic yogurts properties.

Studies have been conducted to change rheological properties of yogurt samples produced by using fermentation starters F-DVS YF-LX700 and the control sample (*Str. thermophilus* and *Lbm. delbrueckii subsp. bulgaricus*) upon the destruction (loading) and subsequent reconstitution (unloading) of the structure (Fig. 4). During the stress phase in sour-milk drinks, the reversible structural decomposition occurred resulting in the decrease in the sample viscosity. During the phase of discharge, the structure of tested samples partially reconstituted.

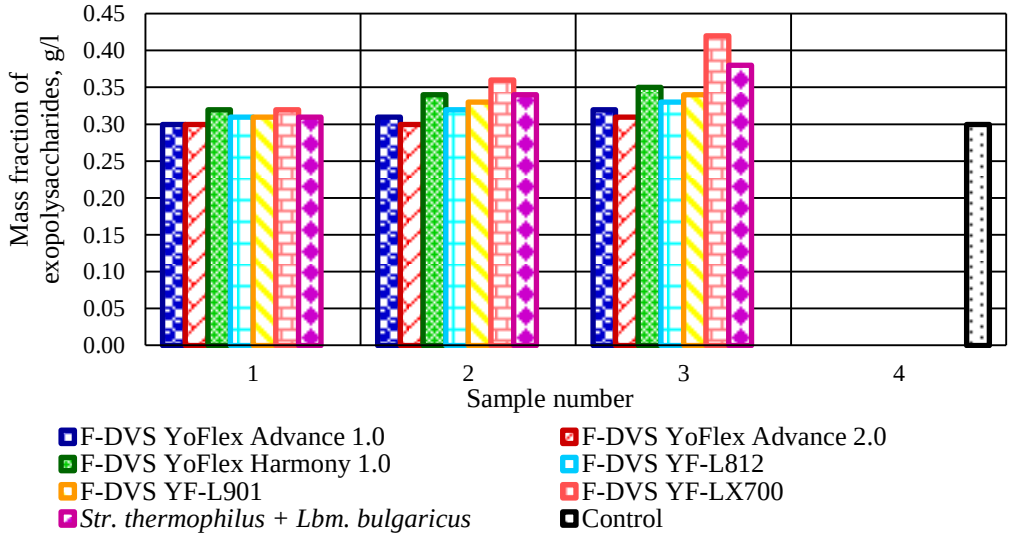


Fig. 1. Content of EPS in yogurt samples.

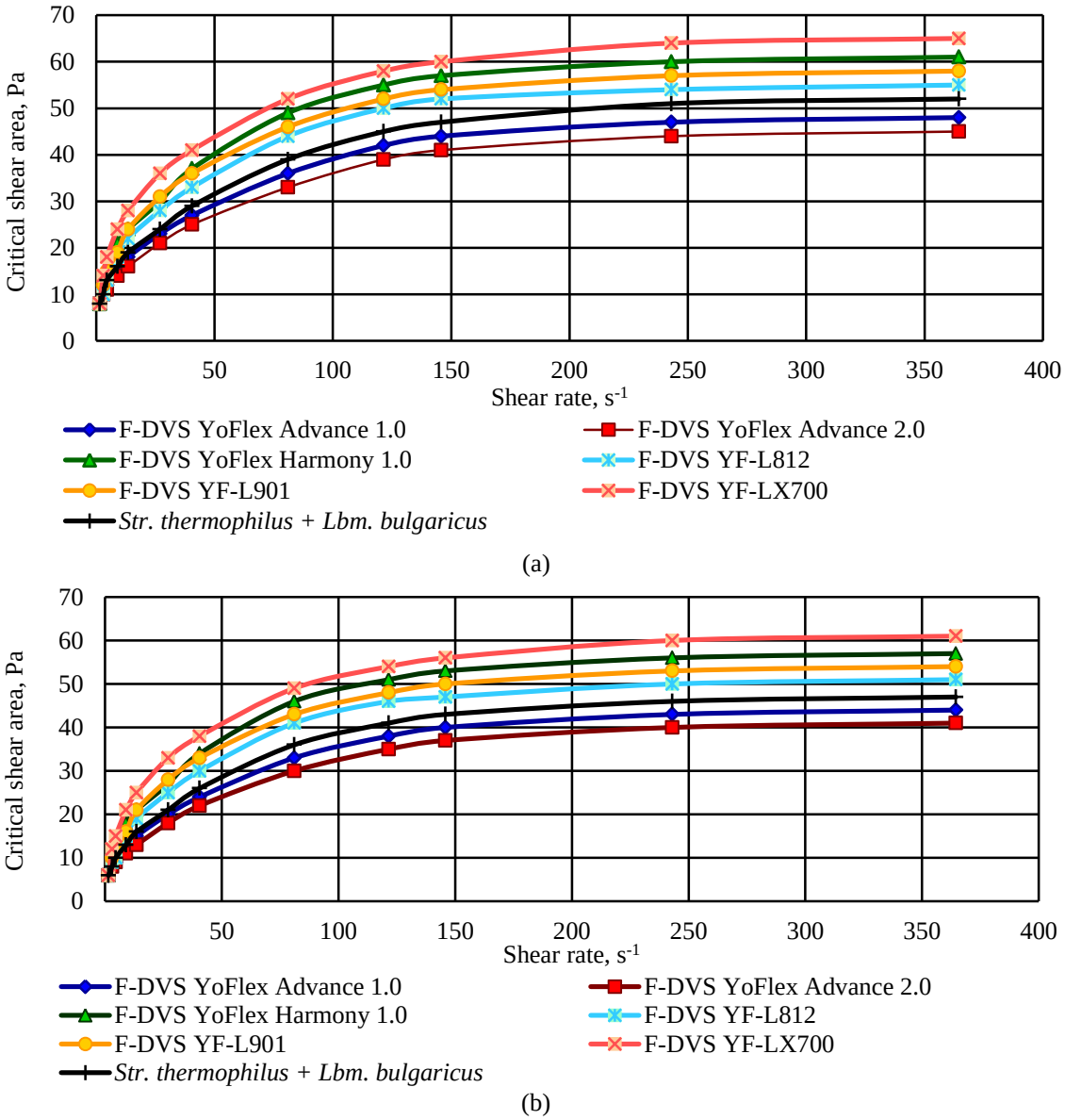
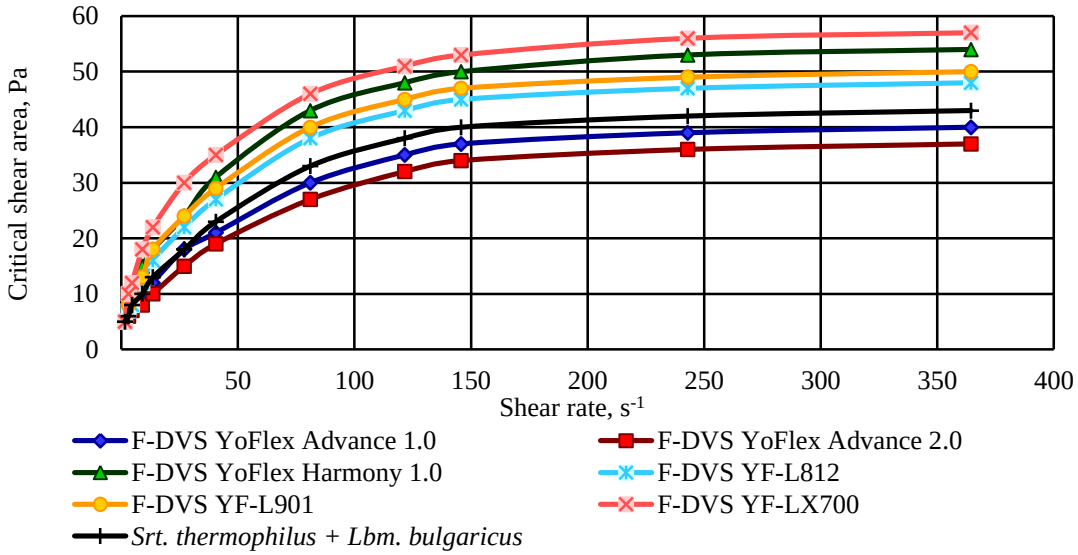
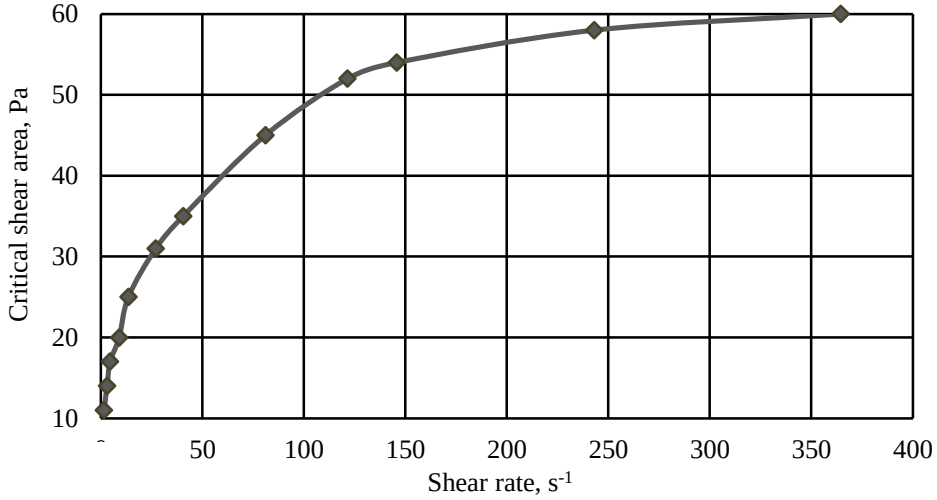


Fig. 2. *Beginning.* Graphic expression of the dependence of critical shear stress on the shear rate of yogurt samples: (a) no. 1; (b) no. 2; (c) no. 3; (d) of control sample.



(c)



(d)

Fig. 2. *Ending.* Graphic expression of the dependence of critical shear stress on the shear rate of yogurt samples: (a) no. 1; (b) no. 2; (c) no. 3; (d) of control sample.

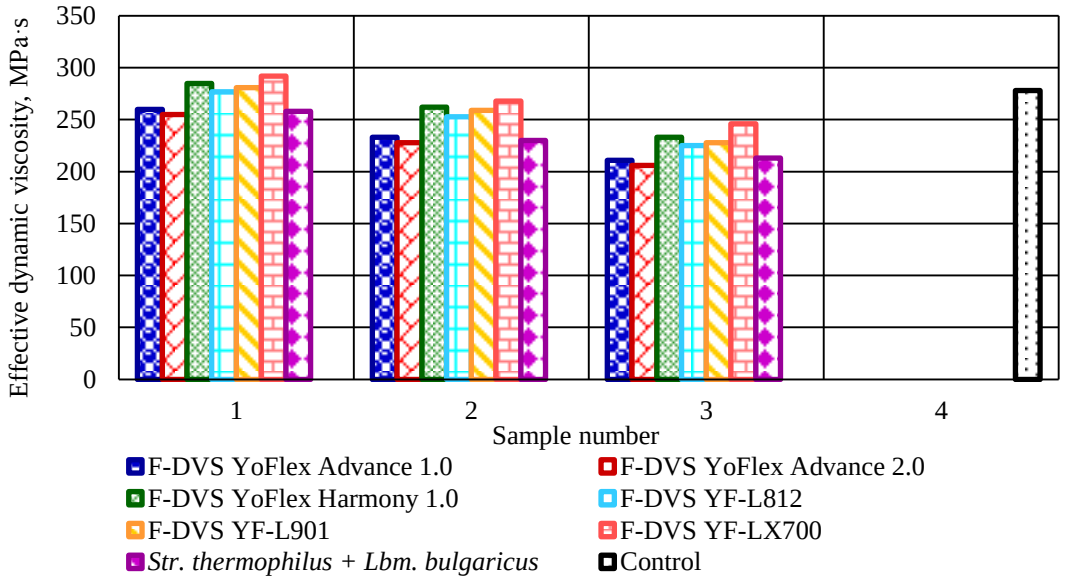


Fig. 3. Effective dynamic viscosity of yogurt samples (at the share rate of 300 s^{-1}).

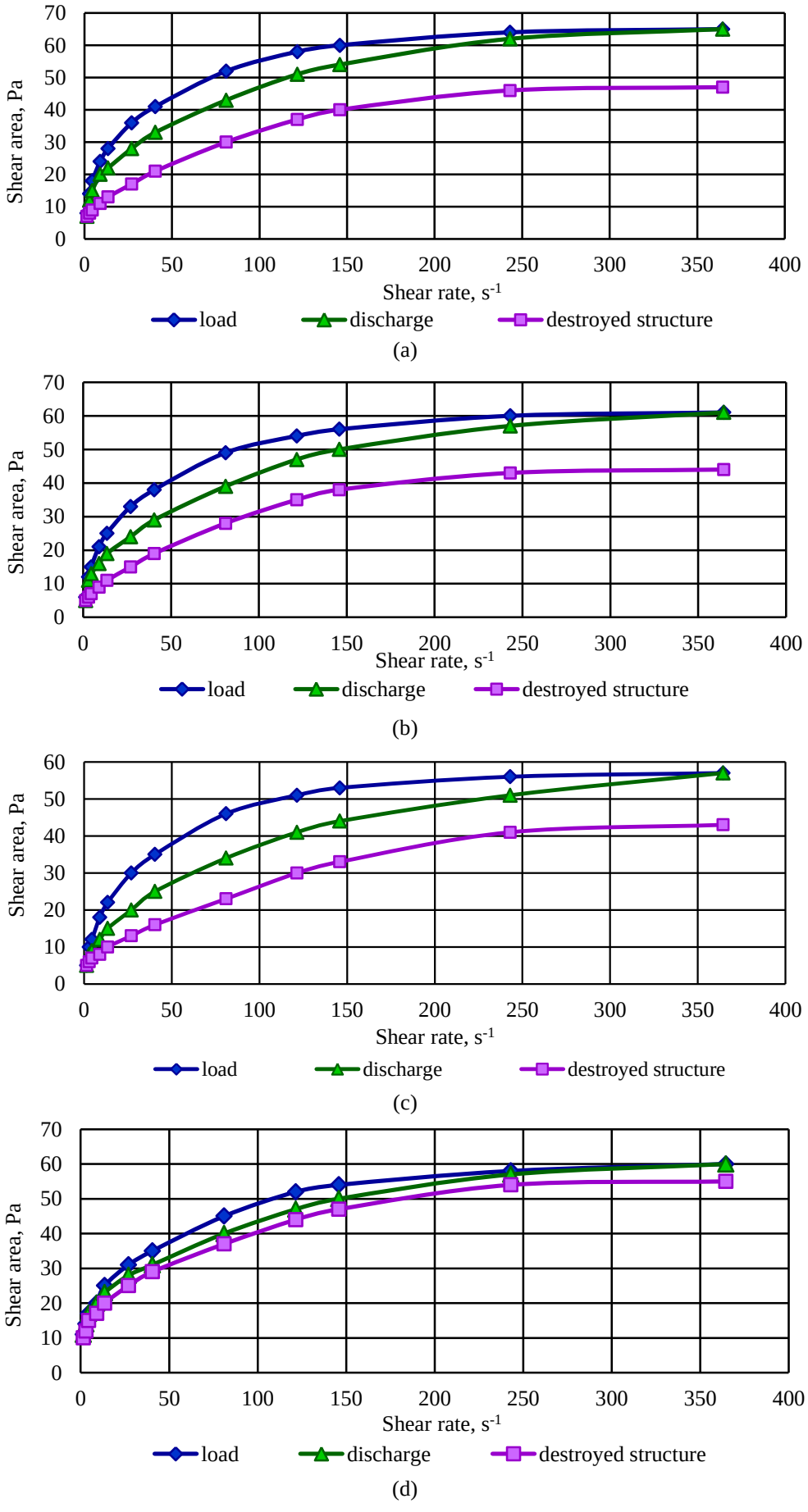


Fig. 4. Graphic expression of the dependence of critical shear stress on the shear rate of yogurt samples: (a) no. 1; (b) no. 2; (c) no. 3; (d) of control sample.

The area of the hysteresis loop (as a percentage of area under the upper curve) is not a direct physical parameter of viscosity. However, the high level of hysteresis in yogurts containing EPS means that these products are less able to reconstruct the structure upon the failure by shear. The structure of the sour-milk drink partially breaks down in the rheometer and in the mouth when having meal. Thus, in case of shear, EPS are likely to be redistributed into large clusters into the intermediate continuous phase and surrounded by acidified casein helium particles obtained by breaking the initial gel. As a result of thermodynamic incompatibility between the casein and EPS, the intermediate phase slows the protein fragment reassociation. Therefore, yogurts containing EPS are reported to have a higher level of hysteresis with respect to the product obtained by using the traditional technology.

Thus, it is established that an increase in the mass fraction of β-lactoglobulin hydrolysate, and accordingly, in the mass fraction of free amino acids in the normalized mixture will promote an increase in the rate of starter microorganism growth. This is due to the fact that a larger number of amino acids are required for the life of lactic bacteria and the thermophilic streptococcus is known to have the maximum need for this growth factor [21].

However, by the results of studies completed, it was found unreasonable to replace over 20% of whole milk with β-lactoglobulin hydrolysate during normalization, since such replacement resulted in an increase in the mixture acidity and prevented the normal growth and development of *Streptococcus thermophilus*, the basic exopolysaccharide producer. This may be explained by that the optimum of enzyme action triggered by the yogurt ferment varies within pH = (5.7–6.0), and at pH below 5.0 their activity reduces for 50% [21]. At the same time, the length of fermentation increased up to 5–6 h as the mass fraction of β-lactoglobulin hydrolysate increased. The viscosity of the resulting coagulum increased due to the reaction of exopolysaccharides with the protein gel mesh and fixation thereof on the surface of protein matrix.

Based on researches conducted, the component formulation for the low-allergenic yogurt was developed providing for 20% replacement of the normalized milk mixture with β-lactoglobulin hydrolysate, elimination of the stabilizer as compared with the control sample and the reduction of the residual antigenicity of the finished product to 48.5% relative to the sour-milk drinks produced by standard technology.

To validate low-allergenic properties of the yogurt obtained, its clinical and physiological properties were evaluated at the Allergology Department of the Federal Research Centre of Nutrition and Biotechnology. By the results of clinical chemical and immunological studies it has been established that the content of high-molecular protein structures does not exceed 57.5% of the total protein-peptide material, and not more than 44.4% of peptide-amino acid structures of the molecular masses less than 11.3 kDa.

Daily consumption of the yogurt obtained for 14 days by patients with known alimentary allergy did not result in changes in the level of serum concentration of total protein and circulating IgG and IgE antibodies specific for β-lactoglobulin. When the test product was added to the patients' diet, there was no statistically significant changes in the serum concentration of TBA-reactive products, as well as changes in the anion-exchange capacity of blood serum of patients relative to the peroxyl radical. This indicates that the lipid peroxidation is prohibited due to the high concentration of peptides with antioxidant properties in the finished product.

In compliance with the report by Federal Research Centre of Nutrition and Biotechnology, the sour-milk drink obtained was classified as the dietary (preventive) food product for children over 3 years of age with the known alimentary allergy to milk proteins.

The physical and chemical and microbiological properties of the finished product met requirements of TR TS No. 027/2012 "On safety of certain types of specialized food products, including dietary health food and dietary preventive nutrition", and TR TS No. 033/2013 "On safety of milk and dairy products". The low-allergenic yogurt was reported to have the mass fraction of physiologically functional ingredients, including vitamins, so it may be classified as the food of the functional product group.

Based on the study of microbiological, physical and chemical properties of the developed sour-milk drink during its storage, it has been established that its shelf life is no longer than 14 days at the temperature of 4 ± 2°C and the relative humidity of not more than 83%.

To produce the new sour-milk drink, the standard scheme was taken as the basis that specifies the use of additional operations to obtain the β-lactoglobulin hydrolysate. Due to application of commercially available equipment, the presented process scheme does not complicate the production process.

This process scheme is adapted to the HACCP system: the temperature, time intervals of temperature influence and residual antigenicity were taken as critical control points using the "decision tree" method. The application of this scheme in the technological process will ensure high quality, reduction of residual antigenicity, sanitary and hygienic safety, biological value and stability of properties of the finished product.

The technical documentation (TU 9222-512-00419785-13 “Lactic food products for dietary preventive nutrition”) was developed and approved, and the state registration certificate was obtained for the yogurt with the reduced allergenicity.

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