International Food Research Journal 24(6): 2652-2659 (December 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Yeast glucan and glucan-containing mushroom biopolymer complexes – stimulators of microflora growth

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Article history

Received: 18 September 2016
Received in revised form: 10 October 2016
Accepted: 11 October 2016

Keywords

Glucans
Biopolymer complex
Lactobacillus acidophilus
Bifidobacterium bifidum
Fermentation
Prebiotic effect

Abstract

Functional activity of human body systems depends on qualitative and quantitative composition of intestinal microflora. However, action of many exogenous and endogenous factors leads to the destabilization of normal microflora. In order to correct such disorder probiotics and prebiotics are used. Among the latter the dietary fibers are the most perspective. They promote not only the growth of intestinal flora but also show other physiological properties. β-glucans and glucan-containing mushroom biopolymer complexes plays a significant role among these substances. They are known as effective immunomodulators. However, there is no information in literature about their prebiotic effect. The aim of this work was to compare prebiotic effect of yeast β-glucan and glucan-containing mushroom biopolymer complexes. The article shows that yeast glucans and glucan-containing mushroom biopolymer complexes are able to stimulate the growth and development of *Lactobacillus acidophilus*-Ep-317/402 and *Bifidobacterium bifidum*. The duration of fermentation process is reduced by 2 – 3 hours while their addition to the ferment system. Including yeast glucans and glucan-containing mushroom biopolymer complexes to the milk mixture provides for fermented clots. They contain *L. acidophilus* 3.5 – 7.4 times higher and *B. bifidum* 25.1 – 269.2 times higher than clots without these preparations. Prebiotic effect of yeast glucans and glucan-containing mushroom biopolymer complexes depend on glucan content in their composition. Moreover, order degree of samples (the number of amorphous polysaccharides) significantly affects this indicator. A high growth-stimulating activity is typical for the preparations with the amorphous structure.

Introduction

Symbiotic relationships between human body and its microorganisms, formed during ontogenesis and phylogeny, led to the formation of vital regulatory system – microbiota. Depending on the colonization of microbial associations there are microbiocenosis of skin, oral cavity, guttur, stomach, intestines etc. (Ley, Peterson and Gordon, 2006; Arumugam *et al*., 2011). The latter is the biggest, following the number of families, genera and species of microorganisms. Microorganisms are divided into three groups: obligate microflora *(Bifidobacterium, Lactobacillus, Bacteroides, Eubacterium, Fusobacterium)*; facultative (*Escherichia, Staphylococcus, Enterococcus*, Fungi); transient – random bacteria that do not belong to the permanent representatives of the intestinal microflora (Berg, 1996; Holzapter *et al*., 1999; Xu *et al*., 2003).

The composition of intestinal microflora depends on many exogenous and endogenous factors. Short-term action of adverse agents leads to the minor destabilization of intestinal microbiota that usually corrects itself over time. The long-term exposure of these factors results to dysbiosis – disoder of qualitative and/or quantitative composition of normal microflora, which is accompanied by translocation of microorganisms in atypical biotypes and their overgrowth (Hawrelak *et al*., 2004; Sekirov *et al*., 2010). Presently dysbiosis is considered as one of the main etiological and pathogenetic factors contributing for emergence and development of various diseases because the intestinal microflora is involved in maintaining biochemical, metabolic and immune balance of human body (Borchers *et al*., 2009; Brown *et al*., 2012). Taking into account the widespread prevalence of dysbiosis among the population, it is necessary to correct the quantitative and qualitative composition of intestinal microbiota.

Recover intestine microflora it is usually used bacterial products – probiotics. They contain lyophilized non-pathogenic microorganisms – normaflora agents. These drugs can be mono- or polycomponent. They mostly include *Bifidobacterium, Lactobacillus, Escherichia* and Colibacillus (Cordina *et al*., 2011). It is believed that due to their high

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adhesive and colonizing ability some probiotic microorganisms can overcome the resistance of intestinal mucosa to colonization and settle down in the wall layer and thus become part of the intestinal microbiota. This, in turn, leads to stabilization and optimization of the intestine microecological status and positively affects some physiological functions, the biochemical and immune response of organism (Guarner *et al*., 2010; Cordina *et al.*, 2011).

However, the available data of scientific works point out temporary positive effect of taking probiotics and there is no any effect in some cases. Probably it is caused by two factors. First, the probiotic microorganisms of the certified drugs hardly survive in the gastrointestinal tract, so the minimum amount of viable microorganisms reach the intestines. Reduction in population under the critical level leads to the death of the all population so it is impossible to restore the composition of indigenous microflora. Second, every person has specific composition of intestinal microflora. Consequently artificially cultivated organisms are mostly incompatible with it; in other words they are failed, adversely affecting the immune system and the whole organism. These microorganisms are eliminated from the intestine in a short time after discontinuation of the drug. It is also considered that the use of probiotics can cause the development of infectious processes, metabolic disorders, and formation of new clones of bacterial strains that are unfavourable for the body (Glushanova *et al*., 2005; Millette *et al*., 2013; Fredua-Agyeman *et al*., 2015).

Low efficiency of probiotics at dysbiosis stipulated the necessity for new approaches to normalize microflora. According to modern concepts the most effective and safe method of prophylaxis and correcting the intestinal microecological violations is to recover its own microflora by using prebiotics. They are substances non-microbial origin that selectively stimulate the growth and/or metabolism of one or more existing groups of microorganisms providing the normal composition of intestinal microbiota (Younes *et al*., 2001; Gibson *et al*., 2004).

Their impact is that they are not hydrolyzed under the influence of the gastrointestinal enzymes and absorbed in the upper gastrointestinal tract. However, these substances are absorbed by beneficial colonic microflora. That promotes the reproduction of bifidobacteria and lactobacilli, reduction in pH and inhibition of the pathogenic flora growth. Low-molecular carbohydrates such as fructo-, isomalto-, xylo-, galacto-, manno-oligosaccharide and semisynthetic disaccharide – lactulose have a high prebiotic effect. However, recently much attention has been paid to the high-molecular carbohydrates – dietary fibers. They promote not only the growth of intestinal flora, but also show up enterosorption properties. They positively affect lipid metabolism and improve the intestinal motility (Rowland *et al.*, 1993; Simmering *et al*., 2001; Gibson *et al*., 2004).

β-glucans play a significant role among these substances because they are known as effective immunomodulators (Chen *et al*., 2007). Their sources are cereals, yeast and mushrooms. Cereals glucans are polysaccharides with linear structure composed of β-D-glucopyranose residues linked by (1→4) and (1→3) glycosidic bonds. Glucans of yeast and mushroom are described by another structure. Their macromolecules have branched structure, which main chain consists of β-D-glucopyranose residues linked by (1→3)-glycosidic bonds. The side branches with different lengths are joined to the places of O-6 monosaccharide residues of the backbone (Chen *et al*., 2007; Rop *et al*., 2009).

It is proved that the grain glucans can stimulate the growth of the beneficial intestinal microflora (Huth *et al.*, 2000). However, there is no information about these properties of yeast and mushroom polysaccharides. Taking into account that the latter are more active immunomodulators than cereals glucans and the fact that dysbiosis leads to the immune system disorders; it is necessity to study prebiotic properties of yeast and mushroom polysaccharides. The aim of this work was to compare prebiotic effect of yeast β-glucan and glucan-containing mushroom biopolymer complexes.

Materials and Methods

The samples preparation

The glucan-containing mushroom biopolymer complexes (samples No.1–8) were obtained by sequential treatment of raw materials with hot water and 3.7% hydrochloric acid (HCl) solution at room temperature. The solid residue was treated with 3.0 and 7.0% sodium hydroxide (NaOH) solution at 98°C, the time of treatment were 1.5 and 4.5 h.

The method described in (Whister *et al*., 1965) was used for obtaining yeast β-glucan – the sample No.9. 6% NaOH solution was added to the yeast (moisture content 72%) and the mixture was heated to 60°C. Then it was diluted with water and the solid phase was separated, treated with 3% NaOH solution for 3 hours, and diluted with water. The solid residue was separated, suspended in water, the mixture was heated up to 80°C, acidified to pH=4.5. The residue was treated with 3% NaOH solution at 80°C for 2 hours. The solid phase was separated, suspended in

water; pH of the mixture was adjusted to 4.5. The residue was separated and treated with 3% acetic acid (CH_3COOH) solution at 75°C. After cooling the gelatinous mass was separated from the supernatant, washed, homogenized in 0.16% CH_3COONa solution and autoclaved at 135°C for 1 hour. After cooling, the reaction mixture was diluted with water, the solid phase was separated. Then it was again suspended in water and autoclaved at 135°C. The solid phase – β-glucan was washed with water, ethanol and ether.

For obtaining yeast β-glucan (the sample No.10) yeast (moisture content 72%) was treated with 24% H_2O_2 solution (water/yeast ratio is 1.5) at the room temperature for 135 minutes. The solid residue was separated, washed, sequentially treated with solutions of 3% NaOH at 25°C for 30 minutes, 6% NaOH solution at 60°C for 60 minutes. The residue was washed, suspended in the water. The mixture was acidified to pH 4.5. The solid phase was separated and treated with 3% CH_3COOH solution at 75°C. After cooling the mixture insoluble β-glucan was separated, washed with water, ethanol and ether.

The chemical composition analysis of samples

The samples were hydrolyzed with solutions of mineral acids (Whister *et al*., 1965). The identification of monosaccharides of hydrolysates was performed using chromatograph Hewlett Packard 5890 (Laine *et al*., 1972). Glucose was determined by the anthrone method with glucose as standard (Sigma-Aldrich, USA) (*et al*., 1954). The glucoasamine content was evaluated with 3-methyl-2-benzothiazolone-hydrazone-hydrochloride according to (Smith and Gilkerson, 1979). The melanin level was calculated from the calibration curve on the basis of the photometry of the solution. Wavelength is 490 nm. Melanin (Sigma-Aldrich, USA) was used for the calibration curve (Selvakumar *et al*., 2008).

Analysis of total nitrogen content was done according to the Kjeldahl method (Manzi *et al*., 1999). Chitin nitrogen level was obtained by multiplying the chitin content by the coefficient 0.069 (in the natural chitin there is 6.9% N) (Ofenbeher-Miletic *et al*., 1984). Total protein nitrogen content was calculated as the difference between total nitrogen level and chitin nitrogen values. Total protein content was calculated by multiplying the protein nitrogen content by 6.25 (Manzi *et al*., 1999).

Characteristic of the samples order degree

Comparative evaluation of the samples order degree was carried out by determining the degree of their hydrolysis with 2% HCl solution for 4 hours. The soluble carbohydrates content was determined by the method reported by Bikales *et al.* (1971).

Activation of probiotic strains

Lactobacillus acidophilus-Ep-317/402 was provided in freeze-dried form by the biochemistry, microbiology and nutrition physiology department in Odessa National Academy of Food Technology (Ukraine). The strain was activated in MRS medium and incubated anaerobically at (37±2)°C to reach pH=4.6.

Bifidobacterium bifidum was supplied in freeze-dried form (PC "Biopharma", Ukraine). The strain was activated by its dissolving in sterilized cow milk (milk company "Peasant", Ukraine, pH=6.8, 20.0°Th).

Cultivation conditions

2% inoculum of *Bifidobacterium bifidum* or *Lactobacillus acidophilus* 2% of the preparation were added to the sterilized milk. Instead of study preparations the reference sample (sample No.12) contains lactulose solution in an amount of 2% (Duphalac, "Solvay Pharmaceuticals B.V.", The Netherlands) Control sample was cow milk (sample No 13). Cultivation was carried out at (37±2)°C. During the cultivation we controlled the pH values and titratable acidity. The pH was measured by means of HI9020pH meter with a glass electrode (Hanna Instruments, RI, USA). The titratable acidity was estimated the method reported by Spreer (1998). The results were expressed as Therner degree (°Th). Sensory analysis of obtained clots was carried out according to the method reported by Robinson (2002).

Microbiological analysis

The viable count of the bacteria was evaluated. 1.0 mL of samples was added to 9.0 mL of sterile normal saline. *Lactobacillus acidophilus* was counted in MRS medium by the pour-plate method (Vinderola, Bailo, Reinheimer, 2000). Enumeration of Bifidobacterium bifidum was carried out in thioglycollate medium (Robinson, 2002). All plates were incubated anaerobically at (37±2)°C for 72 hours. The results were expressed as CFU/ml.

Statistical analysis

Statistical processing of research data was performed by variance, correlation analysis. Statistical differences were considered significant at $p<0.05$. All calculations were performed using a standard software package application of Microsoft Office Excel 2003 (license № 74017-640-0000106-57490). Results are expressed as the mean ± standard

Table 1. The chemical composition of samples (% of the dry weight)

Sample number	Name	Content of components (%)				
		Glucan	Chitin	Total polysaccharides	Protein	Melanin
1	Glucan-containing biopolymer complexes of *Agaricus bisporus*	58.0±2.3	14.5±0.5	72.5±2.7	11.0±0.4	13.0±0.5
2		60.0±2.2	15.3±0.6	75.3±2.6	9.3±0.3	12.4±0.4
3		46.4±1.9	19.3±0.7	65.7±2.5	11.0±0.4	19.8±0.7
4		39.3±1.4	39.7±1.5	79.0±2.7	3.5±0.1	14.1±0.5
5	Glucan-containing biopolymer complexes of *Pleurotus ostreatus*	81.3±3.3	7.5±0.3	88.8±3.5	3.5±0.1	2.5±0.1
6		78.6±3.1	8.1±0.3	86.7±3.3	3.0±0.1	3.4±0.1
7		73.9±3.0	10.0±0.4	83.9±3.2	3.8±0.2	7.9±0.3
8		66.4±2.7	12.7±0.5	79.1±3.1	3.2±0.1	10.3±0.4
9	β-glucan of *Saccharomyces cerevisiae* obtained by the method described in (Whister and Wolfrom, 1965)	90.3±4.5	0.3±0.02	90.6±4.6	3.8±0.2	–*
10	β-glucan of *Saccharomyces cerevisiae* obtained by the peroxidic method	97.3±2.5	0.2±0.01	97.5±2.5	traces	–*
11	Chitin	–*	98.2±0.5	98.2±0.5	traces	

*are not identified Results shown as means ± SD (n=3).

deviation.

Results and Discussion

The chemical composition of samples

For researching work there were selected the samples of structural β-glucan of *Saccharomyces cerevisiae* obtained by the peroxidic method and method described in (Whister *et al*., 1965). In the mushroom cell wall β-glucans form complex with chitin, protein and melanin. Isolating pure β–glucan leads to uncontrolled modification of these polysaccharides (Cherno *et al*., 2013). The attempt to obtain the pure glucan from mushrooms led to very low yields of glucan samples. Due to their modification during isolation these samples did not practically show the prebiotic properties. That is why we investigated the samples glucan-containing biopolymer complexes of *Agaricus bisporus* and *Pleurotus ostreatus*. The chemical composition of the samples is given in Table 1. It is shown that glucan content in insoluble biopolymer complexes of *Agaricus bisporus* was 39.3 – 58.0%, in biopolymer complexes of *Pleurotus ostreatus* – 66.4 – 81.3% and samples of *Saccharomyces cerevisiae* – 90.3 – 97.3.

Effect of samples on the process of milk fermentation

It was found that in the presence of β-glucans and biopolymer complexes *L. acidophilus* and *B. bifidum* adapt to cultivation conditions faster than the control does (Figure 1 and Figure 2). Thus, the phase of active growth for microorganisms of milk mixture with samples starts earlier than the phase of the control sample does. At this stage titratable acidity indices increase while active acidity reduces. Depending on the type of bacteria it continues 10 - 21 hours for test samples, and 12 - 24 hours for the control. During this period carbohydrates transformed into organic acids – final products of metabolism. It results in change of pH medium to the value corresponding to the isoelectric point of milk proteins, and a clot is formed. Further, lactobacilli and bifidobacteria stop their reproduction and transit into the stationary growth phase, as low pH value inhibits their growth.

While cultivation of *L. acidophilus* the medium with biopolymer complexes of *Agaricus bisporus* (No.1) shows a high rate of acid production. It has a high content of glucan. The system enriched with biopolymer complexes of *Pleurotus ostreatus* with minimum level of glucan – 66.4% has the maximum acidity (sample No.8). Milk mixture with biopolymer complexes of *Pleurotus ostreatus* containing 73.9% of this polysaccharide is characterized by a lower intensity of fermentation. When the preparation with a high content of glucan (81.3%) is used, the index of accumulated organic acids increases, but not to the value of the sample No.8.

According to the index of active and titratable acidity the sample No.8 come close to a system that contains a classical prebiotic – lactulose and exceed the most pure preparation of glucan – No.10. The best result is observed for the yeast glucan No. 9. It is almost equal to that of lactulose. It should be noted that the smallest change in active and titratable acidity occurs when chitin (No.11) is added to the milk. As it is not only inferior to the yeast glucan preparations (No.9, No.10), that practically have no related substances, but also to the mushroom biopolymer complexes in which the total content of polysaccharides amounts to only 65.7 - 88.8%, it can be assumed that mainly glucan component influences on rise in indices that were mentioned. Moreover it is

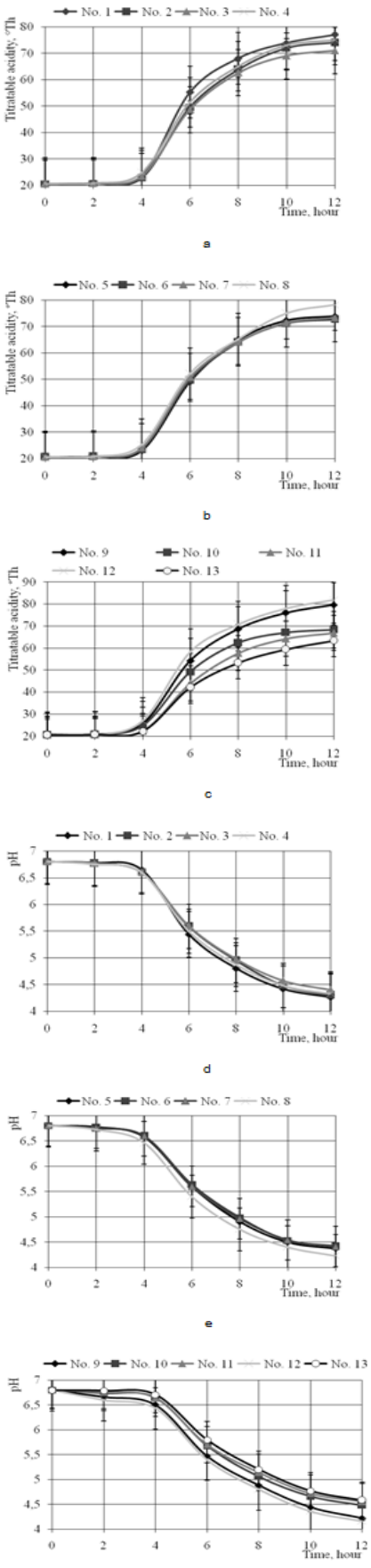

Figure 1. The titratable acid (a, b, c) and pH (d, e, f) of clots obtained by milk fermentation *L. acidophilus* with the samples

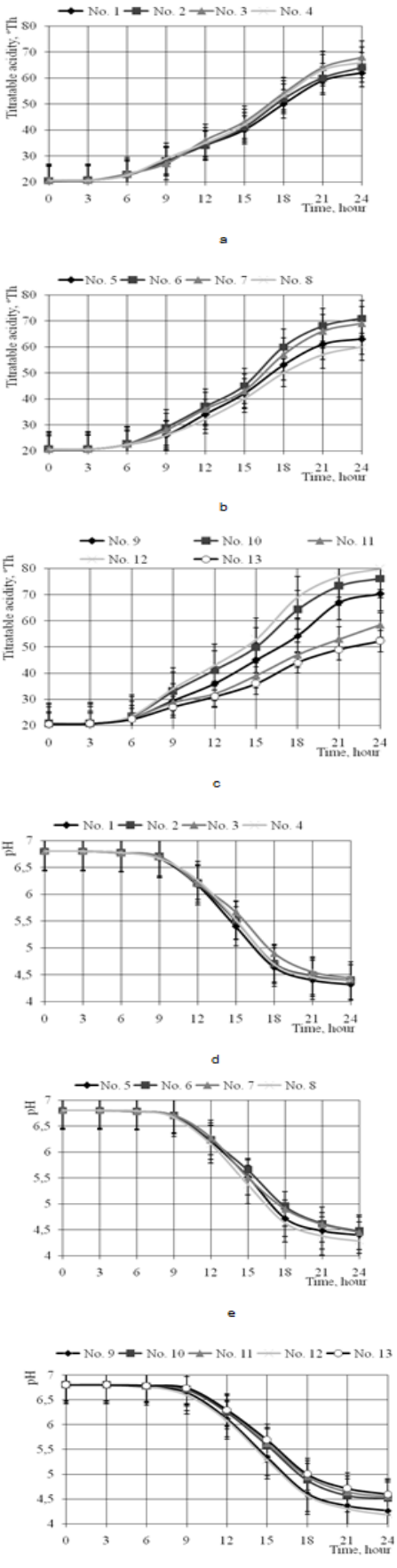

Figure 2. The titratable acid (a, b, c) and pH (d, e, f) of clots obtained by milk fermentation *B. bifidum* with the samples

the dominating component in the complexes.

The all obtained clots are almost identical to each other according their sensory properties. They have a thick, smooth chewy texture without separating lactoserum. The chitin shows the smallest intensity of fermentation among the studied samples while the process fermentating in milk mixture by the culture B. bifidum with that sample goes on (Figure 2). The glucan sample (No.9) and certain mushrooms biopolymer complexes (No.1, No.8) have almost the similar result to lactulose (No.12). Systems differ from each other in sensory properties – homogeneity, chewy texture. However, the correlation with carbohydrate was not observed.

Effect of the samples on count of viable cells in obtained clots

The data in Figure 3 show that the samples of biopolymer complexes significantly exceed the control according the quantity of viable cells in the composition of fermented systems. They content 3.5 – 7.4 and 25.1 – 269.2 times more lactobacilli and bifidobacteria respectively. But they are inferior to the reference sample according that index. The chitin has the least influence on the accumulation of microorganisms in comparison with other studied preparations (No.1-10). The yeast glucan No.9 shows the best result. It comes close to that of lactulose. However, the glucan isolated by the peroxide method, is inferior to all samples of mushrooms biopolymer complexes.

Besides the only sample (No.1) biopolymer complexes of *Pleurotus ostreatus* more active stimulate the growth of the *B. bifidum* than preparations of *Agaricus bisporus.* Their including to the milk mixture provides for fermented clots in which the microorganism concentration is 1.1 – 4.9 times higher in comparison with preparations of *Agaricus bisporus.* We can suppose that this is caused by the fact that biopolymer complexes of *Pleurotus ostreatus* have higher content of glucan than mushroom preparations. However, researching the samples, obtained by microscopy method, showed that the clots with biopolymer complexes of *Pleurotus ostreatus* (except for the sample No.8) had atypical forms of bifidobacteria. They have swollen, involution, tabular form, some of them existed in the form of rods, connected in long thin chain. However it is noted the intensive growth of typical microorganism forms primarily on mediums with biopolymer complexes of *Agaricus bisporus.* That indicates that medium with biopolymer complexes of *Agaricus bisporus* are more favorable for microbial growth.

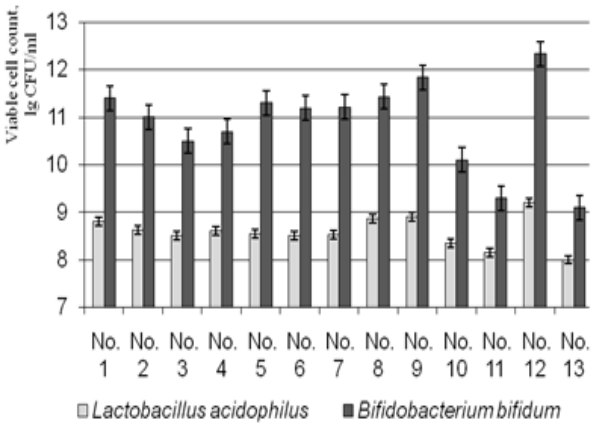

Figure 3. The viable count of *L. acidophilus* and *B. bifidum* in clots obtained by milk fermentation *L. acidophilus* and *B. bifidum* with the samples

Dependence of the order degree of samples and their probiotic effect

Analyzing the obtained results it should be noted that the common patterns during the process of lactobacilli and bifidobacteria cultivation on milk mixtures with the samples can be observed. All the samples (No.1-10) contribute to the acidity rise and accumulation *L. acidophilus* and *B. bifidum* cells compared with the control (No.13). That agrees with the idea that non-digestible polysaccharides (β-glucan and chitin belonging to this group) show prebiotic properties.

The results, describing the influence of glucan samples prepared from yeast by various methods on the accumulation of bacteria cells and change in the acidity of milk mixtures, attract attention. One of them (No.9) comes close to lactulose (No.12) according the all investigated indices. The other (No.10) has considerably lower rates.

Therefore, it is correct to compare data of the samples obtained in the same conditions: No.1 with No.5, No.2 with No.6, No.3 with No.7, No.4 with No.8 while discussing the results of comparative evaluation of prebiotic properties of biopolymer complexes isolated from different species of raw materials. The data from Table 1 show that biopolymer complexes of *Agaricus bisporus* have less total polysaccharide content and, in particular, glucan than those of *Pleurotus ostreatus*. However, it not observed the direct correlation between the preparations chemical composition, in particular their glucan content, and the level of their prebiotic effects. Beta-glucans and chitin are structural polysaccharides. Therefore, the order degree is an important factor in determining of their ability to chemical and biochemical transformations (Bikales and Segal, 1971). This index was estimated according the content in the composition of the samples of

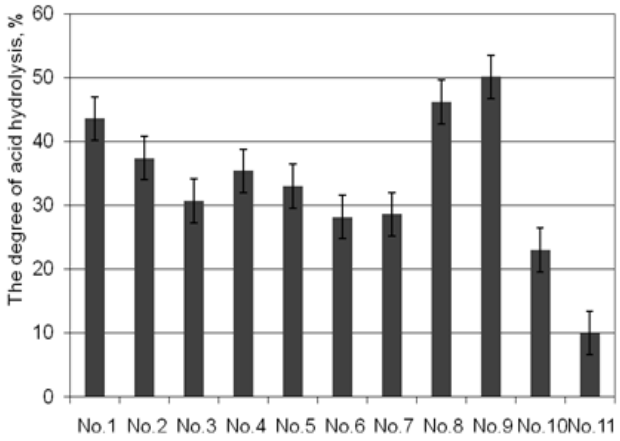

Figure 4. The degree of polysaccharides acid hydrolysis of samples

available amorphous fraction.

The data in Figure 4 show the correlation existing between the content of polysaccharide amorphous fractions in preparations and their ability to stimulate the growth and development of microorganisms. So, the least order structure and a high growth-promoting effect have the yeast glucan No.9. Among biopolymer complexes of mushrooms the samples containing low-order glucans are characterized by significant indices of probiotic culture concentration and vice versa. The high content of crystalline structures in the *Pleurotus ostreatus* can cause the availability of atypical bifidobacteria forms at the systems enriched by that preparations against the background of active growth of cells. Yeast glucan, isolated by the peroxide method, and chitin are characterized by the smallest content of the amorphous fraction. Probably, that explains the low prebiotic effect of that polysaccharides, even when the related substances are not available in their composition.

Conclusion

The article shows that yeast β-glucan and glucan-containing mushroom biopolymer complexes have prebiotic properties. They are able to stimulate the growth and development of *Lactobacillus acidophilus*-Ep-317/402 and *Bifidobacterium bifidum*. The duration of fermentation process is reduced by 2 – 3 hours while their addition to the ferment system. Including yeast glucans and glucan-containing mushroom biopolymer complexes to the milk mixture provides for fermented clots. They contain *L. acidophilus* 3.5 – 7.4 times higher and *B. bifidum* 25.1 – 269.2 times higher than clots without these preparations. Yeast β-glucan shows the highest prebiotic effect among the preparations. The effectiveness of its use is close to lactulose. It is determined that the prebiotic effect of the preparations depend on carbohydrates containing in them and their order degree, i.e. the number of amorphous polysaccharides available for biotransformation. A high growth-stimulating activity is typical for the preparations with the structure that has the amorphous β-glucan.

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