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ORIGINAL RESEARCH PAPER

INFLUENCE OF PLEUROTUS OSTREATUS β-GLUCANS ON THE GROWTH AND ACTIVITY OF CERTAIN LACTIC ACID BACTERIA

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Abstract: Over the last few decades, polysaccharides of fungi, namely beta-glucans captivated researchers' attention for they hold many significant properties; the most important ones are immunomodulating and antitumor properties. Although, beta-glucans are widely represented in nature, the most biologically active form of beta-glucans is beta-1,3 / 1,6-glucan. Most often, this type of beta-glucans is found in some yeast, bacteria, and mycelial fungi.
Thus, in recent years, fungi have been the focus of scientists around the world as a source of biologically active compounds with beneficial effects on human health. Some fungi and derived from them biologically active substances were included in the product group, defined as "functional foods", i.e. as food, whose health benefits were documented by scientific research and whose beneficial effects can not be attributed only to the availability of nutrients.
The use of functional foods containing beta-glucans leaves no doubt. With the aim of imparting new properties to functional fermented products by introducing into their composition beta-glucans of fungal origin, the effect of water-soluble beta-glucans obtained from the deep mycelium of oyster mushroom (*Pleurotus ostreatus*) on the activity of lactobacillus bacteria (*Lactobacillus acidophilus, Streptococcus salivarius* subsp., *thermophilus, Lactobacillus bulgaricus*).

Keywords: *bioactive polysaccharides, functional food, lactic acid bacteria, mushroom, prebiotic activity*

INTRODUCTION

Medical action of fungi has been known in east traditional medicine for years, especially in China and Japan [1]. Fungi had recently become attract more and more attention from scientists worldwide as a source of biologically active compounds with medical applications. Some of the fungi and its biologically active compounds were included in a list of functional foods, in other words, food, which was scientifically recognized to have positive health effects [2].

Many fungal polysaccharides have anti-inflammatory and immunomodulating properties, and therefore researchers consider it to be a treatment for many health problems [3].

Carbohydrates most frequently encountered in fungi are arabinose, mannose, fucose, galactose, xylose, glucuronic acid and glucose [4]. The primary source of fungal carbohydrates is an outer membrane of the fungal cell. It is described by many researchers [5, 6], that from the biotechnological perspective, one of the most valuable forms of fungal polysaccharides is a group of *β*-glucans. *β*-glucans are glucose polymers linked together by a 1→ 3 linear *β*-glycosidic chain core, and they vary by the length and branching arrangements [7]. Most common sources of *β*-glucans are some species of plants, algae, yeasts, bacteria and fungi (specifically *Pleurotus ostreatus*) [8]. The latter seems to be an appealing option for obtaining polysaccharides since *Pleurotus ostreatus* is rich in *β*-glucans and easily accessible as an edible mushroom.

According to [9], during food digestion, *β*-glucans are releasing into the small intestine where they are immediately captured by the macrophages. The *β*-glucans are then fractured into small particles and are carried to the marrow and endothelial reticular system. The particles are then delivered by the macrophages and taken up by the circulating granulocytes, monocytes and dendritic cells. Blood and tissues of the human body almost do not content glucans. Therefore, glucans are recognized by the immunity as foreign substances and hence cause a response of the immune system [10].

Based on the above, the positive effect of including *β*-glucans in food leaves no doubts. In the present study, *β*-glucans derived from *Pleurotus ostreatus* were added in cow milk yoghurt making this product more beneficial for human health.

MATERIALS AND METHODS

Fruiting bodies of *Pleurotus ostreatus* mushroom were purchased at local market in St. Petersburg, Russia, dried at 60 °C and crushed into powder. To obtain beta-glucans, powderized mushroom fruit-bodies were initially boiled in 80 % ethanol twice for 3 hours each time, to increase permeability of cell walls. After that water soluble beta-glucans were extracted from the residue of filtration by boiling in water for 3 hours each time and precipitated from the extract by the addition of 5 volumes of 96 % ethyl alcohol at the chilling temperature (4 °C).

Precipitate was filtered and dried at 55 °C in air flow and powdered. Obtained powder preparation was used for studies of its prebiotic properties.

The *β*-glucan content of the crude extract was estimated on dry weight basis using the *β*-glucan enzymatic assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) [11].

Immunological studies:
For immunological studies fresh donor blood, Ficoll-Paque (density 1.078 $kg \cdot m^{-3}$), Hank's salt solution (Biolot), luminol, phorbolmyristate acetate (PMA), formyl-peptide FMLP 1640, cow fetal serum, zymozane, agarose EEO, prodigiozan, concanavalin A (all "Sigma") were used.
Blood cell isolation: Mononuclear and granulocyte cells were isolated from the donor blood stabilized with heparin solution (20 ME per 1 mL of blood) in Ficoll-Paque gradient using standard technique described by Boyum [12]. Blood plasma was layered on to Ficoll-Paque gradient and centrifuged for 40 min at 4000 g. The mononuclear cells were collected from the interphase between plasma and Ficoll-Paque. The neutrophilic granulocytes were gathered from the precipitate after isolation of mononuclear cells by a hypotonic shock with distilled water for 30 seconds.
Study of the cytokine-inducing activity of the preparation: Mononuclear cell suspension (concentration of 5×10^6 mL^{-1}) resuspended in RPMI 1640 medium including 10 % fetal serum was placed in a 96-well flat-bottom microplates (Costar) by 0.1 ml in each hole. Simultaneously, the investigational preparations placed in appropriate solutions were added in the same holes. The Russian preparation of the natural lipopolysaccharide prodigiosan, a strong inducer of pro-inflammatory cytokines, was used as the positive control. The microplates were placed in the CO_2-incubator for 24 h, after that the number of cytokines in the culture medium was detected by an immunoenzymatic method [12]. Test systems made by the company Cytokine (St. Petersburg, Russia) were used.
Study of the influence of the preparation on the production of oxygen active forms by phagocyting cells: The formation of active oxygen by whole blood cells was detected with luminol dependent chemiluminescence method (LDCL) [13]. The detection of LDCL was carried out in 96-hole white non-transparent microplates (Costar) with the use of the device Victor-2 (Finland) connected with PC which provided sequent registration of chemiluminescence. The reaction result was termed as the light sum (sum of impulse accumulated during the certain time). The base luminol solution (10^{-2} M) was prepared in dimethyl sulfoxide (DMSO) and stored at 4 °C. The working solution (10^{-4} M) was prepared fresh diluting the base solution with Hanks solution (*p*H 7.2).
The experiment was carried out by the following scheme: 120 mL of Hanks solution, 20 mL of fresh donor blood, 40 mL of the working luminol solution and 20 mL of the studied preparation in the appropriate dilutions were placed in each hole of the 96 well microplates. Each test was carried out in 3 parallels. The solution of phorbol-myristate acetate (PMA) with the end concentration of 10 $mg \cdot mL^{-1}$ was used as the standard positive control. The reaction was fixed in 1 h.
Strains of lactic acid bacteria were obtained from the culture collection of the department of Chemistry and Molecular Biology, of Saint Petersburg ITMO University, Russia. Each bacteria strain was estimated for growth ability after adding β-glucans. For the positive control, cultures without additives were used. Bacterial cultures were added in the volume of 10 % of 1.5 % cow milk. 0.2 g of β-glucans were added to each sample. The liquid thermostat "LOIP LT-117b" was used for bacteria incubation. The incubation process lasted for 5 hours at 40 ± 0.1 °C. Oxidation-reduction potential (ORP, mV), *p*H and unit linear electrical conductivity X, ($mS \cdot cm^{-1}$) was measured every hour. "Expert-001" (Russia) ion meter combined with two electrodes "ESK-10601/7" (Russia) and "ERP-105" (Russia) was used to measure ORP and *p*H values

respectively, while X values were measured by conductometer «Expert-002» combined with the conductometric sensor type «UP-P-C» (UralChimLab, Russia). The first determination of *p*H, E and X were estimated 2 hours after the incubation started and then the measurements were conducted each hour. The results are represented in the Tables 1 - 3. The bacteria strains were as following: *Lactobacillus acidophilus, Streptococcus salivarius subsp. thermophilus, Lactobacillus bulgaricus*. All the measurements were done in triplicate and the confidence interval is 95 %.

RESULTS AND DISCUSSION

As a result of extraction process from 1000 g of Oyster mushroom fresh fruit bodies 6.7 g of water-soluble grey-brown colored powder was obtained, containing 23.8 % of beta-glucans.

Primary evaluation of biological activity of studied extracts was made by test analysis of the reactive oxygen species generated by cells of peripheral human blood by a method of luminal-dependent chemiluminescence. The test was selected based on supposition about stimulating effect of extracts of *Pleurotus ostreatus* on the system of phagocytosis. When activating the phagocytes of the blood, a cascade of chain reactions is started accompanied by the increased oxygen consumption from the environment. The cells pass on hexose monophosphate path of respiration. The final products of the reaction contained the reactive oxygen species, which, being combined with luminol, caused luminescence registered at a wave-length of 425 nm.

The extracts from fruit bodies of *Pleurotus ostreatus* were studied at concentrations of 0.001-1.0 mg·mL^{-1}. Against the background of the whole blood and PMA as a positive control, tested samples of mushroom aqueous extracts have increased production of reactive oxygen species by neutrophils of peripheral human blood. The maximum effect was observed at a dose of 0.1 mg·mL^{-1} (Table 1).

Table 1. *The effect of extracts from mushroom fruit bodies on production of reactive oxygen species by human blood neutrophils*

Preparation	**Concentrations, mg·mL^{-1}**			
	1	**0.1**	**0.01**	**0.001**
	Light-sum, impulses in 30 minutes			
Extract from mushroom fruit bodies	380 ± 5	392 ± 2	128 ± 5	87 ± 3
PMA, 0.1 mg·mL^{-1} (positive control)			179 ± 5	
Whole blood (background)	35 ± 2			

The ability of obtained mushroom extracts to induce the synthesis of cytokines, immunoregulating proteins performed by human blood cells were studied. The data obtained are shown in Table 2.

Table 2. *Influence of mushroom fruit body extracts on production of inflammatory cytokines by human blood cells*

Preparation Concentration ($mg \cdot mL^{-1}$)	**Production of cytokines (% of control)**	
	IL-1β	**IL-8**
5.0	340	230
0.5	290	270
0.05	200	130
0.005	170	120
0.0005	130	100

Level of spontaneous production of IL-1β: 670 $ng \cdot mL^{-1}$; IL-8: 2169 $ng \cdot mL^{-1}$. Level of production induced by LPS of: IL-1β: 250 % of control, IL-8: 310 % of control.
The preparations studied showed pronounced immunostimulating action involving almost various immunocompetent cells. The results obtained in the *in-vitro* experiments have indicated the potential for extracts of *Pleurotus ostreatus* to be used as a functional ingredient to develop functional food products.
Addition of beta-glucans to milk, inoculated with starter cultures results in more rapid increase of acidity compared to a reference sample for all studied strains (Table 3).
Moreover, with the growth of lactic acid bacteria in milk in the presence of *β*-glucan, the specific linear electrical conductivity (X, $mS \cdot cm^{-1}$) increases faster than in milk without additives (Table 4). This fact is probably due to sorption of microorganisms on *β*-glucan - which in itself contributes to an increase in the activity of the investigated microorganisms [14].

Table 3. *Dynamics of changes in pH as a result of lactic acid bacteria growth in milk (control samples) and in milk with β-glucan*

Microorganism		***p*H**				
		Duration, [h]				
		0	**2**	**3**	**4**	**5**
Lactobacillus acidophilus	control	6.67±0.004	6.29±0.01	5.67±0.002	5.06±0.001	4.78±0.002
	with *β*-glucan	6.33±0.002	5.79±0.001	5.00±0.004	4.50±0.002	4.25±0.001
Lactobacillus bulgaricus	control	6.71±0.006	6.35±0.002	5.71±0.002	4.96±0.003	4.59±0.002
	with *β*-glucan	6.31±0.003	5.76±0.004	5.05±0.004	4.12±0.003	3.72±0.002
Streptococcus salivarius subsp. thermophilus	control	6.80±0.001	5.84±0.001	5.15±0.001	4.94±0.001	4.61±0.001
	with *β*-glucan	6.31±0.001	5.05±0.003	4.41±0.002	4.14±0.001	3.76±0.001

Table 4. *Dynamics of changes in electrical conductivity X [mS·cm^{-1}] as a result of the lactic acid bacteria growth in milk (control) and in milk with β-glucan*

Microorganism		X [mS·cm^{-1}]				
		Duration, [h]				
		0	2	3	4	5
Lactobacillus acidophilus	control	12.22±0.01	13.48±0.04	14.37±0.03	15.22±0.03	15.98±0.04
	with *β*-glucan	15.22±0.06	17.92±0.09	19.03±0.08	19.73±0.09	20.14±0.09
Lactobacillus bulgaricus	control	13.43±0.06	13.99±0.06	14.43±0.06	14.94±0.07	15.20±0.08
	with *β*-glucan	14.23±0.06	15.22±0.06	15.82±0.05	16.35±0.06	16.66±0.06
Streptococcus salivarius subsp. thermophilus	control	13.25±0.01	14.48±0.03	15.37±0.03	16.22±0.03	16.98±0.04
	with *β*-glucan	16.26±0.06	18.92±0.09	20.03±0.07	20.70±0.09	21.14±0.09

From the other hand, there was no significant difference in the change in the redox potential (E, mV) for both variants, with or without addition of beta-glucans, and apparently, the presence of water-soluble *β*-glucans in the medium does not affect the activity of the redox enzymes of lactic acid bacteria (Table 5).

Table 5. *Dynamics of the change in the redox potential ORP (mV) as a result of the vital activity of the lactic acid bacteria in milk (control) and in milk with β-glucan*

Microorganism		ORP [mV]				
		Duration, [h]				
		0	2	3	4	5
Lactobacillus acidophilus	control	275±0.1	281±0.3	288±0.2	293±0.1	298±0.1
	with *β*-glucan	263±0.1	279±0.1	291±0.1	297±0.1	300±0.1
Lactobacillus bulgaricus	control	278±0.1	284±0.3	291±0.2	297±0.2	301±0.1
	With *β*-glucan	265±0.1	282±0.1	294±0.1	300±0.1	304±0.1
Streptococcus salivarius subsp. thermophilus	control	281±0.2	288±0.4	296±0.3	302±0.2	306±0.1
	with *β*-glucan	268±0.1	286±0.1	299±0.1	305±0.1	309±0.1

CONCLUSION

It was found that the addition of the preparation, containing water soluble *β*-glucans significantly increases the electrical conductivity of milk, fermented by lactic acid bacteria. This indicates a more rapid growth of microorganisms in comparison with control samples. In addition to that, lower *p*H values may indicate greater glycolytic activity of lactic acid bacteria in test samples compared to control samples.
It is also shown that the introduction of *β*-glucans does not lead to a notable change in the oxidation-reduction potential of fermented milk. Therefore, *β*-glucans do not have an inactivating effect on the enzyme system of lactic acid bacteria and do not have a negative effect on the vital activity of the bacterial cultures.

The obtained results confirm the possibility of using β-glucans of the *Pleurotus ostreatus* fungus in fermented milk products as a biologically active additive, prebiotic additive, having a positive effect on the growth of lactic acid bacteria and imparting additional functional qualities to the probiotic product.

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