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Studying Biological Activity of Lactobacillus Hydrolysates

A.G. Koshchaev[1], Y.A. Lysenko[1], A.V. Luneva[1], A.N. Gneush[1], M.V. Aniskina[1], V.I. Fisinin[2], I.P. Saleeva[2]
[1]Kuban State Agrarian University, Krasnodar, 350044, Russia
[2]All-Russian Research and Technological Institute of Poultry, Sergiev Posad, 141311, Russia

Abstract:

This work is aimed at studying pharmacological activity of lactobacillus hydrolysates obtained by fermentative and thermal acid method. The hydrolysates were produced of Lactobacillus intermedius, which were derived and identified by molecular genetic methods from gastrointestinal tract of quails. The subject matter of the article was the influence of biologicals on organism immune status, lipid peroxidation, indices of endogenic intoxication and general functional indices of laboratory animals upon intoxication by carbon tetrachloride. The experiments were performed with laboratory rodents. Biologicals were added orally. The laboratory animals were on the medications for 14 days. The amount of added liquid in all groups was the same equaling to 10.0 ml/kg. On the basis of biochemical analysis, it was established that hydrolysate obtained by thermal acid method exerted the best effect on organism immune status of laboratory animals both upon addition toxicant and without it. In total, the experimental results evidence reasonability of further experiments of application of lactobacillus hydrolysate derived by thermal acid method from Lactobacillus intermedius cells for treatment and prevention of immune deficiency.

Keywords: body weight, dose, hydrolysate, immunity, intoxication, laboratory rodents, liver, spleen.

INTRODUCTION

In order to produce medications modern Russian industry usually applies medical herbs and products of chemical synthesis; bacteria are used for these purposes much less frequently [1-4]. Nevertheless, application of bacteria as raw stuff can be sufficiently promising due to high content of important biologically active substances. In addition, high rate of bacterial growth permits to cultivate them in high amounts in rather short time which provides economic feasibility of bacteria application as raw stuff [5-8].

The most valuable bacterial biologically active substances are proteins, peptides, amino acids as well as amino sugars, organic acids, vitamins, etc. Bacterial protein substances can be used for dietetic therapy, and the products of their complete hydrolysis (combination of all amino acids) are applied for intravenous feeding [9-11]. Especially important are biopolymers or peptidoglycans where comparatively short oligopeptide fragments are attached to polysaccharide chain. This can be exemplified by peptidoglycan of bacterial cell wall built on the basis of residuals of N-acetylglucosamine and N-acetylmuramic acid attached by glycoside bonds. Linear polysaccharide is attached to oligopeptide chains via residual lactides of muramic acid, thus creating protecting frame of bacterial cell [12, 13]. It should be also mentioned that these structures and substances are contained only in bacterial wall, they do not exist in animals and plants. In addition, for Gram positive bacteria (*Lactobacillus*, *Bifidobacterium*, *Streptococcus* and others) the share of peptidoglycan reaches 70% on dry basis of cell wall, whereas for Gram negative bacteria this index is not higher than 10% [14-16]. Proteoglycan splitting provides combination of glycopeptides characterized by adjuvant and antitumor activities [17-19].

The smallest structural unit exhibiting such activity is muramyl dipeptide. Muramyl dipeptide and its derivatives are characterized by wide range of biological effects, the most important of them is pleiotropic immunomodulatory action [20-22]. The immunomodulatory action is related with application of derivatives of muramyl dipeptide for stimulation of natural protecting reaction of organism against tumors and pathogenic organisms [23-25]. In addition to immune adjuvant activity, muramyl dipeptide is characterized by antitumor and anti-infectious activity [26-28]. Due to this properties glycopeptides of bacterial cell attract careful attention of researchers as potential components of medications. In this regard experiments devoted to development of biologicals based on lactobacillus hydrolysate, analysis of its qualitative and quantitative composition, stability and biological activity are very important.

This work is aimed at study of biological activity of laboratory animals affected by lactobacillus lysates obtained by various methods.

MATERIALS AND METHODS

The subject matter of the study was lactobacillus hydrolysates (*Lactobacillusintermedius*) derived and identified by molecular genetic methods from gastrointestinal tract of quails [29]. The experiments were performed with lactobacillus hydrolysates obtained by thermal acid method and biological (fermentative) method.

We studied the influence of biologicals on organism immune status, lipid peroxidation (LPO), endogenic intoxication indices and general functional performances of laboratory animals upon intoxication by carbon tetrachloride.

The experiments were carried out with laboratory rodents [30]. Using paired comparison method three groups of animals, ten heads each, were formed: group 1 – reference animals receiving physiological salt solution; group 2 – test animas receiving hydrolysate produced by thermal acid method in amount of 2.0 ml/kg; group 3 – test animals receiving hydrolysate produced by fermentative method in amount of 2.0 ml/kg.

The biologicals were added orally. The doses were calculated with accounting for published data on effective therapeutic amounts of Licopid and human-to-rat conversion factor. The test animals received the biologicals during 14 days. Healthy reproductive animals after 14-day quarantine were selected for the experiments. The animal management was carried out in accordance with the regulations on arrangement, equipment and maintenance of experimental biological clinics (vivariums). The amount of added liquid in all groups was the same and equaled to 10.0 ml/kg.

The experiments were comprised of two stages:

Stage 1 – study of influence of course biologicals' addition on weight of animals before and after intoxication.

Stage 2 – study of efficiency of course biologicals' addition aiming at correction of violations caused by intoxication by carbon tetrachloride.

High toxicity of carbon tetrachloride characterizes it as stressing factor of chemical essence. It is known that under stress a set of adjustments appears known as general adaptive syndrome. Organism adaptation to harmful factor is accompanied by weight decrease of thymus and spleen as a consequence of death of differentiated lymphocytes.

Experimental intoxication was achieved by intragastric injection of carbon tetrachloride in amount of 1.5 mg/kg in the form of 50% solution in sunflower oil. Prior to simulation of pathology the rats of test groups received the studied substances in

10 days. The animals of reference group received physiological salt solution according to similar schedule. After simulation the animals received either the studied substances (test groups), or physiological salt solution (reference group).

In five days after intoxication the animals were decapitated under ether anesthesia. Biochemical indices were determined in the obtained serum as follows:

– LPO activation (content of TBA-reactive substances in liver);

– middle molecules;

– variation of relative weight of immune competent organs (spleen, thymus);

– humoral immunity indices: activity of lysozyme, β-lysine, serum bactericidal activity (SBA).

– cell immunity indices: phagocytic activities of leucocytes (PAL), phagocytic index (PhI), leucocyte phagocytic index (LPI).

Integrative indices were as follows: variations of body weight as well as relative weight of immune competent organs (spleen, thymus) – organ weight, mg to body weight, 100 mg ratio.

Variation of LPO intensity was estimated by variation of level of LPO secondary product − malondialdehyde (MDA). The method is based on the fact that upon heating in acid medium MDA reacts with 2-TBA forming colored pink trimethine complex with maximum adsorption at 535 nm.

Protein in serum of laboratory animals was detected by biuret method.

Lysozyme activity in serum was detected using nephelometric method by lysis of *Micrococcuslysodeicikus*. Activity percent was calculated by difference of light transmittance of the considered mixture after incubation and that of initial mixture.

Activity of β-lysines in serum was detected using rapid nephelometric method by reduction of optical density of the considered mixture in *Bacillus subtilis* before and after incubation.

SBA was determined using photonephelometric method by difference between death percent of *E. coli* daily culture in test sample with physiological salt solution and test sample with serum.

Phagocytic activity of neutrophilic leucocytes was determined using the technique based on incubation of heparinized blood with billionth suspension of *E. coli* in meat infusion broth with subsequent deposition using centrifugation of leucocyte bulk and obtaining fixed and colored films on specimen glass on its basis according to Romanovsly–Gimsa. One hundred cells were counted in a film and phagocytosis indices were calculated:

– PAL − percent of neutrophilic leucocytes participating in phagocytosis;

– PHI − number of cells with completed phagocytosis per 100 segmentonuclear neutrophils participating in phagocytic activity;

– LPI, Wright's index − average number of phagocytic microbes per one neutrophil;

– opsonic index of absorption − PAL of test animal group to PAL of intact animals.

The experimental results were processed using statistical methods detecting arithmetic average (M), mean error of arithmetic average (m) and error probability according to Student's table.

RESULTS AND DISCUSSION

Preliminary selection of lactobacillus hydrolysis based on biologically active substances

Previous experiments on selection of lactobacillus hydrolysis providing maximum yield of biologically active substances revealed that thermal acid method made it possible to obtain lysates with maximum content of total protein, peptides with molecular weight < 1500, protein and peptides with molecular weight > 1500, amino acids as well as GMDP (glucosaminylmuramyl dipeptide) (Table 1).

It was established that the best results were obtained upon lysis of *Lb. intermedius* cells. Upon thermal acid method the content of amino acids was higher than upon hydrolysis of *Lb. Agilis* and *Lb. salivarius* by 8.3 and 6.3%, peptides with low molecular weight - by 14.5 and 12.6%, peptides with high molecular weight - by 3.8 and 5.1%, respectively. GMDP content should be especially mentioned as the major compound providing immune stimulation, its amount was higher in hydrolysate derived from *Lb. Intermedius* cells in comparison with *Lb. agilis* and *Lb. Salivarius* by 1.3 and 1.9 times, respectively.

Analysis of hydrolysates obtained by fermentative method demonstrated that in terms of quantitative content of the considered substances the best results were obtained in lysate derived from *Lb. intermedius*. Herewith, the content of amino acids was higher than that upon hydrolysis of *Lb. Agilis* and *Lb. Salivarius* by 11.1 and 8.7%, peptides with low molecular weight - by 1.8 and 1.3 times, peptides with high molecular weight - by 13.2 and 8.6%, respectively. GMDP content should be especially mentioned which was higher in hydrolysate derived from *Lb. Intermedius* cells in comparison with *Lb. Agilis* and *Lb. Salivarius* by 2.3 and 1.8 times, respectively.

Therefore, the thermal acid method makes it possible to obtain hydrolysates with high content of biologically active substances. The best results were obtained in analysis of hydrolysate derived from *Lb. intermedius*. The obtained hydrolysate was used as initial substance upon development of immune stimulator of microbial origin for poultry breeding. Despite the fact that in hydrolysate obtained by thermal acid method, the content of biologically active substances was higher than that of fermentative technique, it was decided to determine their biological impact on laboratory animals in order to select the best hydrolysate.

Table 1. Analysis of the best hydrolysates lactobacillus obtained by thermal acid and fermentative methods

Lactobacillus hydrolysate	Index				
	Amino acids, g/100 ml	Total protein, g/100 ml	Peptides with molecular weight < 1500, g/100 ml	Peptides with molecular weight > 1500, g/100 ml	GMDP, g/100 ml
Thermal acid method					
Lb. agilis	3.25	5.68	2.35	2.38	0.063
Lb. intermedius	3.52	5.21	2.69	2.47	0.082
Lb. salivarius	3.31	5.45	2.39	2.35	0.043
Fermentative method					
Lb. agilis	2.36	4.63	1.31	1.67	0.023
Lb. intermedius	2.62	4.13	2.45	1.89	0.054
Lb. salivarius	2.41	4.33	1.82	1.74	0.030

Table 2. Effect of biologicals on growth and development of laboratory rodents without intoxication

Group	Body weight, g		Increment per test, g	Survivability, %
	Start	End		
Reference group 1	188.36±1.01	203.52±1.03	15.16	100
Test group 2	186.72±1.12	211.36±1.15*	24.64	100
Test group 3	186.56±1.07	206.38±1.02	19.82	100
* Relevant difference with reference (P < 0.05)				

Influence of course addition of biologicals per body weight of test animals under experimentally normal conditions

Body weight is an integrative index reflecting state of living organism. Hence, the first stage was devoted to analysis of influence of biologicals on variation of body weight of experimental animals without abnormalities.

It has been determined experimentally that in ten days addition of lactobacillus hydrolysates results in weight increase of test laboratory rodents without abnormalities (Table 2).

The experimental results demonstrated that in test groups the body weight was higher than in reference group. However, it should be mentioned that only in test group 2 the relevant difference (P < 0.05) with reference group was observed, and in test group 3 there was a trend to weight increase. At the end of experiments the body weight of laboratory rodents in test group 2 was higher than that in reference group by 7.84 g or 3.9%. Whereas in test group 3 this index in comparison with the reference group was 2.86 g or 1.4%. The difference between test groups was 4.98 g or 2.4% in favor of group 2 where lactobacillus hydrolysate was used obtained by thermal acid method.

Influence of course addition of biologicals per body weight of test animals upon intoxication by carbon tetrachloride

In order to analyze the influence of course addition of biologicals on body weight upon intoxication, toxicant was added to laboratory rodents (carbon tetrachloride). The influence of biologicals upon toxicant addition is summarized in Table 3.

Table 3. Effect of biologicals on growth and development of laboratory rodents upon intoxication

Group	Body weight, g	
	Start	End
Reference group 1	181.65±1.03	171.24±1.01
Test group 2	182.21±1.06	178.60±1.02
Test group 3	181.42±1.02	175.42±1.02

It is established that upon addition of carbon tetrachloride the body weight of rodent rats in reference group decreases. However, application of biologicals constrained to various extent the weight loss of test animals caused by toxic agent. Thus, in test group 1 at the end of experiment the body weight decreased in comparison with initial value by 10.41 g or 5.7%; in test group 2 it decreased by 3.61 g or 1.9%, and in test group 3 - by 6.0 g or 3.3%. Herewith, it should be mentioned that the use of hydrolysate obtained by thermal acid method in test group 2 demonstrated the best influence on organism of laboratory rodents.

Studying efficiency of preventive addition of hydrolysates aiming at correction of violations caused by intoxication by carbon tetrachloride

It is established experimentally that the animals of test groups after injection of hydrolysates were characterized by constraints of hyperalbuminosis which was detected in animals of reference group. In addition, injection of biologicals constrained accumulation of TBA-reactive substances in liver: the target organ for carbon tetrachloride (Table 4).

Table 4. Effect of biologicals on biochemical blood properties of laboratory animals

Group	Total protein, mmole/l	TBA-reactive substances, μmole/g	Middle molecules
Reference group 1	96.7±1.0	8.3±0.2	0.058±0.010
Test group 2	85.4±1.0	7.1±0.1	0.051±0.007
Test group 3	89.2±0.9	7.4±0.1	0.053±0.008

The obtained results evidence capability of the considered biologicals to exert membrane protecting effect on liver cells upon injection of intoxicant. It is established that the considered hydrolysates to various extent prevent increase in middle molecules. Thus, in test groups 2 and 3 the number of middle molecules was lower than in reference group 1 by 12.1 and 8.6%, respectively.

While analyzing the influence of the considered substances on weight of immune competent organs upon intoxication by carbon tetrachloride, it has been established that in the reference group injection of carbon tetrachloride resulted in decrease in relative weight of spleen, thymus involution, whereas addition of biologicals prevented decrease in relative weight of both spleen and thymus (Table 5).

Table 5. Relative weight of organs (mg/100 g)

Group	Spleen	Thymus
Reference group 1	353.3±11.2	34.2±1.2
Test group 2	413.2±12.1	41.3±1.3
Test group 3	416.3±12.4	42.1±1.3

Steady weight of spleen upon addition of biologicals evidences the absence of significant variations caused by this organ. This is very important since it is known that phagocytizing mononuclears of spleen capture xenobiotics and its lymphocytes and plasma cells participate in immune response promoting removal of foreign agents from organism. In addition, spleen cells produce immunoglobulins.

It is also established that application of biologicals constrains decrease in relative weight of thymus: the organ which controls both cellular (differentiation of lymphocytes) and humoral immunity (generation of antibodies), which evidences less pronounced exhaustion of immune system of rats in test groups 2 and 3.

While estimating the influence of lactobacillus hydrolysates on immune reactivity of laboratory rodents, it has been established that course application of the considered substances does not have significant influence on SBA, activity of β-lysine and lysozyme in comparison with the reference group upon simulation of intoxication (Table 6).

Table 6. Influence of biologicals on immune status of laboratory animals

Group	SBA,%	Lysozyme, % activity	β-lysine, % activity
Reference group 1	35.1±1.1	49.5±1.5	36.7±1.0
Test group 2	34.2±1.2	50.1±1.4	35.7±1.3
Test group 3	34.8±1.1	49.9±1.7	38.2±1.1

However, significant variations of test animals with regard to reference animals were observed concerning cell immunity (Table 7).

Table 7. Influence on phagocytic activity

Group	PAL,%	PhI,%	LPI, units
Reference group 1	28.3±1.0	31.1±1.1	0.32±0.01
Test group 2	41.3±1.2*	41.4±1.1*	0.44±0.01*
Test group 3	32.1±1.1	39.5±1.0*	0.39±0.01*
*Relevant difference with reference (P < 0.05)			

Thus, the course addition of all considered substances activates PAL, and hydrolysate in group 2 has more pronounced impact on this property than in test group 3; this index increased statistically relevant in comparison with reference group. In addition, it is established that the course addition of the considered hydrolysates has higher statistically valid influence on PhI. It should be mentioned that this index in reference group was significantly lower than that of animals of test groups at $P < 0.05$. Positive distinctions upon the use of the considered substances aiming at correction of violations caused by addition of toxic agent were revealed also upon analysis of LPI. Thus, it was experimentally established that upon addition of hydrolysate obtained by thermal acid methodб its influence in test group 2 was more efficient on phagocytic activity of neutrophils in comparison with hydrolysate obtained by fermentative method.

CONCLUSIONS

The performed studies demonstrated that lactobacillus hydrolysate derived by thermal acid method from *Lactobacillus intermedius* contained biologically active substances, in particular GMDP to the highest extent. Thus, the GMDP content in the best lactobacillus hydrolysate was 0.082 g/100 ml, in average by 11.5% higher than in other obtained biologicals. On the basis of pharmacological (biological) tests, the hydrolysate obtained by thermal acid method exerted the best influence on immune status of laboratory animals both upon addition of toxicant and without it. Oral addition of hydrolysates in the range of experimental norm promoted weight increase of test animals by 1.4 and 3.9%. Upon addition of carbon tetrachloride, the weight of test animals decreased, however, addition of hydrolysates constrained weight loss of test animals caused by toxic agent. Therefore, the experimental results evidence reasonability of further experiments of application of lactobacillus hydrolysate derived by thermal acid method from *Lactobacillus intermedius* cells for treatment and prevention of immune deficiency.

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