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RESEARCH ARTICLE

INFLUENCE OF LYSOZYME ON ADHESION OF *BIFIDOBACTERIUM BIFIDUM 791, BIFIDOBACTERIUM BIFIDUM LWA-3, BIFIDOBACTERIUM ANIMALIS SUBSP. LACTIS BB-12* IN EXPERIENCE IN VITRO ON HUMAN ERYTHROCYTES

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ABSTRACT

Aim: To determine the adhesive properties of strains of *Bifidobacterium bifidum* 791, *Bifidobacterium bifidum LVA-3*, *Bifidobacterium animalis subsp. lactis BB-12* and the effect of egg lysozyme on their adhesive activity in vitro experiments on human erythrocytes. **Material and Methods:** The bacterial adhesion and the effect of lysozyme in the concentrations range from 3.13 to 1500 μg ml is determined on human erythrocytes I (0) Rh (+) blood group. The average adhesion index (AAI) was determined - the average number of bacteria adhered on one erythrocyte, "K" - the percentage of erythrocytes adhering the bacteria. **Results:** *B. bifidum 791* is highly adhesive (AAI = 4.1 ± 0.113), *B. bifidum LVA-3* is low-adhesion (AAI = 2.43 ± 0.073) and *B. animalis BB-12* is medium-adhesiv (AAI =3.16 ± 0.053). The "K" was 65.4 ± 3.13%, 51.6 ± 2.16%, 47.0 ± 2.28%, respectively Lysozyme was enhanced the adhesion of these strains by 21-80.4% in a wide range of concentrations and with different intensity, individual for each strain. "K" was increased with concentrations of lysozyme from 6.3 to 1000 μg / ml, and AAI was increased for *B. bifidum 791* at 100 - 1500 μg / ml, the increase of "K" was for *B. bifidum LVA-3* at 100-1000 μg ml and the increase of AAI was at 25-1500 μg ml of lysozyme, and for *B. animalis BB-12* was "K" at 50-1000 μg/ml and AAI at 12.5-1500 μg/ml. **Conclusions:** Inclusion of lysozyme in the composition of probiotics can enhance their pharmacological effect.

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INTRODUCTION

Bifidobacteria. are the main protective beneficial microorganisms of the intestine, which include probiotics (Blokhin, 2016; Deshpande *et al*., 2017; Di Mauro *et al*., 2013; http://www.fao.org/tempref/ docrep/fao/009/ a0512e/ a0512e00.pdf). They have a very high aggressiveness against pathogenic bacteria, participate in the digestion process, reduce cholesterol, stimulate the immune system of the human's organism (Banai, 1978; Brilis, 1986; Montville *et al*., 2003). Normal microflora forms a protective "screen" on the surface of the mucous membrane, which prevents the adhesion of pathogenic microorganisms and the incipience of the inflammatory process (Banai, 1978; Gagnon *et al*., 2016; Ewaschuk *et al*., 2008; Rao, 2013). However, probiotics do not always have a rapid therapeutic effect.

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This may be due to the fact that strains of bifidobacteria are weakly attached to the surface of the intestinal mucosa, i.e. microbes have insufficient adhesiveness (Andryushchenko, 2015; Baranov, 1999). It is known that lysozyme stimulates the adhesion of some strains of bifidobacteria. The inclusion of lysozyme in the probiotic gives the combined therapeutic effect to the drug against pathogens (Andryushchenko *et al*., 2015; Baranov, 1999). It is important to study the adhesive properties of bifidobacteria, namely *Bifidobacterium bifidum 791 (B. bifidum 791)*, *Bifidobacterium bifidum LVA-3 (B. bifidum ЛВА-3,)*, *Bifidobacterium animalis subsp. lactis BB-12 (B. animalis BB-12*), included in the composition of medicines, and the possible influence of lysozyme on their adhesion to enhance the therapeutic effect.

Aim of the study: To study the adhesive properties of strains *B. bifidum 791*, *B. bifidum LVA-3*, *B. animalis subsp. lactis BB-12* and the effect of lysozyme on their adhesive process in vitro experiments on human erythrocytes.

MATERIALS AND METHODS

Various models can be used to assess the level of adhesion of microorganisms: erythrocytes, epithelial cells, tissue cultures, animals-gnotobionts, polymeric carriers (Banai, 1978; Ewaschuk *et al*., 2008; Vazquez-Gutierrez *et al*., 2016). Currently, erythrocytes are a universal model for studying the adhesion of microorganisms. They have glycophorin on their surface - a substance identical to the glycocalyx of epithelial cells, on which the receptors for the adhesins of microbes are located (Banai, 1978; Brilis *et al*., 1986). Adhesive activity of bifidobacteria strains *B. bifidum 791*, *B. bifidum LVA-3*, *B. animalis BB-12* studied by the method on native human erythrocytes O(I) Rh(+) blood group (Brilis, 1986). This method is a modification of the earlier method (Banai, 1978). Erythrocytes were washed three times with 0.1 M phosphate buffer solution (pH 7.2) by centrifugation (at 370 x g for 5 min) and prepared suspension of erythrocytes at a concentration of 10^8 cells/ml. A slurry of each lyophilized strain of bifidobacteria was prepared in physiological saline with a concentration of microbes 10^9 per ml.

The amount of 0.45 ml of a suspension of erythrocytes and a suspension of microorganisms were mixed in a test tube. Then 0.1 ml of physiological solution (control) was added to the control tube, and 0.1 ml of one of the lysozyme solutions (test) was added to the test tubes. The experiments used lyophilized egg lysozyme in the final sub-therapeutic and therapeutic concentrations: 3.1; 6.25; 12.5; 25; 50; 100; 200; 400; 800 and 1500 μg / ml. The tubes were incubated in a thermostat at 37 ° C on a shaker for 30 minutes. Then preparations for microscopy were prepared. A thin smear from a suspension of erythrocytes and microorganisms from control and experimental tubes was made on the slide. The drug was air-dried, fixed with a mixture of diethyl ether and ethanol 96% in a 1: 1 ratio, washed with water, and then stained for 20 minutes at room temperature with the coloring of Romanovsky-Giemsa (Chernoshey, 2016). According to the method, adhesive activity of each experimental culture of microorganisms was assessed by immersion microscopy in two respects: I) by the average adhesion index (AAI) - the number of adherent microbes on one erythrocyte participating in the adhesive process. In this case, the microorganism was considered unadhesed if the AAI ≤1.75, low-adhesion - AAI - from 1.76 to 2.5, medium-adherent - AAI from 2.51 to 4.0 and highly adherent at a AAI above 4.0; II) by the coefficient ("K") of the participation of erythrocytes in the adhesive process, which is the percentage of erythrocytes having on their surface adhered microbes. For each concentration of lysozyme experience, as well as the control, were set in 5 parallels and the average value of "K" and "AAI" was calculated. The inhibitory or stimulating effect of the drug on the adhesion of the microorganism was also determined by the formula:

$E_{AAI=}(AAIe - AAI_K) \times 100/AAI_K$;
$E_{K=}(Ke-K_K)x100/K_K$,

where: E_{AAI} and E_K - indices of the inhibitory or stimulating effect of the lysozyme preparation, AAIe and Ke - the adhesion of the experience, AAI_K and K_K - the adhesion of the control. The inhibitory effect is characterized by a negative sign of the numbers "$-E_{AAI}$" and "$-E_K$", the stimulating effect is a positive sign of the numbers "$+E_{AAI}$" and "$+E_K$". Essential differences were considered between the parameters of the control experiment with the addition of physiological saline and lysozyme supplementation, which differed by 20% or more ($p < 0.05$) (6). The statistical analysis was performed using Microsoft Excel computer program. The calculation of statistical indicators “M”, “σ”, “m”, “t” and “p”. The critical value of significance level was considered p=0.05.

RESULTS

The results of the investigation of the adhesive properties of microorganisms are presented in Tables 1-4. As can be seen from Table 1, *B. bifidum 791* strain was highly adhesive, *B. bifidum LVA-3* was low-adhesion and *B. animalis BB-12* was medium-adhesive. As can be seen from Table 2, the highest "K" was in *B. bifidum 791*, and the smallest was in *B. animalis BB-12*. As can be seen from Tables 1-4, lysozyme exerted a stimulating effect on the adhesion of all strains studied. This process depended on the dose of the preparation and the individual properties of the strains of *Bifidobacterium spp*. As can be seen from Table 1, a significant increase in AAI for *B. bifidum 7*91 was in the range of lysozyme concentrations from 50 to 1500 μg / ml, for *B. bifidum LVA*-3 - from 25 to 1500 μg / ml, for *B. animalis BB-12* - from 6.25 to 1500 μg / ml. According to the AAI index, the strengthening of adhesion by lysozyme was at concentrations from 100 to 1500 μg / ml for all studied strains of *Bifidobacterium spp.* The *B. animalis BB-12* strain became highly adhesive (AAI≥4.0) within the concentration of lysozyme from 50 to 1000 μg / ml. There was no significant decrease in the AAI index in the presence of the investigated concentrations of lysozyme. It should be noted that there was the same dynamics of changes in AAI in the presence of lysozyme for all strains. Specifically, at first there was a gradual increase in the AAI with the raise in the concentration of lysozyme and with the maximum of the AAI.

The concentration of lysozyme (with AAI maximum) was depended on the type of strain. It was 1000 μg / ml for *B. bifidum 791*, 200 μg / ml for *B. bifidum LVA-3* and 500 μg / ml for *B. animalis BB-12*. Then there was a gradual decrease in the AAI with an increase in the concentration of lysozyme, but this index remained significantly higher than the initial level in the control. As can be seen from Table 2, a significant increase in the AAI (more than 20%) for *B. bifidum 791* was in the range of lysozyme concentrations from 100 to 1500 μg / ml, for *B. bifidum LVA-3* from 25 to 1500 μg / ml , for *B. animalis BB-12* - from 12.5 to 1500 μg / ml. As can be seen from Table 3, a significant increase in "K" for *B. bifidum 791* was in the range of lysozyme concentrations from 3.1 to 1000 μl / ml, for *B. bifidum LVA-3* - from 100 to 1000 μg / ml, for *B. animalis BB-12* - from 12.5 to 1000 μg / ml. Strengthening of adhesion by lysozyme on the "K" index was at concentrations from 100 to 1000 μg / ml for all the investigated strains of *Bifidobacterium spp*. The dynamics of the change in "K" with an increase of lysozyme concentration was similar to the dynamics of changes in the AAI. The maximum "K" was for *B. bifidum 791* and *B. animalis BB-12* at the lysozyme concentration of 500 μg / ml, for *B. bifidum LVA-3* - 100 μg / ml.Noteworthy is the fact that at a lysozyme concentration of 1500 μg / ml, the "K" of all strains decreased to the initial control value. There was no significant reduction in the "K" indicator relative to control in the presence of the studied concentrations of lysozyme. As can be seen from Table 4, a significant increase in "K" (more than 20%) for *B. bifidum 791* was in the range of lysozyme concentrations from 6.25 to 1000 μg / ml, for *B. bifidum LVA-3* - from 100 to 1000 μg / ml, for *B. animalis BB-12* - from 50 to 1000 μg / ml.

Table 1. The average adhesion index (AAI) of Bifidobacterium spp. in the presence of various concentrations of lysozyme (M ± m)

N p/p	Concentration of lysozyme μg / ml	B. bifidum 791	B.bifidum LVA-3	B.animalis BB-12
1	Control	4.10±0.113	2.43±0.073	3.16±0.053
2	Lysozyme 3.13 μg / ml	4.11±0.433	2.42±0.124	3.21±0.139
3	Lysozyme 6.25 μg / ml	4.11±0.119	2.58±0.183	3.56±0.053*
4	Lysozyme 12.5 μg / ml	4.28±0.226	2.71±0.120	3.80±0.156*
5	Lysozyme 25 μg / ml	4.27±0.120	3.05±0.069*	4.10±0.154*
6	Lysozyme 50 μg / ml	4.47±0.030*	3.12±0.058*	4.10±0.088*
7	Lysozyme 100 μg / ml	5.40±0.103*	3.80±0.183*	4.19±0.078*
8	Lysozyme 200 μg / ml	5.40±0.171*	3.48±0.160*	4.69±0.131*
9	Lysozyme 500 μg / ml	5.88±0.264*	3.46±0.111*	4.98±0.096*
10	Lysozyme 1000 μg / ml	5.91±0.345*	2.94±0.048*	4.52±0.138*
11	Lysozyme 1500 μg / ml	5.38±0.162*	2.94±0.183*	3.79±0.174*

Note. The symbol "*" indicates indicators that reliably (p≤0.05) exceed the initial indices of adhesion (in the absence of lysozyme).

Table 2. The stimulating effect of "+ EAAI" different concentrations of lysozyme on the adhesion of Bifidobacterium spp. (change in the average value of the AAI in % of the initial value of the AAI)

N p/p	Concentration of lysozyme μg / ml	B. bifidum 791 «EAAI»%	B. bifidum LVA-3 «EAAI»%	B. animalis BB-12 «EAAI»%
1	Control	(AAI=4.1)	(AAI=2.43)	(AAI=3.16)
2	Lysozyme 3.13 μg / ml	+0.24	0	+1.6
3	Lysozyme 6.25 μg / ml	+0.24	+6.2	+12.7
4	Lysozyme 12.5 μg / ml	+4.4	+11.5	+20.3*
5	Lysozyme 25 μg / ml	+4.4	+25.5*	+29.7*
6	Lysozyme 50 μg / ml	+9.0	+28.4*	+29.7*
7	Lysozyme 100 μg / ml	+31.7*	+56.4*	+32.6*
8	Lysozyme 200 μg / ml	+31.7*	+43.2*	+46.5*
9	Lysozyme 500 μg / ml	+43.4*	+42.4*	+57.6*
10	Lysozyme 1000 μg / ml	+44.1*	+21.0*	+43.0*
11	Lysozyme 1500 μg / ml	+31.2*	+21.0*	+38.7*

Note. The symbol "*" indicates indicators that are 20% or more higher than the initial indices of adhesion (in the absence of lysozyme).

Table 3. The adhesion coefficient "K" of Bifidobacterium spp. in the presence of different concentrations of lysozyme (M ± m) in %

N п/п	Concentration of lysozyme μg / ml	B. bifidum 791	B. bifidum LVA-3	B. animalis BB-12
1	Control	65.4±3.13	51.6±2.16	47.0±2.28
2	Lysozyme 3.13 μg / ml	78.0±1.38*	48.6±2.73	48.4±1.54
3	Lysozyme 6.25 μg / ml	81.6±0.61*	48.8±0.996	52.6±1.73
4	Lysozyme 12.5 μg / ml	83.6±2.07*	47.8±2.29	55.5±0.83
5	Lysozyme 25 μg / ml	83.8±1.11*	54.2±2.50	55.8±0.66*
6	Lysozyme 50 μg / ml	84.4±2.21*	52.8±2.00	57.4±1.25*
7	Lysozyme 100 μg / ml	85.4±1.78*	75.2±0.87*	71.0±1.66*
8	Lysozyme 200 μg / ml	85.8±1.79*	73.8±4.00*	82.4±1.00*
9	Lysozyme 500 μg / ml	91.8±0.71*	73.8±0.87*	84.8±0.66*
10	Lysozyme 1000 μg / ml	85.8±2.53*	73.6±1.08*	76.0±2.46*
11	Lysozyme 1500 μg / ml	68.0±1.09	49.0±0.49	50.2±1.18

Table 4. The stimulating effect of "+ EK" " different concentrations of lysozyme on the adhesion of Bifidobacterium spp. (change in the average value of the adhesion coefficient in % of the initial value "K")

N p/p	Concentration of lysozyme μg / ml	B. bifidum 791	B. bifidum LVA-3	B. animalis BB-12
1	Control	(K=65.4)	(K=51.6)	(K=47.0)
2	Lysozyme 3.13 μg / ml	+19.3	-5.8	+3.0
3	Lysozyme 6.25 μg / ml	+24.8*	-5.4	+11.9
4	Lysozyme 12.5 μg / ml	+27.8*	-7.4	+18.1
5	Lysozyme 25 μg / ml	+29.1*	+5.0	+18.7
6	Lysozyme 50 μg / ml	+30.6*	+2.3	+22.1*
7	Lysozyme 100 μg / ml	+40.4*	+45.7*	+51.1*
8	Lysozyme 200 μg / ml	+31.2*	+43.0*	+74.5*
9	Lysozyme 500 μg / ml	+40.4*	+43.0*	+80.4*
10	Lysozyme 1000 μg / ml	+31.2*	+42.6*	+61.7*
11	Lysozyme 1500 μg / ml	+4.0	-5.0	+6.8

The increase of the two adhesion parameters of *B. bifidum 791* and *B. bifidum LVA-3* strains simultaneously was at the concentration of lysozyme from 100 to 1000μg / ml, and the *B. animalis BB-12* strain was 50 to 1000 μg / ml.

DISCUSSION

Mechanisms of adhesion of microorganisms are very complicated. They have both common and distinctive features in different species. The adhesion of bacteria depends on many factors: 1) various electrostatic forces of attraction or repulsion between the layer of ions surrounding the bacterium and the surface charge; 2) chemical forces of short range (Van der Waals link, hydrogen link, ionic link and covalent link, hydrophobic interaction) (Montville *et al*., 2003). Some bacteria are retained on the surface with filamentous appendages – pilli. Other bacteria produce adhesins.

These are surface proteins that promote the adhesion of bacteria to other cells or surfaces (Coutte *et al*., 2003). Adherent bacteria are retained on the surface and form biofilms. Bacteria inside the biofilm can resort to complex forms of information exchange. The biofilm structure is an ideal environment for gene exchange, intercellular interaction and bacterial growth (Coutte *et al*., 2003; Deshpande, 2017). It is known that the adhesion of bifidobacteria depends on the hydrophobic properties of the cell membrane: the higher the hydrophobicity, the stronger the adhesiveness. Thus, the content of a large number of unsaturated fatty acids (oleic acid and linoleic acid) and branched fatty acids (isoforms) increases the fluidity of the cell membrane of these bacteria, promotes the formation of high hydrophobicity and autoagglutination, provides resistance to peroxide oxidation (Gleinser *et al*., 2012; Reis, 2012; Wang *et al*., 2010; Zakharova *et al*., 2015). The ability of bifidobacteria to produce bioactive factors (polysaccharides, Sialidase enzyme), which are supposedly involved in the adhesion of these microbes (Inturri *et al*., 2017; Nishiyama *et al*., 2017). It can be assumed that lysozyme, possessing antioxidant properties, affects the stability of the bacterial membrane of *Bifidobacterium spp*. (Alimova *et al*., 2016; Andryushchenko, 2015). It is known that lysozyme stimulates the repair of morphological structures and cellular composition of the mucosa of the digestive tract (Deshpande, 2017). However, the mechanism of the influence of lysozyme on adhesion of *Bifidobacterium spp.* is not known and require further study.

Conclusion

Therefore, the egg lysozyme stimulates the adhesion of commercial strains of *B. bifidum 791, B. bifidum LVA-3 and B. animalis BB-12* in vitro in a wide range of subtherapeutic and therapeutic concentrations. The effect of lysozyme on the adhesion of the studied strains of *Bifidobacterium spp.* has a general tendency, specifically, as the concentration of this drug increases, the parameters of AAI, "K", E_{AAI} and E_K, which gradually increase, reach a maximum, and then decrease. However, the intensity of changes in these indicators and the maximum values of the indicators, the range of active concentrations of lysozyme, enhancing adhesion are individual for each strain.The inclusion of lysozyme in the composition of probiotics and synbiotics (drugs, dietary supplements, dietary foods) can enhance their microbiological and clinical effect.

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