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RESEARCH ARTICLE

Effect of Ozone on Antagonistic Activity of Lactobacilli

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Abstract

This article describes study results that confirm the effect of ozone on the antagonistic effect of *Lactobacillus plantarum 8P-A3* against *Bacillus subtilis, Escherichia coli* and *Candida albicans* test cultures. A stable (within 48 hours) dose-dependent effect on lactobacilli antagonistic activity was proven; it was maintained in all used ozone concentrations (from <0.29 mg/L, 0.29 mg/L, 0.38 mg/L). It was found that the intensity of lactic acid formation which is the most important criterion for lactobacilli antagonistic activity is significantly higher at the ozone concentration of <0.29 mg/L than in control samples; herewith the bacterium retains the properties of a strong antagonist. Level of lactic acid at the doses of 0.29 mg/L and 0.38 mg/L is reduced in comparison to control samples, and lactobacilli can be characterized as weak antagonists.

Keywords: *Antagonistic activity of lactobacilli, Antagonistic factors, Ozone, Effect of ozone on microorganisms, Lactic acid.*

Introduction

Much attention is paid currently to the study of the biological properties of lactic acid microorganisms that are widespread in nature and have applied significance of prime importance. It is lactobacilli that are the most important component of most biogeocenoses inhabiting the most diverse biotopes. Moreover, one cannot overestimate the role of lactobacilli as a part of microbial communities that ensures their stability, qualitative and quantitative composition and properties [1, 2].

The most important feature of lactobacilli that allows their surviving in biotopes, also being affected by various exogenous factors, is their antagonistic activity. Phenomenon of antagonistic activity is very common in microorganisms. In the course of the long history of evolution, in the struggle for existence, different types of microbes have developed certain means of struggle against their competitors. These means are very different. Some species displace others with the help of abundant and very fast reproduction. Other species produce specific and nonspecific substances that inhibit the growth of many microbes [3, 4]. Being an antagonist, one species of microbes inhibits the development of other ones, and sometimes completely destroys them.

It is known that the antagonistic activity of bacteria is performed using different molecular mechanisms, and its presenting features depend on a number of factors, first of all, on the diversity of interactions between antagonist and its victim in specific environmental conditions [5, 6]. One of the functions of lactobacilli as a part of the normal microflora of human body is to prevent occupation of biotopes by pathogenic microorganisms; it happens due to their bactericidal and bacteriostatic effect caused

by formation of antibiotic substances, first of all, lactic acid [7].

Mechanism of the antagonistic action of lactic acid is due to the fact that lactic acid by itself has a certain bactericidal effect and, in addition, causes a decrease in medium pH to the values that are unfavorable for many types of microorganisms [8, 9].Over recent years, studying the role of factor variability of the antagonistic activity of microorganisms, including lactobacilli, generates more and more research and practical interest. These factors include temperature, different chemicals, electromagnetic waves, etc. [10, 11, 12].

In this regard, ozone seems to be not without interest. It is increasingly used in different spheres of medicine and for economic purposes [13, 14, 15, 16, 17].Despite the fact that there are many reports on the effect of ozone on various microorganisms, the information is contradictory and points at the fact that ozone in different doses can have exactly opposite effect on the same microbe (from full tolerance to stimulating or depressing) [18, 19].

Considering the unique properties of ozone to initiate the multidirectional variability of the properties of microorganisms depending on dose [38, 39], one cannot exclude its positive or negative effect on lactic acid bacteria. In our opinion, this fact is of particular relevance when using ozone as an exogenous factor that has an effect, by direct contact, on body tissues contaminated with lactobacilli, as well as in the future use of ozone when creating new strains of lactobacilli with predictable beneficial production characteristics. As for the effect of ozone on lactobacilli, no reliable scientific information on this issue could be found in available literature. We set a goal-to study the effect of ozone on the antagonistic effect of lactobacilli against test microorganisms (*Escherichia coli; Bacillus subtilis; Candida albicans*)

Materials and Methods

We used *Lactobacillus plantarum 8P-A3* strain isolated from Lactobacterin drug (Federal State Unitary Enterprise "Microgen Research and Production Association", Russia). This drug is a microbial mass of a live lactobacilli strain with antagonistic activity *Lactobacillus plantarum 8P-A3* lyophilized in cultivation medium which forms part of a probiotic widely used for the treatment of diseases of gastrointestinal tract and of female genital sphere what caused the interest for choosing this strain.

To determine the effect of ozone on the antagonistic activity of this strain, a series of experiments was performed; these tests included preliminary ozonation in selected mode and pouring 4.5 mL of physiological saline into tubes (experiment-60 for each mode, 20 for each strain and control) and adding 0.5 mL of bacterial suspension diluted according to the optical turbidity standard to a value of 0.5 McFarland standard. OZON-OViV universal ozonizer (Institute of Ozone Therapy and Medical Equipment, Ukraine) was used to generate ozone. Ozonation regimes were defined by sparging time (1, 2, 5 minutes) while maintaining a constant rate of 0.5 L/min, power of 100% and temperature (20ºC), specified in user instruction for the ozonizer as values required for water disinfection [20].

Mode-1. Time-1 min, rate-0.5 L/min, power – 100%, temperature – 20°C (± 1°C).

Mode-2. Time-2 min, rate-0.5 L/min, power – 100%, temperature – 20°C (± 1°C).

Mode-3. Time-5 min, rate-0.5 L/min, power – 100%, temperature – 20°C (± 1°C).

Antagonistic activity of studied culture, both ozonated and non-ozonated, was evaluated by the degree of growth inhibition of *Escherichia coli, Bacillus subtilis* and *Candida albicans* test strains, on a Mueller Hinton agar solid medium (Pharmacotherapy Research Center, St. Petersburg, Russia) and Sabouraud Dextrose Agar (Hi Media, India). The choice of test cultures was due to their widespread occurrence in the biotopes of microorganisms, as well as to their frequent use for production (biotechnological) purposes what is of practical importance. Antagonistic properties of both ozonated and non-ozonated (control) lactobacilli cultures were studied by cup test (H.K. Jalali 2016) [21, 22]. In the course of previous studies on defining the amount of residual ozone in saline, it was found that ozone concentration during sparging at a constant rate of 0.5 L/min, power of 100%

and temperature of 20°C for 5 min was 0.38 mg/L, for 2 min-0.29 mg/L. Ozone concentration for sparging during 1 minute was not determined since its values were below the detection limit of iodometric method [23]. Assessment of lactobacilli survival in different modes of ozonation in our previous studies revealed that ozone concentrations exceeding 0.38 mg/mL caused the death of lactobacilli, therefore, further experiments on studying ozone effect on the antagonistic activity of lactobacilli were carried out only in modes 1, 2, 3; in our opinion, concentrations below 0.29 mg/L are of no practical interest [32]. Ozone concentration in saline was measured using iodometric titration [34].

For interpretation and understanding the mechanism of variability of antagonistic activity, we additionally studied the level of lactic acid (Table 2) which directly reflects antagonistic ability of lactic acid bacteria. Lactic acid was determined with spectrophotometric method. 50 µL of studied milk were added to 2 mL of 0.2% solution of iron (III) chloride, then mixed, and then optical density was measured at λ=390 nm. Reaction and measurements were carried out at 25±5°C. Colored solution remains stable for 15 min [24].

Milk + *Lactobacillus plantarum 8P-A* was used as a control for ozonated milk in all cases. 0.5 mL of bacterial suspension diluted according to the optical turbidity standard to 0.5 McFarland standard (manufacturer-HiMedia, India) were added to 4.5 mL of physiological saline previously ozonated in the selected mode and poured into test tubes (Eskom Research and Production Concern OJSC, Russia) (experiment-60 for each mode, 20 for each strain and control).

Lactobacilli contact with ozonated physiological saline lasted 30 seconds since additional and intermediate studies proved that a longer contact of bacteria with ozonated 0.9% NaCl results in its death. Test cultures were preliminarily prepared by inoculation and subsequent selection. After 30 seconds, 0.5 mL of both ozonated and non-ozonated suspension was taken from test tube and transferred to 4.5 mL of MRS-Bouillon liquid medium (Basis) (SIFIN, Berlin). Culture was grown at 39.5±0.5°C for 26 - 28 hours. Growth activity of cultures was monitored.

Isolation and identification of microorganisms was carried out by common methods based on their morphological, tinctorial, cultural and biochemical properties [25, 26, 27].*Escherichia coli, Bacillus subtilis* and *Candida albicans* were cultivated by plating on appropriate medium for the subsequent selection of colonies. After 24 hours, suspensions from the selected bacterial cultures were prepared in physiological saline and brought to 0.5 units of optical turbidity standard [32].

Candida albicans was isolated from rat intestine by cultivation on Candida Agar medium (HiMedia, India) prepared according to the appropriate formulation of manufacturer and intended for quick (within 48 hours) preliminary identification of fungi that were widely met in studies in mycological and clinical microbiological laboratories.

Selective properties of this medium allow identifying *Candida albicans* fungi by color and colony morphology due to the presence of chloramphenicol -it makes *Candida albicans* form green smooth colonies [28, 29]. *Escherichia coli* was isolated from Bioflor liquid complex preparation (Russia) that contains biologically active extracts from soy, vegetables and propolis fermented according to a special technology with *Escherichia coli* bacteria. DS-DIF-ENTERO -1 2 diagnostic test system manufactured by "Diagnosticheskiye Sistemy" Research and Production Association, Russia, was used for biochemical identification and differentiation of *Escherichia coli* [30].

Bacillus subtilis was isolated from BETOM 1.1 N50 CAPS dietary supplement ("Issledovatelskiy Tsentr" Research and Production Company LLC, Russia) by cultivation using the method proposed by Shirshikov (2016) with the use of grain-based nutrient media [31]. Prepared suspensions of *Escherichia coli, Bacillus subtilis* were added to molten and cooled Mueller Hinton agar; 2.5 - 3 mL of suspension per 100 mL of agar.

Then it was poured into Petri dishes with a bottom diameter of 9 cm (30 - 35 ccm of agar in each dish) placed on a strictly horizontal surface; then they were placed in refrigerator for setting solid; after solidification of agar, wells were made with a sterile piercer 7 mm in diameter.

We worked with Candida albicans culture in the same manner using Sabouraud Dextrose Agar solid medium. 0.2 mL of MRS broth with both ozonated and non-ozonated *Lactobacillus plantarum 8P-A3* was added to each well. Cups were placed in TC-1\80 SPU aerobic thermostat (Russia) for 24 hours at the temperature of (39.05±0.5°C); after 24 hours, growth inhibition zones were measured with an accuracy of 1 mm taking into account the diameter of well itself using Axio ZOOM.V16 macroscope (Carl Zeiss Microscopy) and Zena 2012 Pro software (Austria). Amount of lactic acid was determined by spectrophotometric method proposed by L.N. Borschevskaya (2016).

Milk contains all the substances required for the development of lactic acid and other microorganisms: milk sugar, protein, fat, vitamins, amino acids, salts. When using milk as a medium for lactic acid bacteria, their need for nitrogen nutrition and vitamins is largely satisfied; this fact resulted in the choice of milk instead of a nutrient medium. 0.5 mL of bacterial suspension diluted and titrated to 0.5 McFarland turbidity standard were added to experiment and control samples; then they were cultivated at 37.1 +/- 0.1°C for 24 to 48 hours. After 28-48 hours, the level of lactic acid in milk was assessed [24].

Results

Results of this study are shown in Table 1. Study of the effect of both ozonated in selected modes and non-ozonated culture of *Lactobacillus plantarum 8P-A3* on growth inhibition of test cultures demonstrated no antagonistic activity against *Escherichia coli* and *Candida albicans*. However, studied Lactobaci*llus plantarum 8P-A3* strain showed a definite antagonistic effect against *Bacillus subtilis* test culture, both in control and in all experimental modes of ozonation. It was found that inhibition zone in millimeters in mode 1 was larger than in control samples by 3.03%. In mode 2, inhibition zone was 2.30% less compared to the control group. In mode 3, it was 4.35% less compared to the control group; validity of data obtained was defined using Student's test.

When assessing the degree of antagonistic activity of lactobacilli by the criteria of antagonistic activity, we took into account recommendations [34] that suggested that bacteria whose metabolites form growth inhibition zones of test cultures from 10 to 15 mm can be considered as weak antagonists, from 15 to 20 mm-as medium ones, and more than 20 mm-to strong ones. Thus, analysis of data (Table 1) shows that lactobacilli retain their properties of a strong antagonist under the effect of ozone at the dose of <0.29 mg/L as well as in the control group. At the same time, despite retaining antagonistic properties at the concentration of 0.29 mg/L, lactobacillus can be characterized as a weak antagonist at ozone concentration of 0.38 mg/L [33].

Table 1: Antagonistic activity of studied *Lactobacillus plantarum 8P-A3* strain against *Bacillus subtilis, Escherichia coli, Candida albicans* when exposed to ozone in selected modes, M ± m

Mode	n=	Ozone concentration in saline, µg/mL	Ø of test culture growth inhibition zone, mm both in control and in experiment samples.		
			Bacillus subtilis	*Escherichia coli*	*Candida albicans*
1	60	–	15.36±0.37*	-	-
2	60	0.29± 0.0085	14.82±0.53*	-	-
3	60	0.38± 0.0063	14.51±0.62*	-	-
Control	**56**	No ozone	15.17±0.46	-	-

Note: "-"no inhibition; *-difference compared with control samples is statistically significant at P<0.05.

After treating the test milk containing bacterial suspension with ozone-oxygen mixture, a variation in acid activity was registered depending on ozonation mode. Values of lactic acid level obtained in different modes indicate that with an increase in ozone dose within used concentrations (from 0.29 mg/L to 0.38 mg/L) this parameter decreases while at ozone concentrations less than 0.29 mg/L there is a sharp spike in the volume of lactic acid in sample. So, lactic acid values in ozonation mode 1 (<0.29 mg/L) in 24 hours after inoculation amounted to 0.125 ± 0.0081 mg/mL, in 48 hours – 0.449 ± 0.0097 mg/mL. Amount of lactic acid in ozonation mode 2

(0.29 mg/L) in 24 hours was 0.053 ± 0.0059 mg/mL, and in 48 hours – 0.353 ± 0.0083 mg/mL; in mode 3, the content of lactic acid in 24 hours was 0.024 ± 0.0014 mg/mL, and in 48 hours it reached 0.365 ± 0.0037 mg/mL while the concentration of lactic acid in control samples for all selected ozonation modes was 0.109 ± 0.0021 mg/mL in 24 hours, and 0.365 ± 0.0037 mg/mL in 48 hours. Concentration of lactic acid in milk at the time of bacterial suspension adding was 0.043 ± 0.0082 mg/mL. When comparing with the control group, the increase of lactic acid content for mode 1 was 14.77% in 24 hours, and 23% in 48 hours. In mode 2, lactic acid amount was in 24 hours – 51.1% lower, and 48 hours – 3.29% lower than in the control group. In mode 3, the level of lactic acid was lower by 77.34% in 24 hours, and by 14.24% in 48 hours compared with control samples. Thus, the level of lactic acid during ozonation in all concentrations increases up to 48 hours of cultivation what allows observing retaining antagonistic activity when exposed to ozone. Moreover, the amount of lactic acid in mode 1 was significantly higher than in control samples what confirmed the stimulating effect of this ozone concentration on lactobacilli antagonistic activity mechanism.

Table 2: Acid activity of studied *Lactobacillus plantarum 8P-A3* strain in milk, exposed to ozone in selected modes, M ± m

Time, h	Sample	Content of lactic acid, mg/mL, depending on ozonation modes		
		Mode 1	Mode 2	Mode 3
24	Milk+O_3+ L.plantarum 8P-A3	0.125±0.0081	0.053±0.0059	0.024±0.0014
	Control Milk+ L.plantarum 8P-A3	0.109±0.0021	0.109±0.0021	0.109±0.0021
48	Milk +O_3+ L.plantarum 8P-A3	0.449±0.0097	0.353±0.0083	0.313±0.0049
	Control Milk + L. plantarum 8P-A3	0.365±0.0037	0.365±0.0037	0.365±0.0037

* – difference compared with control samples is statistically significant at P<0.05

Discussion

When comparing our data with recent studies of ozone effect on the antagonistic activity of microorganisms, it can be said that when studying ozone effect at the concentrations of 20, 40, and 60 µg/L on the antagonistic activity of lactobacilli against urogenital pathogens, the author *Alia A. Shoeib (2014)* found that ozone has a detrimental effect on normal vaginal flora, especially on the vaginal isolates of *Lactobacillus*.

In our opinion, the author of this study had chosen in this case too high ozone concentrations which have a detrimental effect on bacteria. These doses can be used as an alternative antibacterial therapy as confirmed by the study by *Mingsheng Song (2017)* on the antibacterial effect of ozone in the treatment of skin infections. This study confirms that ozone is one of the strongest antiseptics against most microorganisms [35, 37].

According to *Qiong-Qiong Zhang (2019)*, studying the effect of ozonated water (ozonation at the dose of 80 mg/L) for vagina washing in non-menstrual period revealed that ozonated water used for vaginitis treatment has minor side effects on vaginal microbial ecology and lactobacilli [36]. Comparing ozone concentrations that were used in previous experiments on defining ozone effect on the antagonistic activity and survival of microorganisms, we can conclude that ozone concentrations specified in our study (from <0.29 mg/L, 0.29 mg/L, 0.38 mg/L) have no detrimental effect on lactobacilli. These doses can be considered high-potential for use in medical, scientific and economic purposes and for conservation or targeted modification of lactobacilli (creation of new lactobacilli strains with high antagonistic activity, ensuring the survivability of lactobacilli and controlling its properties in natural biotopes of the body).

Conclusion

Thus, the study performed demonstrated that ozonation of lactobacilli culture in selected doses (from <0.29 mg/L, 0.29 mg/L, 0.38 mg/L) contributed to the retaining antagonistic activity of bacteria. In addition, ozonation of lactobacilli culture at ozone concentrations (from <0.29 mg/L) not only retained the antagonistic activity of studied *Lactobacillus plantarum 8P-A3* strain, but also significantly increased it what was confirmed by the results of measuring growth inhibition zones on Mueller-Hinton agar against *Bacillus subtilis* test culture and by the changes in the level of lactic acid which is the most important factor in lactobacilli antagonistic activity mechanism. In the future, a study of the antagonistic activity of

lactobacilli exposed to ozone can be continued in deeper and more detailed way.

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