Research Article
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Biologically active compounds as a means of changing the metabolic activity of pathogenic clinical strains of *E. coli* and *S. aureus*

Abstract

Microorganisms require nutrients to maintain their physiological functions. Changes in the availability of free amino acids affect the expression of virulence factors, the degree of activity of bacteria, their ability to undergo genetic transformation, and the formation of persistent cells. This study demonstrates that bioactive compounds, such as tryptophan, zinc aspartate, arginine, tatipacin, and taurine, have a modulating effect on the metabolism of microorganisms *in vitro*.

The study found that the metabolic activity of microorganisms increased when exposed to concentrations of bioactive compounds ranging from 0.5–50 µg/ml, particularly in the presence of arginine. Additionally, the presence of BAS in the nutrient medium impacted the growth kinetics of microorganisms. Statistically significant changes were observed compared to the control (without the addition of BAS) after 4 hours of incubation and continued until 36 hours of incubation. The study revealed a significant difference between BAS and control during the 16–24 hour incubation period ($p<0.001$).

The modulation of bacterial activity by BAS can be achieved through various targets and interaction points, such as BAS concentration, the unique cellular structure of the microorganism, its metabolism, and the bacterial pathogen's resistance mechanisms. These findings have broad implications, including the potential for future studies to overcome antibiotic resistance in bacteria.

Keywords: amino acids, *S. aureus*, *E. coli*, antibacterial activity, time-kill analysis

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Introduction

In the last decade, much attention has been paid to optimizing the micronutrient composition for the functioning of body systems. The main regulatory capabilities of biologically active substances (BAS) in relation to the human body are well understood.[1] However, there is little information about the use of key metabolites by microorganisms in a healthy human body and in the presence of various pathological processes.[2]

Amino acids are structural components of the bacterial cell wall, and also regulate cell wall remodeling in the stationary phase.[3] Another aspect of amino acids was recognized in the study of reactivation of *C. difficile* spores. The regulation of spore germination by pseudo proteases is carried out most effectively in the presence of bile acids in combination with amino acids.[4] Recent studies have shown the great role of intercellular communication systems in the development of infectious processes.[5] It's been reported that peptides included in the intercellular microbial communication system named as quorum sensing (QS) consist of various amino acids such as tryptophan, serine, tyrosine, isoleucine, leucine, cysteine, threonine, glutamic acid, glycine, phenylalanine, proline and valine. Peptides-auto-inductors of gram-positive bacteria play a key role in the control of various microbial properties, such as biofilm build-up, antimicrobial resistance etc.[6]

On the other hand, there are reports that amino acids have antibacterial properties.[7] In particular, D-amino acids are reported to exhibit direct broad-spectrum antimicrobial activity. The target for antimicrobial amino acids, as well as for many antibiotics, is the peptidoglycan of the cell wall. Amino acids such as D-methionine, D-tryptophan and D-phenylalanine, in the process of exogenous incorporation into peptidoglycan, replace L-alanine at position 1 and D-alanine at positions 4 and 5 in the terminal position, which leads to modification of peptidoglycan and inhibition of bacteria.[8]

From the above, it can be concluded that microorganisms utilize amino acids as a source of nutrients and energy for their survival.[9] At the same time, amino acids are considered as antimicrobial and antibiofilm agents.[10–13]

The aim of the study was to determine and comparatively analyze the effect of biologically active compounds (tryptophan, zinc aspartate, arginine, tatipacin, taurine) in the concentration range of 0.5-50 µg/ml on the daily growth of *S. aureus* and *E. coli*; to analyze the effect of the presence of different concentrations of these compounds on the metabolic activity of planktonic forms of microorganisms *in vitro* over time.

Materials and methods

Bacterial strains

The object of the study was gram-negative isolates of *Escherichia coli* 2646, and gram-positive isolates of *Staphylococcus aureus* 2738, isolated in clinical trials at Health care institution «Grodno University Clinic» in surgical patients from the wounds. Typing of microorganisms was carried out using such a microbiological analyzer as Vitek 2 Compact produced by bioMérieux.

Biologically active compounds collection

The peculiarities of the metabolism of the examined microorganisms were studied in combination with tryptophan (Trp), zinc aspartate (Zn), arginine (Arg), tatipacin (Tat), taurine (Taur). L-tryptophan ($C_{11}H_{12}N_2O_2$) is an aromatic alpha amino acid in the form of a white powder. Zinc asparaginate ($C_8H_{12}N_2O_8Zn$) is a zinc salt of aspartic acid in the form of a white powder. The mass fraction of zinc in zinc asparaginate is at least 27.5%. Arginine ($C_6H_{14}N_4O_2$) is a partially replaceable aliphatic basic α–amino acid in the form of a white powder. Taurine ($C_2H_7NO_3S$) is a sulfonic acid formed in the body from the amino acid cysteine, which is a white crystalline powder. Tatipacin is a composition of substances (leucine, isoleucine, valine, taurine, thiamine, calcium panthate, zinc sulfate) in the form of white powder. The manufacturer is the State Scientific Institution "Institute of Physical Organic Chemistry of the National Academy of Sciences of Belarus" (Minsk, Belarus).

Microdilution method

Tryptophan, zinc aspartate, arginine, tatipacin, and taurine were investigated in the concentration range of 50-0.5 μg/ml. For this purpose, working solutions were prepared using Mueller-Hinton liquid nutrient medium. The concentration of the working solution was calculated based on the required maximum concentration in a series of serial dilutions.

For inoculation, a daily culture of microorganisms grown on mown meat-peptone agar at a concentration of 1.5×10^8 CFU/ml (0.5 units according to McFarland), diluted 100 times, was used. The final concentration of bacteria in each tube, taking into account the dilution factor of the nutrient medium, was approximately 1.5×10^5 CFU/ml. The test tubes were incubated at a temperature of 35 ± 10° C for 24 hours. To determine the microbial growth, test tubes with crops were viewed in transmitted light, and the optical density of solutions was measured on the turbidity detector of Biosan McFarland DEN-1 suspensions before and after incubation. Also, seeding from test tubes on meat-peptone agar into Petri dishes was carried out to register CFU and exclude possible undesirable turbidity of the studied compounds (24 hours at 35 ± 1°C).

Time-kill analysis

Initially, growth curves were constructed to confirm that the strains reach a stable early or medium logarithmic phase after 4 hours of pre-incubation in Muller-Hinton broth.

The growth kinetics of the bacteria were studied in the presence of 0.5 μg/mL of the test compounds after 0 hours, 4 hours, 8 hours, 16 hours, 24 hours, 36 hours, 2 days, 3 days, 5 days and 8 days. After the microorganisms were exposed to the compounds for a certain time, the bacterial suspension was serially diluted 1:100 and sown on an agarized medium. The number of colonies was counted in all time intervals, and used to calculate back the number that was in the original suspension. The CFU value was compared with the data when measuring turbidity on a densitometer and between groups of the studied substances, expressed in % increase or decrease from the control. The results were visualized on a graph "time-lethal effect."[14]

Statistical analysis

All experiments were conducted by repeating them four times. For statistical analysis of the results obtained in the experiment, the Student's criterion with separated variance estimates (Welch's criterion), as well as descriptive statistics data were used. Comparison of bacterial growth data in groups of the studied substances was carried out using the Kraskell-Wallis analysis of variance. The effects of substances in the dynamics of time by ANOVA analysis. Differences between groups were considered statistically significant at the level of $p<0.05$.

Results and discussion

Results of the micro-dilution method

The studied microorganisms had a different structure of the cell wall [9]. However, the average growth values between the gram-positive bacterium *S. aureus* and gram-negative *E. coli* under the influence of the studied compounds were not significantly different in statistical terms (t=0.28; df=29.36; p=0.80). Among the tested compounds, the mean growth values between *S. aureus* and *E. coli* when analyzing the effects of the sum of mean tenfold dilutions compared to the control group were statistically significantly different only for zinc aspartate t=2.84; df=17.20; p=0.010.

The amino acids arginine, tatipacin, and taurine at a concentration of 50 μg/ml had an inhibitory effect on the tested bacteria. Tryptophan at a concentration of 5-50 μg/ml had modulating effects: it increased the metabolism of *E. coli* and slowed down the metabolism of S. aureus. Zinc aspartate dose-dependently decreased the kinetics of bacterial growth, which is consistent with the data on its antibacterial activity.[15,16]. At concentrations of 0.5 μg/ml, the studied compounds (tryptophan, arginine) didn't show a significant inhibitory effect, but on the contrary contributed to the growth of microorganisms ($p<0.05$). This pattern is observed to a more pronounced extent for arginine and requires study on a larger sample (Table 1).

Table 1 The mean ± SD of bacterial strains under the influence of different concentrations of the investigated substances ($p<0.05$)

Concentration [μg/ml]	Bacterial strains, *S. aureus*, CFU/ml				
	Trp	**Zn**	**Arg**	**Tat**	**Taur**
50	3.9 ± 0.03 #	2.4 ± 0.08 #	5.0 ± 0.06 #	4.2 ± 0.06 #	5.4 ± 0.07 #
5	4.5 ± 0.09 #	3.5 ± 0.06 #	5.4 ± 0.01 #	4.5 ± 0.05 #	5.7 ± 0.01 *
0.5	5.8 ± 0.06 ψ	5.3 ± 0.04 #	6.2 ± 0.06 ψ	5.6 ± 0.05 #	5.4 ± 0.06 #
0	**5.7 ± 0.06**	**5.7 ± 0.05**	**5.7 ± 0.02**	**5.7 ± 0.01**	**5.7 ± 0.05**
	Bacterial strains, *E. coli* CFU/ml				
50	4.8 ± 0.05 ψ	3.3 ± 0.03 #	4.3 ± 0.11 #	4.4 ± 0.07 #	4.0 ± 0.06 #
5	4.7 ± 0.02 ψ	3.5 ± 0.01 #	4.5 ± 0.06 #	4.7 ± 0.05 #	4.7 ± 0.06 ψ
0.5	4.8 ± 0.06 ψ	4.9 ± 0.08 ψ	6.9 ± 0.08 ψ	4.8 ± 0.06 ψ	4.5 ± 0.06 #
0	**4.6 ± 0.03**	**4.6 ± 0.05**	**4.6 ± 0.05**	**4.6 ± 0.02**	**4.6 ± 0.01**

Note: # - Inhibitory effect of the drug relative to the control group;

ψ - Stimulating effect of the drug relative to the control group;

* - No statistically significant changes compared to the control.

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It is known that amino acids are demanding in maintaining the optimal concentration to fulfill the necessary functions in the human body. As a consequence, such a tendency may continue for exogenous amino acids as a nutrition factor for microorganisms.[17]

It is important to note that the availability of nutrients affects the number of persistent cells, the level of which is significantly reduced in the environment free of stress. The proportion of persist in the microbial population is low (one persistent cell per 100 thousand ordinary ones), but they play a key role in preserving the population when exposed to bactericidal doses of antibiotics. The awakening of dormant (persistent) cells due to the abundance of a nutrient substrate before exposure to antibiotics can increase its antibacterial effectiveness.[18]

At this stage of the study, it has been established that BAS have dose-dependent effects on the bacterial growth kinetics (*S. aureus, E. coli*). The identity of metabolic flows leads to competition between body cells and microorganisms for nutrient substrates, which is important in the development of the infection process, and also indicates the need for further more in-depth study of the effects of BAS.

The results of time-kill assay for *S. aureus*

The analysis of the elimination time of the five studied compounds at a concentration of 0.5 μg/ml was compared for *S. aureus*. The comparison showed a statistically significant difference in 4 hours ($p < 0.01$), 8 hours ($p < 0.01$), 16 hours ($p < 0.01$) and 24 hours ($p < 0.001$). The effects of BAS according to the "time-kill" analysis at a concentration of 0.5 μg/ml *for S. aureus* are illustrated in Figure 1.

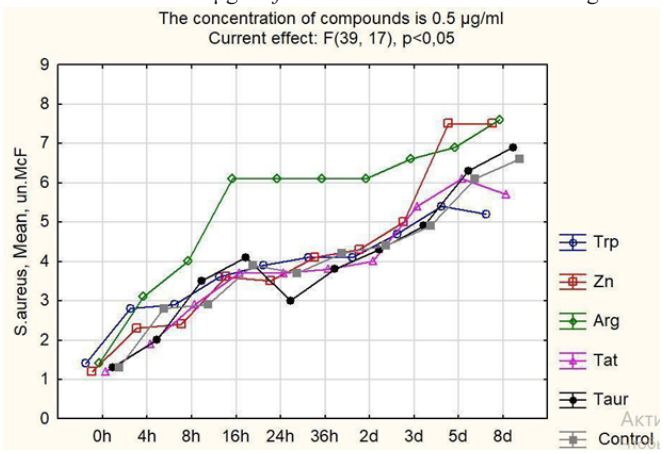

Figure 1 "Time-lethal effect" diagram in the modification of "time-kill analysis" for *S. aureus* (CFU/ml) in the presence of the studied compounds at a concentration of 0,5 μg/ml.

Regarding *S. aureus*, tryptophan exhibits an inhibitory effect of 7.69% compared to the control growth only in 16 hours of incubation; in 24 hours, an increase in growth of 5.41% is observed; within 24 hours – 8 days the growth of *Staphylococcus* in the presence of tryptophan does not differ from the control ($p<0.05$).

In the presence of zinc aspartate, the number of microorganisms decreases by 14.29% of the control value in 4 hours; in 8 hours it decreases by 17.24%; in 16 hours it decreases by 7.69%; in 24 hours it decreases by 5.41%; in 36 hours it decreases by 2.38%; within 3 – 8 days an increase in the growth of microorganisms is observed up to 20% ($p<0.05$).

In the presence of arginine, the growth of *S. aureus* increases by 10.81 % in 4 hours; in 8 hours, the growth increases by 37.93%; in 16 hours, it increases by 56.41%; in 24 hours, it increases by 64.86%; in 36 hours, it increases by 45.24%; within 36 hours – 8 days it increases by 15% ($p<0.05$).

In the presence of tatipacin, bacterial growth has been ±5% on all timescales ($p<0.05$). In the presence of taurine, the growth of *S. aureus* has been 14.29% less than the control value in 4 hours; in 8 hours, the growth has been 13.79% more than the control value; in 16 hours, 5.13% more; in 24 hours, 18.92% less; in 36 hours, 9.52% less; within 36 hours -8 days the growth has been equal to ±5% ($p<0.05$).

Zinc aspartate prevents the growth of *S. aureus* at a concentration of 0.05 μg/ml of zinc on all timescales; the maximum inhibitory effect is observed in 8 hours; arginine promotes growth on all timescales, the maximum effect is observed in 24 hours; tryptophan, taurine and tatipacin in low concentrations have a modulating effect.

The results of time-kill assay for *E. coli*

The analysis of the elimination time of the five studied compounds at a concentration of 0.5 μg/ml was compared for *E. coli.* The comparison showed a statistically significant difference in 4 hours ($p<0.01$), 8 hours ($p<0.01$), 16 hours ($p<0.01$) and 24 hours ($p<0.001$). The effects of BAS according to the time-kill assay at a concentration of 0.5 μg/ml for *E. coli* are shown in Figure 2.

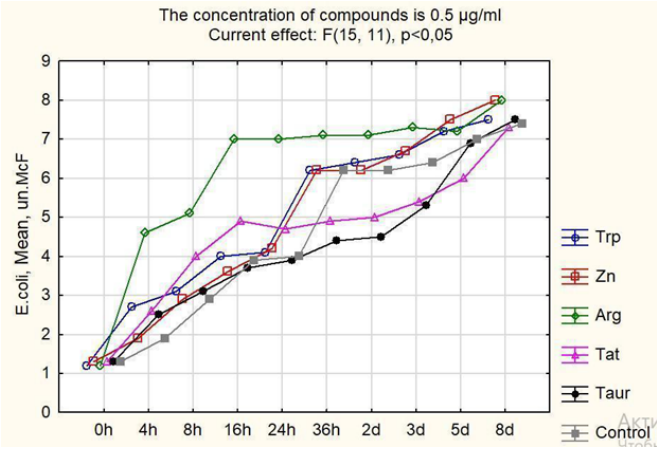

Figure 2 "Time-lethal effect" diagram in the modification of "time-kill analysis" for *E. coli* (CFU/ml) in the presence of the studied compounds at a concentration of 0.5 μg/ml.

In the presence of tryptophan, the growth of *E. coli* increased by 21.05% compared to the control in 4 hours; in 8 hours by 6.90%; in 16 by 2.56%; in 24 hours by 2.50%; in 36 hours by 3.23%; within 36 hours – 8 days the growth was ± 5% ($p<0.05$).

In the presence of zinc aspartate, the growth decreases by 7.69% in 16 hours; in the remaining time periods, the growth is equal to the control ($p < 0.05$).

In the presence of arginine, the growth of *E. coli* increased by 78.95% compared with the control in 4 hours; in 8 hours by 75.86%; in 16 by 79.49%; in 24 hours by 75.00%; in 36 hours by 14.52%; within 36 hours – 8 days it increased by 8% more than the control ($p<0.05$).

In the presence of tatipacin, the growth of *E. coli* increased by 36.84% compared to the control in 4 hours; in 8 hours by 37.93%; in 16 by 25.64%; in 24 hours by 17.50%; in 36 hours, the growth of bacteria in the presence of tatipacin was equal to the control growth; within 36 hours – 8 days the growth was less than the control up to 15% ($p<0.05$).

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In the presence of taurine, the growth of *E. coli* increased by 15.79% compared to the control in 4 hours; in 8 hours by 6.90%; in 16 hours it decreased by 5.13%; in 24 hours it decreased by 2.50%; in 36 hours it decreased by 24.19%, within 36 hours – 8 days the growth was less than the control up to 7% ($p<0.05$).

All the studied BAS increased *E. coli* metabolism at a concentration of 0.05 µg/ml within 0–24 hours. There was a decrease in growth in the presence of tatipacin and taurine in 24 hours.

Based on the results of the "time-kill" analysis, it can be concluded that the most pronounced changes in the effects of BAS in comparison with the control were observed within 16-24 hours ($p<0.001$). After 36 hours of exposure, bacterial growth rates for all the studied substances increased up to the control culture (without adding amino acids).

The data obtained from the time-kill assay confirmed the results of the broth microdilution method: low concentrations of BAS increased the growth kinetics of the bacteria tested, which was truer for *E. coli*. This is consistent with the results of a study on the ability of E. coli to utilize 7 out of 20 amino acids in a compound of equal molarity without other carbon or nitrogen sources.[19]

Bacterial activity modulation by BAS can occur through various targets and interaction points, including the concentration of low molecular weight compounds, the unique cellular structure of the microorganism (level of cell wall permeability), its metabolism (presence of enzymes), and the resistance mechanisms of the bacterial pathogen (efflux mechanisms, biofilm formation), among others.

Conclusion

Tryptophan, taurine, arginine, and tatipacin at concentrations of 50 – 5 µg/ml modified bacterial metabolism ($p>0.05$). At a concentration of 0.5 µg/ml, the studied compounds increase metabolism by increasing the microbial population ($p<0.05$). For a detailed consideration of the effect of low concentrations on the metabolic activity of bacteria, further studies for each individual substance are also needed. Although, many previous studies have shown that BAS have dose-dependent effects on bacterial metabolism, these results require further experiments to confirm their validity and authenticity.

The results of "time-kill" analysis showed the expediency of studying the effects of BAS on bacterial growth kinetics not earlier than 4 hours after the introduction of the compound under study into the medium and not later than 36 hours. The most pronounced effects of the compound were observed in the interval of 16h- 24h.

The data obtained on the effect of BAS on bacterial metabolism suggest a potential impact on the sensitivity of clinically important multidrug-resistant pathogens to antibacterial drugs. It is critical to study both high concentrations of BAS (50 µg/ml or more) and low concentrations (0.5 µg/ml or less). High concentrations of BAS can be considered as adjuvants of antibiotics, increasing their efficacy. In turn, pre-incubation with low concentrations of BAS may increase the metabolism of dormant multidrug-resistant pathogens in biofilms prior to antibiotic therapy. This method can be used to improve the efficacy of antibiotic therapy.

Acknowledgments

None.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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