Testing Neurotransmitters for Toxicity with a Luminescent Biosensor: Implications for Microbial Endocrinology

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Abstract: *Background*: The human organism is a complex superorganism including numerous eukaryotic, eubacterial, and archaean cells. The qualitative and quantitative assessment of the *microbiota toxicity* of chemical agents, i.e., their inhibitory effects on the microbial inhabitants of the human organism in health and disease, seems to hold much value in this context. In this work, a bacterial luminescence-based express test system for microbiota toxicity is applied to neurotransmitters such as serotonin, dopamine, norepinephrine, and histamine.

Methods: The biosensor was based on a GM *Escherichia coli* K12 strain (TGI) that contained the *lux* operon of the luminescent soil bacterium *Photorhabdus luminescencens* ZMI. The biosensor was exposed to the action of the tested neurotransmitters for 5 to 60 minutes The intensity of bacterial luminescence (counts·sec^{-1}) was monitored in the control and the experimental samples with a Biotoks 6 ms luminometer (Russia); the toxicity index (T) of the neurotransmitters was determined.

Results: A marked toxic effect on bioluminescence was produced by serotonin, histamine, and dopamine at concentrations exceeding 80 μg/ml, 100 μg/ml, and 1 mg/ml, respectively. At lower concentration, these neurotransmitters were "negatively toxic", i.e. stimulatory in terms of the effect on bacterial luminescence. In contrast, norepinephrine inhibited luminescence at all concentrations tested.

Conclusions: The bacterial luminescence-based testing method is applicable to the assessment of the destructive and stimulatory effects of neurotransmitters; the data obtained are of microbiological and clinical relevance.

Keywords: Bioassay, bioluminescence, toxicity, neurotransmitters, serotonin, dopamine, histamine, norepinephrine.

INTRODUCTION

In light of recent data and concepts, the human organism is to be regarded as a complex "superorganism", i.e. a symbiotic system including numerous eukaryotic, eubacterial, and archaean cells as well as viruses. Symbiotic microorganisms are invariably present on the skin, the eye conjunctiva, and the mucosa of the upper airways and the urogenital tract. However, it is the microbiota of the gastro-intestinal (GI) tract that is particularly abundant and diverse. The GI microbiota is directly involved in nutrient digestion, xenobiotic metabolism, intestinal motility regulation, resistance to gut colonization by extraneous microorganisms including pathogens [1-3]. Detrimental chemical factors and stressors, if applied at destructive (above-threshold) doses and for inadequate

periods of time, may cause various microecological disorders, increasing the risk of a large number of pathological syndromes and diseases including diabetes, obesity, asthma, cancer, and neuro-psychological problems such as, e.g., autism [3]. Therefore, of paramount importance is developing an adequate and reliable test for the purpose of qualitatively and quantitatively assessing the *microbiota toxicity* of chemical agents, i.e., their inhibitory effects on the microbial inhabitants of the human organism in health and disease.

One of the widely used biological tests for the primary assessment of the effects of chemicals, their mixtures, and physical factors is based upon bacterial luminescence [4-6]. Intact cells of luminescent bacteria are used in this test, and one of its advantages is that it enables estimating the response to the tested factor on the basis of luminescence intensity. Its level is directly correlated with the bacterial cells' metabolic state. The inverse relationship between bacterial luminescence

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intensity and toxic agent concentration is monitored [7-8]. The employment of the bioluminescence-based test system as a bioassay tool is facilitated by a number of its advantageous features: the system is technically simple and easy to use, the assay is quick, its costs are low, the results are in conformity with bioassay data obtained using higher animals as test subjects [7, 9]. Therefore, data on microbiota toxicity are to a large extent concordant with those on integral biological toxicity. Of particular importance is the bio-assessment of the group of substances called neurotransmitters with respect to their microbiota toxicity.

Apart from transmitting impulses from neuron to neuron, these biologically active substances perform a wide variety of regulatory functions in diverse biological systems ranging from microbial cells to the human gastro-intestinal (GI) tract. They significantly influence human neurophysiology, health, and psyche [10-12]. The effects of neurotransmitters on microbial systems are also of much intetest. These effects are either inihibitory or stimulatory. Stimulatory activity with respect to microorganisms may be referred to as negative microbiota toxicity*.* Negative toxicity with regard to the bacterium *Escherichia coli* was revealed with nano- and micromolar concentrations of dopamine, norepinephrine, serotonin, and histamine [13]. Increasing the concentration of, e.g., serotonin results in changing the toxicity sign: it becomes positive, i.e., high serotonin concentration suppress, and not stimulate, *E. coli* growth [14].

The importance of rapid reliable assessment methods for neurotransmitters is highlighted by the fact that neurotransmitters are widely used in practice as neurotropic drugs that are exemplified by acetylcholine, norepinephrine, dopamine, and γ-aminobutyric acid (GABA). These assessment methods would enable us to select the most active preparations and to estimate their therapeutically efficient and toxic concentrations. Presently, it is imperative that highly sensitive tests for evaluating the biological activities and elucidating functionally important properties of natural and synthetic pharmacologically active compounds should be developed. Such tests could be applied, apart from pharmaceutical industry, to the bio-assessment of industrially and domestically used chemicals and to the evaluation of the environmental situation.

The goal of this work was to test the applicability of a bacterial luminescence-based express bioassay to the assessment of the effects of various concentrations of neurotransmitters such as serotonin, dopamine, norepinephrine and histamine–in terms of their microbiota toxicity.

MATERIALS AND METHODS

In this work, we used the genetically engineered strain *Escherichia coli* K12 TGI as the biosensor. Its luminescent phenotype was created by cloning the *lux* operon of the luminescent soil bacterium *Photorhabdus luminescencens* ZMI in this *E. coli* strain. The strain was obtained from the Microbiology Department of the School of Biology, Moscow State University. It is known as the biosensor of the *Ecolyum-08* test system [15]. In this study, freeze-dried biosensor cells were rehydrated in cold distilled water for 30 minutes. The working suspension contained 2.3 - 2.7 x 10^7 cells/ml.

The density of the bacterial suspension was determined nephelometrically (λ = 670 nm) with a KF77 photoelectrocolorimeter, and the number of cells per ml was determined by plotting a calibration curve. The pH values of aqueous test samples were determined potentiometrically.

Analytic grade serotonin creatinine sulfate, dopamine hydrochloride, norepinephrine hydrochloride, and histamine hydrochloride (Sigma, USA) were used in this work; their concentrations were calculated based on the content of the base form of the respective neurotransmitter in each of the chemicals.

To determine the toxicity index of the aqueous solutions of the neurotransmitters, 0.1 ml of the bacterial biosensor suspension were applied to 1.5 ml flasks; 0.9 ml of sterile distilled water or of the sample solution was added to the control and the experimental flasks, respectively.

The intensity of bacterial luminescence (counts / sec) was concomitantly monitored in the control and the experimental samples with a Biotoks 6 luminometer (Russia) at room temperature (20-22 °C); the biosensor was exposed to the action of the tested neurotransmitters for 5 to 60 minutes. The toxicity index (T) of the neurotransmitters was determined automatically by the luminometer that was programmed to use the formula:

$$T = 100 \times (I_k - I) / I_k,$$

where I_k and I are the illumination intensity of the control and the experimental samples, respectively. The tested neurotransmitters were classified into three groups according to their toxicity level: T <20, the agent is non-toxic; T >20 < 50, the agent is toxic; T> 50, the

agent is very toxic [7, 15]. In some studies, the stimulation of luminescence of the test organism occurred, and the T value was considered to be negative. In addition to the toxicity index, we measured the concentrations that caused 20% and 50% inhibition of bacterial luminescence (EC, intensity of luminescence, which varies depending on the concentration of the substance or its volume). The value of EC_{20} is usually referred to as the lower "threshold" toxicity value. The EC_{50} value of a substance was found to be correlated with its LD_{50} value with respect to higher animals [7, 9].

The results of the studies were treated statistically. The reliability of the differences between experimental and control values was assessed using the t-Student criterion.

RESULTS AND DISCUSION

The bacterial luminescence-based bioassay enabled us to estimate the effects of neurotransmitters after different exposure periods (Figures **1-4**). Serotonin and dopamine exerted a stimulatory effect (Figures **1** and **2**) at concentrations up to 10 µg/ml, i.e., approximately 50 µM. The serotonin effect on biosensor luminescence proved to be analogous to that on biomass accumulation, colony-forming unit (CFU) generation, and cell aggregation in *E. coli*. All these processes were stimulated by serotonin at concentrations below 20 µM and inihibited by higher serotonin concentrations [13, 14]. Since high serotonin concentrations (> 50 µM) also suppressed the generation of membrane potential by bacterial membranes, the inhibitory effect of serotonin was attributed to its uncoupling activity with respect to membrane phosphorylation [14]. Analogous uncoupling activity is possible for the other tested amines that also contain an ionizable amino group in the vicinity of the aromatic ring.

Histamine stimulated the biosensor's luminescence at a concentration of 25 µg/ml, i.e., 200 µM (Figure **3**). Norepinephrine inhibited bioluminescence at all tested concentrations (Figure **4**). For each of the neurotransmitters, we determined the non-toxic, toxic, and highly toxic concentration ranges. Serotonin was non-toxic within the 1-60µg/ml concentration range; the toxicity index was ~20.

Histamine was not toxic unless its concentrations exceeded 50 µg/ml. Norepinephrine was toxic at all tested concentrations. High toxicity (T> 50) was observed after incubating the biosensor with at least 1 mg/ml of serotonin for 5 min. The toxicity of dopamine and histamine increased gradually with an increase in exposure time; it reached the maximum level after 60 minutes of incubation with the biosensor. Histamine at a concentration of minimally 100 µg/ml was highly toxic.
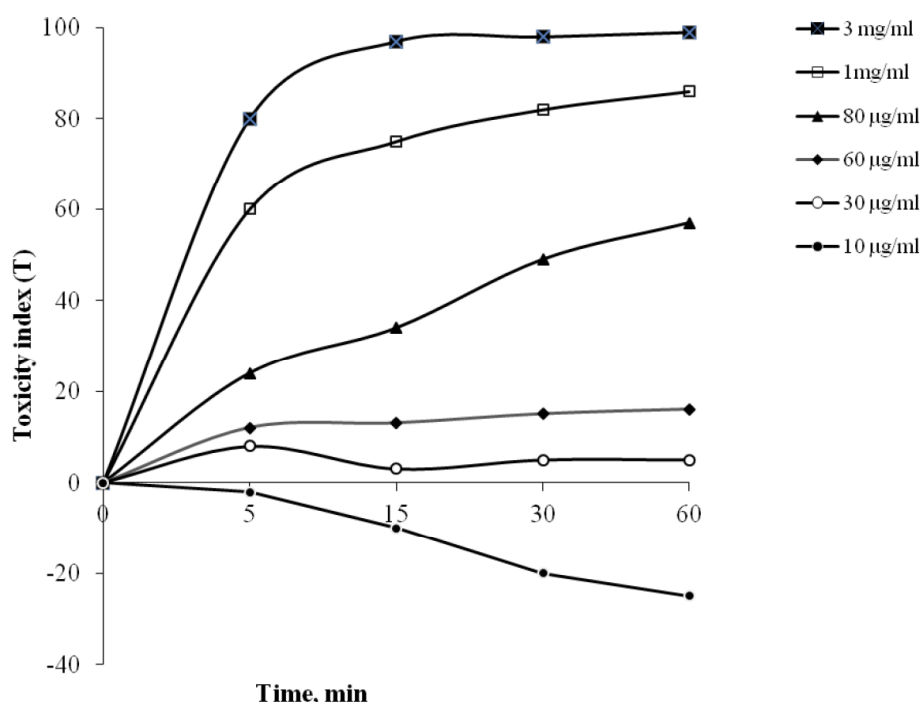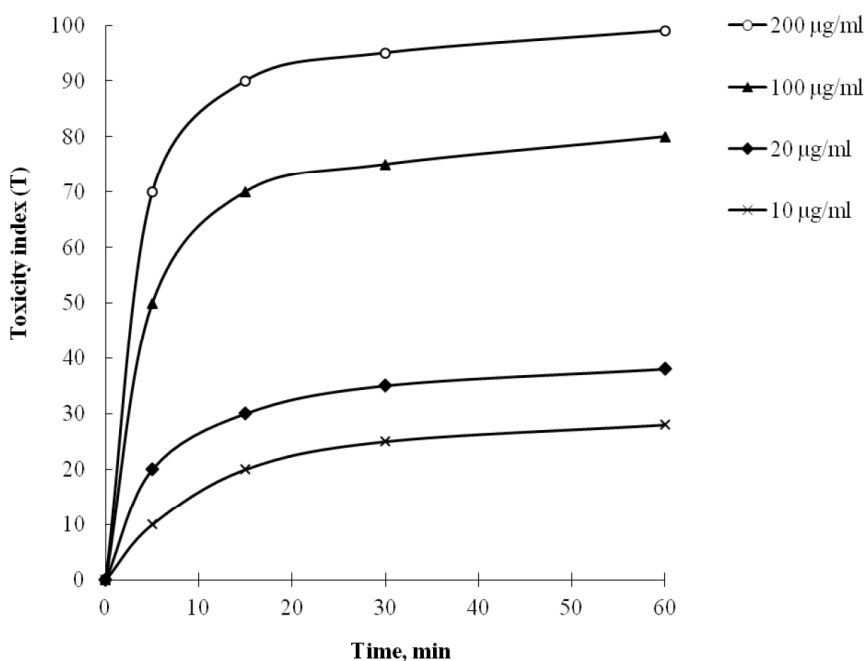

Figure 1: Toxicity of various serotonin concentrations in the bacterial luminescence-based test system plotted against exposure time.
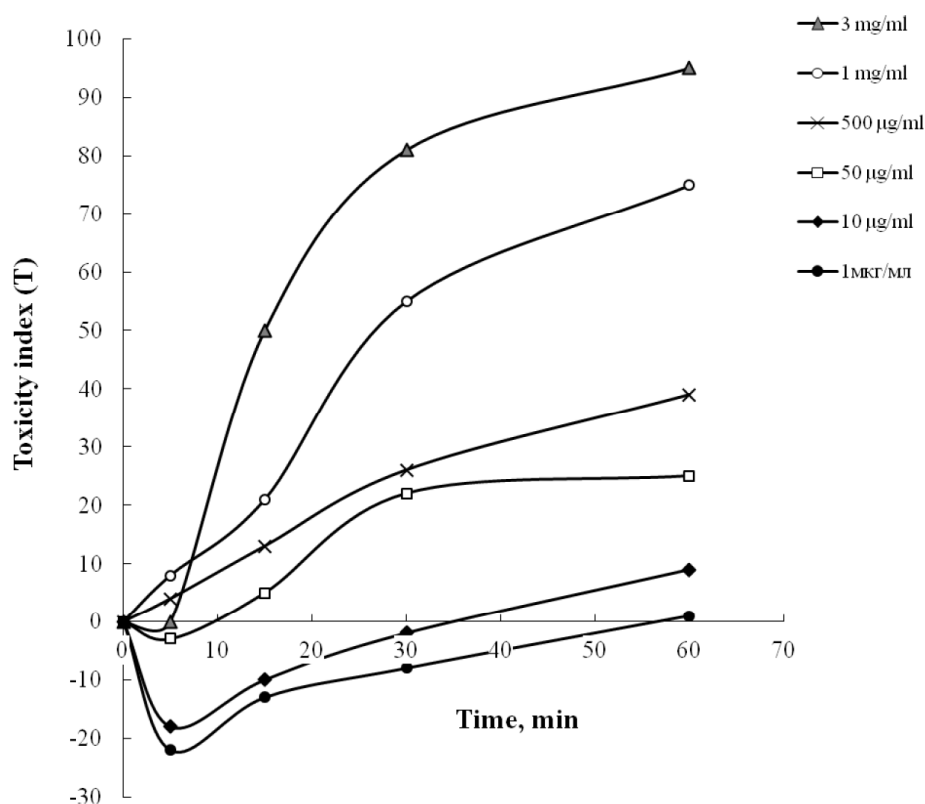
Figure 2: Toxicity of various dopamine concentrations in the bacterial luminescence-based test system plotted against exposure time.
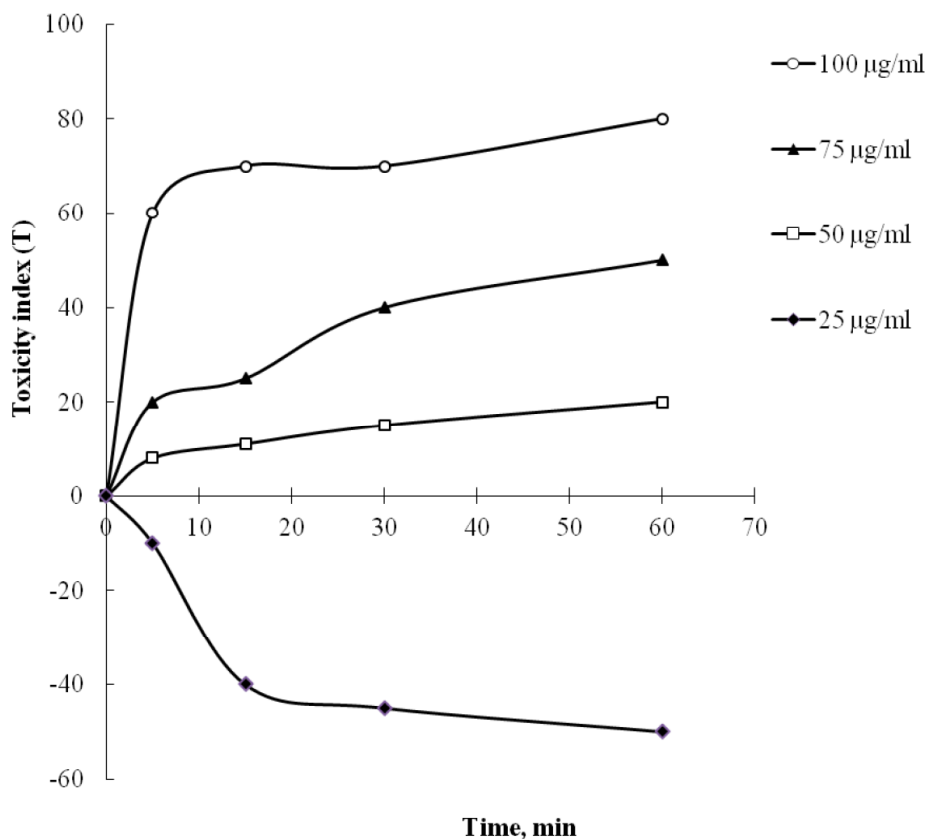
Figure 3: Toxicity of various histamine concentrations in the bacterial luminescence-based test system plotted against exposure time.

Figure 4: Toxicity of various norepinephrine concentrations in the bacterial luminescence-based test system plotted against exposure time.
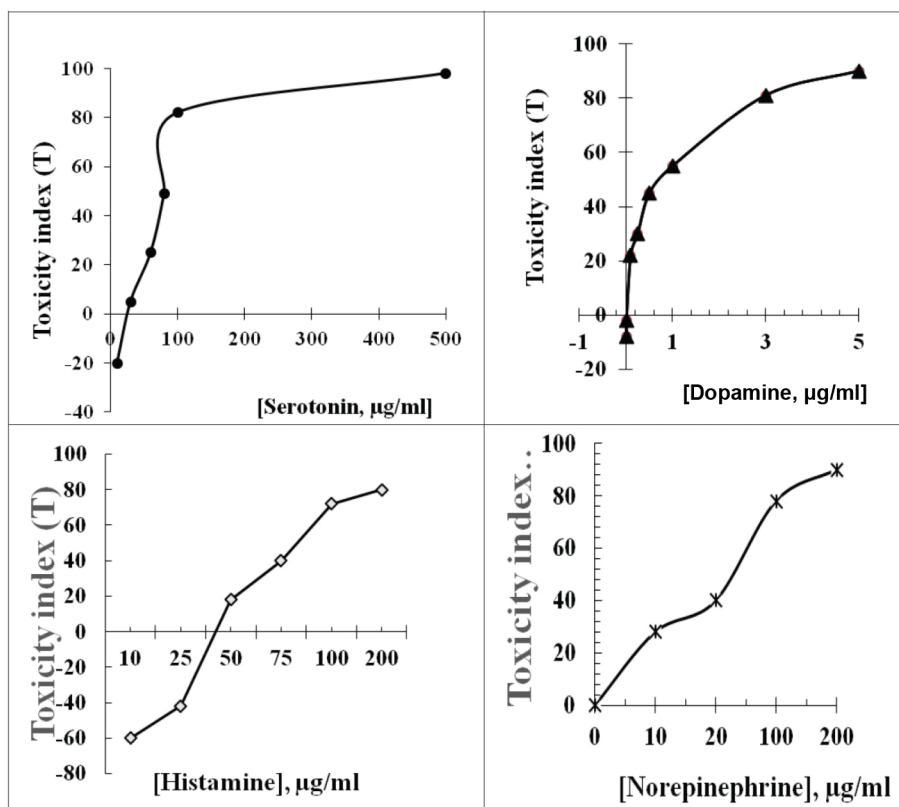
Figure 5: Toxicity indices of the tested neurotransmitters applied at various concentrations after 30 mins of incubation with the biosensor.

The data on the toxicity indices of the tested neurotransmitters at a biosensor exposure time of 30 min are in Figure **5**. They enable us to estimate the activity of the neurotransmitters under study and to compare them.

The EC_{20} and EC_{50} values were calculated for the tested range of neurotransmitter concentrations. It was revealed that dopamine was the least toxic ($EC_{20} \sim 100$ µg/ml; $EC_{50} \sim 500$ µg/ml), and norepinephrine the most toxic agent ($EC_{20} \sim 5$ µg/ml; $EC_{50} \sim 40$ µg/ml). The

corresponding values for serotonin (EC_{20} ~ 45 µg/ml; EC_{50} ~ 80 µg/ml) and histamine (EC_{20} ~ 57 µg/ml; EC_{50} ~ 85 µg/ml) are also shown in Table **1**.

Table 1: EC_{20} and EC_{50} Values of the Tested Neurotransmitters

Neurotransmitters	EC_{20}, µg/ml	EC_{50}, µg/ml
Serotonin	45	80
Dopamine	100	500
Histamine	57	85
Norepinephrine	5	40

The EC_{20} value is routinely referred to as the lower threshold value of toxicity, where as the EC_{50} value is correlated with the LD_{50} level in higher animals. A large number of previous bioassay studies on the toxicity indices of diverse xenobiotics revealed a relationship between the luminescence quenching EC_{50} value in the biosensor model and the corresponding LD_{50} value for eukaryotic organisms. The correlation coefficient is 0.8 to 0.95 [7, 9].

In terms of bio-toxicity, our results indicate that the tested neurotransmitters can be arranged in the following order:

norepinephrine > serotonin ≈ histamine > dopamine [16-18].

For comparison, the literature data on the per os toxicity of biogenic amines for the mouse are as follows (Table **2**).

Table 2: LD_{50} Values of the Neurotransmitters for the Mouse [19-21]

neurotransmitter	LD_{50}, mg/kg (per os administration)
Serotonin	220
Dopamine	1640
Histamine	220
Norepinephrine	26

According to recent literature data, biogenic amines are synthesized and accumulated in microbial cells, including those of *E. coli*, even if the cultivation medium does not contain them. These concentrations are sufficiently high to enable their binding to receptors in animal /human cells [3, 19]. Possibly, the effects that were observed in our test system resulted from the combined action of both exogenous and endogenous (biosensor cell-synthesized) neurotransmitters.

Of much interest are our results on the difference between the toxicity of dopamine and norepinephrine in conjunction with the data on their biological effects [20-21]. Structurally, it is only the presence of an additional hydroxyl radical that distinguishes the molecule of norepinephrine from that of dopamine. Norepinephrine performs important functions in the brain and the sympathetic branch of the peripheral nervous system; there is a long biosynthetic pathway that leads from phenylalanine and tyrosine to dopamine. Under the catalytic influence of β-hydroxylase, dopamine converts to norepinephrine. Taking into account the difference between the effects of the two chemically similar compounds, we suggest that these effects at least partly result from specific interactions involving receptors on the surface of microbial cells that individually recognize neurotransmitters. We demonstrated in our recent studies that dopamine stimulated cell proliferation in the eukaryote *Saccharomyces cerevisiae*, while norepinephrine did not significantly influence this process. The dopamine agonist apomorphine that binds to receptors D_1 and D_2 also was found to stimulate cell proliferation [18, 19].

Thus, the bioassay method that is based on the bacterial luminescence model can be applied for the evaluation of the biological effects of stimulatory and inhibitory concentrations of natural and synthetic neurochemicals. Biogenic amines produce analogous effects on biosensor cell luminescence and on the growth parameters and physiological characteristics of bacterial cells (see the authors' earlier research, [13, 14]), This fact emphasizes that the information obtained with the bioluminescence test is of primary relevance to the physiological effects of neurotransmitters, including their impact on the human microbiota. Therefore, our test technique and the data obtained with its help seem to be of considerable importance with regard to future research concerning human physical and mental health.

CONFLICT OF INTEREST DISCLOSURES

The authors do not have any conflicts of interest.

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