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PROBIOTIC STRAINS OF *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* PRODUCE NEUROACTIVE SUBSTANCES

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Abstract

The symbiotic microbiota of the human organism constantly communicates with the host. An important communication channel is based on neurochemicals including biogenic amines (BAs). Probiotic lactococci with the Generally Recognized as Safe (GRAS) status are involved in producing a variety of fermented dairy products including functional food items for medical purposes. However, their capacity to produce neurochemicals still remains to be explored. In this work, important neuroactive BAs were detected in several strains of *Lactococcus lactis* subsp. *lactis*.

We tested 4 strains of lactococci, 3 of which were isolated from milk and fermented dairy products; strain F-116 was obtained by the cell fusion method. The probiotic activity of these strains was estimated from their antimicrobial effects on pathogenic and opportunistic microorganisms. BAs such as dopamine (DA), noradrenaline (NA), and serotonin (5-HT) were quantitatively determined in the culture liquid and the ultrasonically disintegrated biomass sediment. They were separated using HPLC and detected amperometrically.

NA at nanomolar concentrations was present in the culture liquid and not in the biomass fraction of all tested strains. The two fusion strains with high bacteriocine-producing activity (equivalent to 4200 - 4600 IU/mL nisin) and fungicidal effects (against *Aspergillus* sp., *Candida* sp., and others) contained 50 μM DA in the biomass fraction and low amounts of DA in the culture liquid. The biomass of the fusion strains contained more 5-HT than that of the other tested strains. Almost no 5-HT was detected in the culture liquid.

Thus, the tested probiotic strains produce neuroactive substances that should impact the nervous and the immune system of the host. They can potentially be used as target-oriented functional food items for preventive and therapeutic purposes.

***Key words**: Probiotics, Lactococcus lactis subsp. lactis, bacteriocine, Fungicidal effects, Functional food, neurochemicals, Psychobiotics, Biogenic amines.*

1. Introduction

Lactococcus lactis subsp. *lactis* is a probiotic microorganism. Probiotics were officially defined by FAO/WHO (2001) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". A large number of probiotic microorganisms belong to the genera as: *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*. Besides, representatives of other genera, e.g., *Bacteroides* [1] and various symbiotic (non-pathogenic) strains of *Escherichia coli* (exemplified by the Nissle-1917 strain that was used to develop the probiotic Mutaflor®) conform to the aforementioned definition. More recently, the subgroup of probiotics referred to as psychobiotics has been defined as live organisms that, when ingested in adequate amounts, produce a health benefit in patients suffering from psychiatric illness [2]. A large number of probiotic strains, e.g., the *Lactobacillus helveticus* R0052+ and *Bifidobacterium longum* R0175 combination, mitigate depression and anxiety. The antidepressive effect of *Lactobacillus rhamnosus* on mice is associated with a decrease in the corticosterone level in the host organism; this effect seems to depend on the *vagus* nerve [2].

Needless to say, probiotics including psychobiotics form an important part of the beneficial microbiota of the host organism. In the human (animal) organism, the microbiota represents a peculiar "microbial organ" [3, 4] that is directly or indirectly involved in a wide variety of metabolic, behavioral, and communicative activities of the host. An important communication channel is based on neurochemicals, i.e., "any chemicals

produced by a microorganism that are also recognized within neurobiology as either neurotransmitters, neuromodulators, or neurohormones in a mammalian system" [5]. Importantly, the ongoing microbiota-host communication is bidirectional. "The ability of microorganisms to not only respond to, but also produce the very same neurochemicals that are more typically thought in the context of mammalian systems, means that host interactions with microorganisms are much more interactive than previously envisioned" [5]. Various microorganisms are known to produce neurochemicals, including: neuropeptides, amino acids, biogenic amines, and gaseous substances [6 - 9].

The symbiotic bacterium *Lactococcus lactis* subsp. *lactis* is a promising probiotic/psychobiotic. *L. lactis* belongs to a widespread group of lactic acid bacteria which has been used by mankind for thousands of years as starter cultures in the food industry and are able to produce different kind of bioactive molecules, such as organic acids, bacteriocins, and other antimicrobial agents. Accordingly to the European Commission [10], the genera *Lactococcus* have Generally Recognized As Safe (GRAS) status. In the light of increased antibiotic resistance among pathogens, antibiotic-like substances have attracted attention as an alternative means to prevent infection by pathogens [11, 12]. The lactic acid bacteria isolated from the national lactic acid products of functional nourishment, are of special interest as probiotics that ameliorate the intestinal microbiota of humans and, therefore, play an important role in human ecology. A common property of these probiotic bacteria is the formation of natural antibiotic-like substances [13, 14].

The goal of this work was to detect neurochemicals such as biogenic amines in the biomass and culture liquid of several probiotic strains of *L. lactis* subsp. *lactis*.

2. Materials and Methods

We have used natural strains of lactococci with high antagonistic activity isolated from milk products, and then obtained fusant strains. Based on their microbiological properties and the gene sequence of the 16S rRNA, the novel strains isolated by us, are to be classified as *Lactococcus lactis* subsp. *lactis* [15].

The characteristics of the strains of *L. lactis* subsp. *lactis* used in this work are given in Table 1.

Importantly, strain 729 that was isolated from raw milk in Moscow was employed to produce a mutant (strain 1605) using UV radiation and ethylenimine (the mutant was not directly used in this study); the mutant cells were subsequently fused with strain 729 to produce strain F-116 (see Table 1 for information on other tested strains). The strain K-205 was isolated from Kurunga, a national Buryat dairy product with a therapeutic and a disease-preventing effect from Buryatia. This beverage is obtained using mixed lactic acid and alcoholic fermentation. It is characterized by high biological value in terms of protein and fatty acid composition. It contains vitamins and mineral substances. Its microbiota is physiologically active and includes: lactobacilli, lactococci, bifidobacteria, and yeast.

The strains were stored as lyophilized cells in a household refrigerator. The lyophilized cultures were reconstituted by incubation in sterile non-fat (skimmed) milk. To obtain the inoculum, the cultures from skimmed milk were re-inoculated into the inoculation medium, which contained glucose (1 g/L), yeast autolysate (35 mg% of ammonium nitrogen), and tap water (pH 6.8 - 7.0). Thereafter, the inoculum (OD_{600} 0.14 - 0.19) was added (5 vol%) to the basic fermentation medium, which contained (g/L): KH_2PO_4, (20); glucose (10); NaCl (1); $MgSO_4$ (0.2), and yeast autolysate (35 mg% of ammonium nitrogen), pH 6.8 - 7.0. The cultures were grown under steady-state condition at 28 ^{0}C. The biomass was determined by measuring the absorbance (optical density (OD) at 540 nm (l = 0.5 cm), using a FEK-56 colorimeter-nephelometer (Russia).

The bactericine-synthesizing activity was expressed in nisin activity units and estimated from the suppression of growth of the indicator culture *Bacillus coagulans*, which was introduced into the agar medium as a suspension with a density of 10^9 cell/mL. This substances were extracted from the cells and culture liquid using a 4:1:5 mixture of acetone, acetic acid, and water (55 ^{0}C, 1.5 h). Quantitative determination of the antimicrobial activity was performed by measuring the zones of growth suppression in mm with subsequent calculation involving a calibration plot for standard nisin solutions. Solutions of the preparation Nisaplin served as the standards (the activity was 1 000 000 IU/g) [14].

Table 1. Characterization of the tested strains of *L. lactis* subsp. *lactis*

Strain	Origins	GenBank
K-205	Isolated from Kurunga, a traditional fermented beverage in Buryatia	EF 114305
194	Isolated from milk in Buryatia	DQ 255954
729	Isolated from raw milk in Moscow	EF 102814
1605	Obtained by treating strain 729 with UV light in combination with ethylenimine (not used in this study)	EF 102815
F-116	Obtained by fusing the protoplasts of strains 729 and 1605	EF 100777

The biogenic amines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) were separated by high-performance liquid chromatography (HPLC) [16]. The cells were harvested, and bacterial biomass was separated by centrifugation (8000g; 20 min.). Culture fluid supernatant (CF) was passed through a Millipore filter (pore diameter 0.22 mm). The wet biomass sediment was sonicated (Braunsonic 1510, USA) at 22 KHz. The sonicated biomass (SM) was centrifuged (8000g; 20 min.), and the SM supernatant, the germ-free CF, and the sterile media were used for High Performance Liquid Chromatography (HPLC), with an amperometric detection system. LC-304T chomatographer (BAS, West Lafayette, США) with a Rheodyne 7125 injector was used. The volume of the loop used for applying samples was 20 ml. The tested biogenic amines were separated on a reverse-phase ReproSil-Pur column (ODS-3, 4x100 mm, 3 µ; Dr. Majsch GMBH, Elsico, Moscow). A PM-80 pump (BAS, USA) was used. Elution rate of the mobile phase was 1.0 mL/min. at a pressure of 200 atm. The mobile phase contained 0.1 M citrate-phosphate buffer with 1.1 mM octanesulfonic acid, 0.1 mM EDTA, and 9% acetonitrile (pH 3.0). The measurements were carried out using an LC-4B electrochemical detector (BAS, USA) with a glass-carbon electrode (+0.85 V) against an Ag/AgCl reference electrode. The samples were scanned with the Multichrome 1.5 (Ampersand) hardware-software system. All reagents used for the assay were analytical grade. The chromatographer was calibrated using a mixture of the tested biogenic amines; the concentrations of all these substances were 500 nM. The amine concentrations contained in the samples were calculated by the internal standard method that is based on determining the ratio between the peak area in the standard mixture and that in the samples [16].

The dynamics of growth, bacteriocin and biogenic amines accumulation of strains were followed for 24 h.

All experiments were replicated three times.

Standard deviations were calculated and included in the graphical representation of the data. The results were processed statistically with program OriginPro v.8.1 for Windows (OriginLab, Data Analysis Graphing Software, USA), Statistica for Windows, v.5.0 (StatSoft Inc., USA).

3. Results and Discussion

According to: morphological, cultural, physiological, biochemical properties, and gene sequence of 16S rRNA, novel and most effective strains were identified as *Lactococcus lactis* ssp. *lactis* which have status GRAS (absolutely harmless for human health and animals). The nucleotide sequences of the 16S rRNA genes of studied strains were deposited to the GenBank database under following accession numbers: strain K-205 - EF 114305, 194- DQ255954, 729 - EF102814, 1605 - EF102815, and F-116 - EF100777.

A study of the growth dynamics of the tested strains of *L. lactis* subsp. *lactis* in the fermentation medium with glucose and yeast autolysate demonstrated the mesophilic lactococci-typical growth pattern (Figure 1). Growth in the medium containing glucose caused a decrease in the pH from 6.8 to 3.6. The pH of the medium decreased to 4.8 after 9 h of the growth of strain 194, which was accompanied by an increase in the biomass (OD_{540} from 0.30 to 1.58); in this process, the activity level increased to 3700 IU/mL. The biomass of fusant strain F-116 accumulated gradually to 12 h; nisin accumulated concurrently with the biomass, as demonstrated by an increase in the activity of the culture liquid: after 12 h the pH dropped to 4.1, biomass increased to 1.66, and the level of antibiotic activity elevated 1.2-fold (4500 IU/mL). After 12 h of incubation, the cells passed from the exponential growth phase to the stationary phase, which continued for 3 h. After 15 h, a further decrease in pH, antibiotic activity, and optical density of the culture liquid were observed due to cell lysis. The antibiotic synthesis by *L. lactis* subsp. *lactis* 729 was parallel to the growth of the producer with maximum antibiotic activity (over 3500 IU/mL) obtainable at 10 - 12 hours of cultivation (Figure 1).

Bacteriocins are microbial proteins with antimicrobial activity; they differ from antibiotics in the kinetics of the synthesis and are formed in parallel with the growth of producer microorganisms and DNA and protein synthesis [14]. As secondary metabolites, antibiotics are largely synthesized during the second (steady-state) phase of microorganism growth and subsequently, when the cells start to die. Our experiment, in which the dynamics of growth of the strains was studied in glucose-containing medium, demonstrated that the increase in the antimicrobial activity of the culture liquid paralleled the accumulation of the biomass of lactococci (Figure 1). The amount of the biomass attained its maximum within 9 to 12 h of the incubation. The strain F-116 differed in its growth characteristics from other strains: the amount of the cells are slowly increased during the first 10 h, which was paralleled by a gradual increase in the antibiotic activity of the culture liquid up to 4200 IU/mL.

HPLC with amperometric detection was applied to the culture liquid of the following *Lactococcus lactis* subsp. *lactis* strains (see Table 1 for their characteristics): F-116, 729, 194, and K-205.

The content of biogenic amines (BAs) was determined in the culture liquid against the background of germ-free medium. The medium contained 0.09 µM norepinephrine (NE), 0.63 µM dopamine (DA), and 0.05 µM dihydroxyphenylalanine (DOPA), the immediate DA precursor. The medium lacked epinephrine (E), serotonin (5-hydroxytryptamine, 5-HT), and 5-hydroxytryptophan (5-HTP), the 5-HT precursor, as shown in Table 2. The germ-free medium also contained (data not shown)

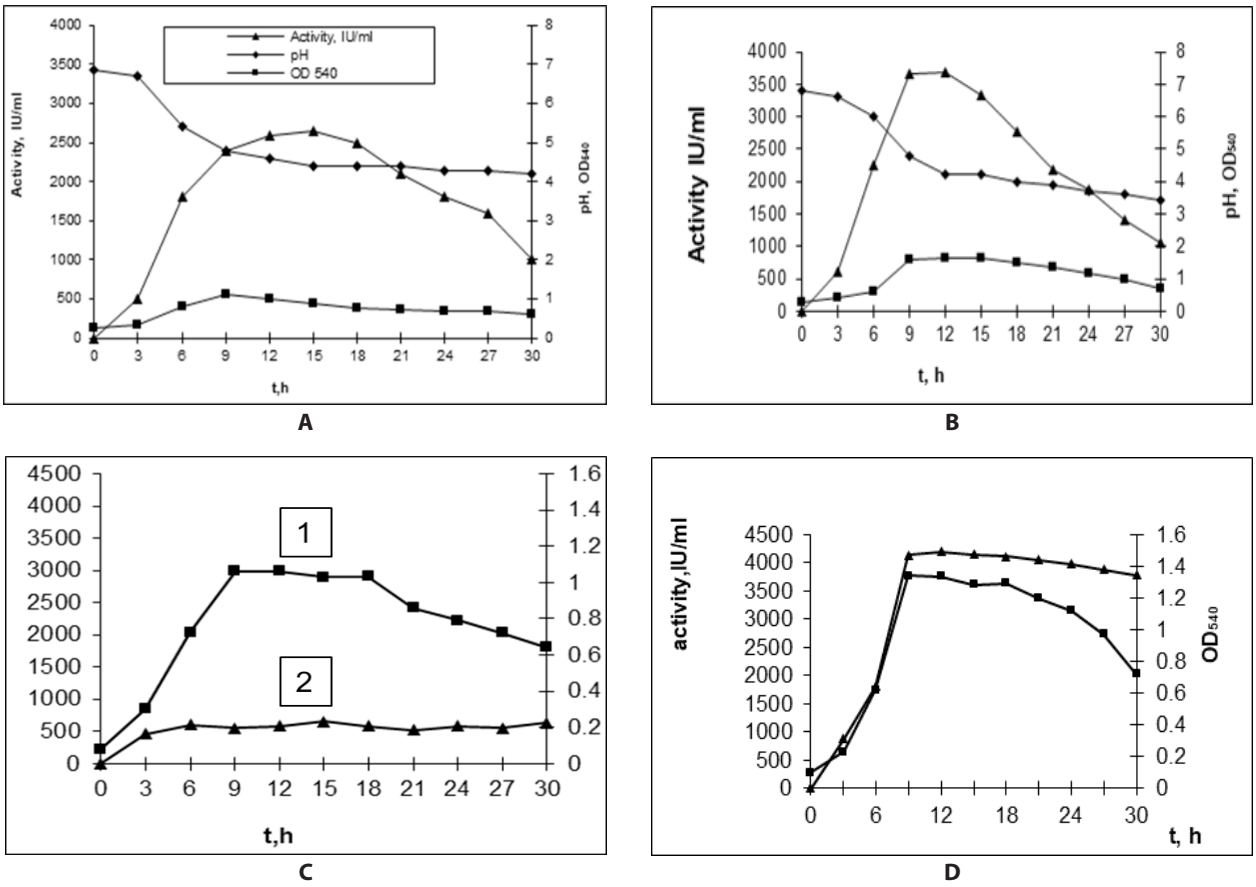

Figure 1. Dynamic of growth (1) and antimicrobial activity (2) of strains *Lactococcus lactis* subsp. *lactis*

Table 2. Concentrations (micromoles/L) of neurochemicals in the culture liquid of the tested strains at various growth stages ($p \leq 0.05$)

Samples	NE	E	DA	5-HTP	5-HT
Culture liquid	0.09	0	0.630	0	0
F-116 lag phase	0.07	0.01	0.49	0	0
F-116 stationary	0.08	0	1.04	0	0
F-116 terminal	0.06	0.01	0.28	0	0
729 lag phase	0.06	0.01	0.49	0	0
729 exponential	0.06	0.01	0.34	0	0
729 stationary	0.02	0	0.18	0	0
194 lag phase	0.06	0.01	0.37	0	0
194 exponential	0.05	0.05	0.30	0	1.87
194 stationary	0.72	0	0.14	0	0.02
K-205 lag phase	0.06	0.01	0.35	0	0
K-205 exponential	0.05	0.03	0.33	0	0.04
K-205 stationary	0.65	0	0.15	0	0
K-205 terminal	0.05	0	0.19	0	0

0.02 µM 5-hydroxyindoleacetic acid (5-HIAA), the 5-HT oxidation product; 0.04 µM homovanilic acid (HVA) and 3-methyltyramine (3-MT), the DA oxidation products.

Table 2 presents the results obtained with the 4 tested strains at the following stages of their growth: (1) the lag phase (6 h of cultivation); (2) the exponential phase (9 h); (3) the stationary phase (17 h); and (4) the end of fermentation (18 h).

It was established that strains 194 and K-205 released significant amounts of NE into the medium during the stationary phase; the other tested strains did not considerably change the NE concentration in the medium, irrespective of the growth stage. Strains 194 and K-205 released low (nanomolar) amounts of E into the medium at the exponential growth stage (Table 2). The implication is that, when used in the capacity of probiotics, strains 194 and K-205 can potentially stimulate the growth of a large number of intestinal bacteria including, unfortunately, potential pathogens such as the enterohemorrhagic strain (EHEC) of *Escherichia coli, Shigella* sp., and *Salmonella* sp. It is known the growth of these bacteria is accelerated in the presence of catecholamines [11-13].

All tested strains failed to enrich the medium in DA (Table 2), DOPA, and 5-HT.

An interesting fact is that ca. 2 µM of 5-HT were released by strain 194 during the exponential growth stage. Since BAs normally do not cross the gut-blood barrier, 5-HT is expected to produce local effects on the enteric nervous system that regulates intestinal motility and perform other important functions. Probiotic-produced 5-HT is also expected to impact the intestinal immune system where "the effects of serotonin are complex. Serotonin can both stimulate and block the functioning of immunocytes, depending on the types of the serotonin receptors involved, their degree of activation, and the impact of the supplementary factors of the cell's micro-environment" [8]. 5-HT stimulates the growth of some benign microorganisms including, notably, symbiotic *Escherichia coli* K-12 [17-20].

Neither of the tested strains of *L. lactis* subsp. *lactis* produced detectable amounts of the 5-HT precursor 5-HTP.

At the end of the fermentation period, the biomass was harvested, sonicated, and tested for BAs. As shown in Table 3, 5-HT was present at very low (nanomolar) concentration in strain 194 that also enriched the culture liquid in this compound (see Figure 2). No significant amounts of other neurochemicals were contained in the biomass of the *L. lactis* strains. Since the medium concentrations of 5-HT substantially exceeded its intracellular concentrations, it seems likely that 5-HT performs some extracellular functions. Presumably, it is implicated in cell-cell communication, in an analogy to its well-known histohormone and neuromediator role in eukaryotic systems. The data obtained raise interesting issues that are of clinical relevance.

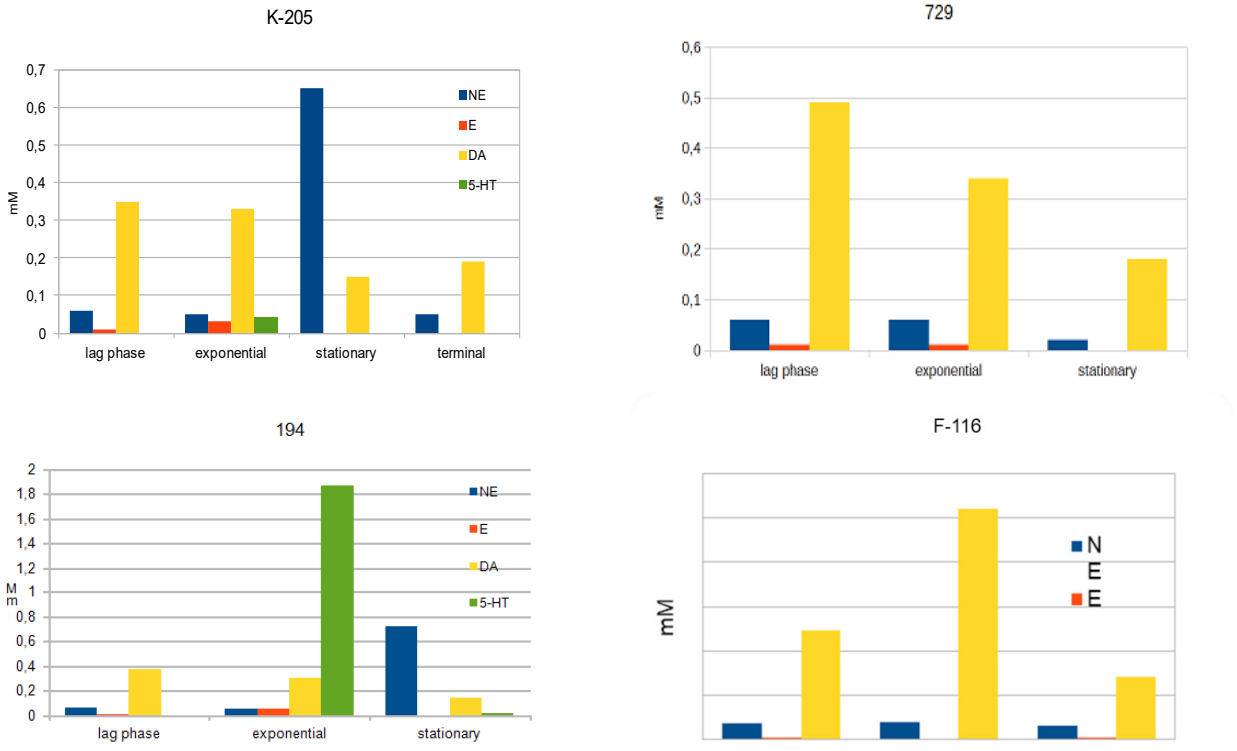

Figure 2. Concentrations of neurochemicals in the culture liquid of the tested strains *L. lactis* subsp. *lactis* at various growth stages

Table 3. BA concentrations (μM /L) in the sonicated biomass of the tested strains of *L. lactis* subsp. *lactis* harvested at the end of the fermentation period (p ≤ 0.05)

Samples	NE	E	DA	5-HTP	5-HT
Culture liquid	0.09	0	0.63	0	0
F116	0	0	0	0	0.01
194	0	0	0	0	0
729	0	0	0	0	0
K-205	0	0	0	0	0

First, we reemphasize that probiotic strains 194 and K-205 that synthesize NE could produce an unwanted side effect by stimulating the growth of opportunistic pathogens.

Second, beneficial bacteria such as some strains of lactobacilli and bifidobacteria [20] increase their growth rate if BAs are present in the medium. Hence, the BAs-producing strains of *L. lactis* subsp. *lactis* are expected to stimulate the growth of other probiotic microorganisms and, therefore, to enhance their beneficial effects on the human organism. Catecholamines-stimulated probiotics include overproducers of an important neurochemical, γ-aminobutyric acid (GABA) such as bifidobacterial strains [21]. It is recommendable, therefore, to administer these bifidobacteria in combination with catecholamines-releasing strains of *L. lactis* subsp. *lactis,* e.g., strain 729, to patients suffering from GABA deficit-related conditions such as irritated bowel syndrome (IBS).

It should be noted that the tested strains of *L. lactis* subsp. *lactis* produce the essential neurochemicals NE, and/or 5-HT in addition to other important biomediators, particularly short-chain fatty acids (SCFAs). Based on our data (unpublished), the predominant SCFA synthesized by the strains of *L. lactis* subsp. *lactis* is acetic acid whose micromolar concentrations are produced by them.

Interestingly, strain 194 that released micromolar 5-HT amounts into the culture liquid, also proved in our previous studies to be highly active as an antagonist. It exhibited high antimicrobial activity, which was equivalent to that of 370 u/mL of levomycetin, 3600 IU/mL of nisin A (Nisaplin), or 2500 IU/mL of the fungicidal antibiotic nistatin [13]. These data raise the question to be addressed in future studies, whether 5-HT (and possible other neurochemicals) perform extracellular communicative functions that are of relevance to the antagonistic activity of the strain involved within the microbiota of the human organism.

4. Conclusions

- Some of the tested probiotic strains of *L. lactis* subsp. *lactis* with the GRAS status produce and release into the medium neuroactive substances, such as norepinephrine (strains 194 and K-205), epinephrine (the same strains), and serotonin (strain 194). These microbially synthesized neurochemicals should impact the nervous and the immune system of the host and, in addition, are likely to perform (auto) regulatory functions inside the microbial community of the host organism, including the gastro-intestinal tract.

- Probiotic microorganisms that produce the tested biogenic amines can potentially be used as target-oriented functional food items for preventive and therapeutic purposes.

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