Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *85* (2) 163–176 (2020) UDC 616.7:615.33:547.466.64:547.466.24:577.2
JSCS–5291 *Original scientific paper*

Characterization of pH resistance and the proteolytic activity of GABA producing *Lactobacillus brevis* BGZLS10-17 in preparation of fermented milk beverage and the effects on the symptoms of the experimental autoimmune encephalomyelitis

SVETLANA S. SOKOVIC BAJIC[1,3], SANJA B. MIHAJLOVIC[1], DUSAN D. RADOJEVIC[1], DUSANKA D. POPOVIC[1], JELENA M. DJOKIC[1], SUZANA M. STANISAVLJEVIC[2], MILICA N. LAZAREVIC[2], DJORDJE M. MILJKOVIC[2], PATRICIA RUAS-MADIEDO[3], NATASA E. GOLIC[1] and MAJA S. TOLINACKI[1]*

[1]*Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade, Serbia,* [2]*Department of Immunology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia and* [3]*Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias – Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Asturias, Spain*

(Received 16 July, revised 22 August, accepted 3 September 2019)

Abstract: Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system. The aim of this work was to study the probiotic effect of γ-aminobutyric acid (GABA)-producer *Lactobacillus brevis* BGZLS10-17 on experimental autoimmune encephalomyelitis (EAE), an experimental animal MS model. Clinical EAE symptoms were monitored in Dark Agouti (DA) rats treated with *L. brevis* BGZLS10-17 strain, or supernatant obtained from 48 h culture of *L. brevis* BGZLS10-17 cultivated in growth medium with or without GABA precursor monosodium glutamate (MSG). The results revealed that oral administration of *L. brevis* BGZLS10-17 alleviates the symptoms of EAE in DA rats. Namely, treatment with BGZLS10-17 and the supernatant of the strain cultivated in medium with MSG delayed the onset, shortened the duration, and reduced the intensity of the disease in the period when the EAE symptoms in controls were most pronounced. The probiotic treated animals were completely recovered after forty days, unlike the control animals. The results indicate that supplementation with live strain or with supernatant containing GABA produced by *L. brevis* BGZLS10-17 could alleviate the EAE symptoms. However, the use of *L. brevis* BGZLS10-17 in functional food as probiotic for autoimmune diseases should be tested in clinical trials.

Keywords: probiotics; γ-aminobutyric acid (GABA); EAE; multiple sclerosis.

* Corresponding author. E-mail: maja_tolinacki@imgge.bg.ac.rs
https://doi.org/10.2298/JSC190716094S

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune, inflammatory neurological disease of central nervous system (CNS) often leading to severe physical or cognitive disability and neurological defects.[1] MS is characterized by multifocal demyelination and axonal damage resulting in dysfunction and death of neurons, mostly targeting the young adults.[2] The cause of the disease is still unknown, although it seems to be a combination of genetic susceptibility and non-genetic and environmental factors.[3] MS was recently linked to gut microbiota dysbiosis.[4,5] The connection between the brain and gastrointestinal tract – GIT (gut brain axis, GBA) is a two-way communication between the central and enteric nervous systems. The link between the GIT microbiota and GBA is also bidirectional and occurs *via* neural, endocrine, immune, and humoral factors.[6] The balance between the microbiota and the intestinal barrier is disturbed during chronic inflammation and autoimmune diseases, and one of the very attractive fields of research is the use of certain microorganisms to restore the balance.

The etiology of MS is related to reduced level of plasma GABA and activities of enzyme glutamic acid decarboxylase (GAD).[7] A number of clinical studies investigated glutamate and γ-aminobutyric acid (GABA) levels in MS patients. The lower glutamate levels in sensorimotor and parietal regions of the left cerebral hemisphere of the brain, as well as higher GABA levels in sensorimotor region were detected in MS patients compared to healthy controls.[8] On the other hand, significantly lower GABA and glutamate levels, associated with verbal and visuospatial memory deficits, were reported in relapsing remitting MS patients compared to healthy controls.[9] The studies indicated that modulation of glutamate and GABA levels could be a novel therapeutic strategy for treatment of early stages of MS.[9]

GABA is a major inhibitory neurotransmitter with antidepressant, antihypertensive, anti-diabetic effects. It regulates pain sensations, anxiety, and serum lipid levels in humans.[10] Apparently, GABA has an immunomodulatory effect and a specific role in autoimmune diseases like multiple sclerosis, type 1 diabetes, and rheumatoid arthritis. It is a non-protein amino acid synthesized by glutamic acid decarboxylase (GAD) in the irreversible reaction of α-decarboxylation of L-glutamic acid.[11] The GAD pathway is encoded by the *gadB* and *gadC* genes regulated by the GadR activator; it is induced by presence of glutamate and/or environmental stresses such as acidity, osmotic stress, or starvation and has a role in a stress-resistance.[12] GABA is produced by various microorganisms including lactic acid bacteria (LAB).[13,14]

In our previous work, the probiotic properties of GABA-producing *Lactobacillus brevis* BGZLS10-17 natural isolate from artisanal cheese from Zlatar Mountain was evaluated.[13] The high adhesion of BGZLS10-17 strain to Caco-2 cells and the exclusion ability of *Escherichia coli* ATCC25922 and *Salmonella*

enterica C29039 was revealed. Interestingly, we have shown in an *in vitro* model of inflammation-induced disruption of the intestinal barrier that GABA-containing supernatant of BGZLS10-17 culture decreased the production of IL-8 and increased the expression of TGF-*β* cytokines and had protective effect on the intestinal epithelial barrier through stimulation of expression of tight junction proteins.[15] As the restraint of exacerbated inflammation and the stabilisation of intestinal barrier was suggested to be very important for MS treatment, in this work the effect of GABA-producing BGZLS10-17 as well as GABA-enriched supernatant of this probiotic strain on clinical symptoms of EAE in DA rats was investigated. Our aim was to test if BGZLS10-17 could be used as adjunct starter culture for production of GABA-enriched dairy functional food in therapy for various inflammatory diseases, including multiple sclerosis.

EXPERIMENTAL

Bacterial strains

Lactobacillus brevis BGZLS10-17, GABA-producing natural dairy isolate from artisanal Zlatar cheese (bacterial collection of Laboratory for Molecular Microbiology, IMGGE) was used in this study. BGZLS10-17 was cultured in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C under anaerobic conditions using Anaerocult A (Merck). In order to stimulate GABA production, the strain was cultivated in MRS broth with addition of 0.6 % monosodium glutamate (MSG, Acros Organics, Morris Plains, NJ, US) for 48 h at 37 °C under anaerobic conditions. Supernatants were neutralised to pH 7 and then filtrated through 0.22 μm filters (Sarstedt, Nümbrecht, Germany). For fermentation purposes strain *Streptococcus thermophilus* BGKMJ1-36 was used. BGKMJ1-36 was grown in M17 medium, pH 7.2 (Merck) supplemented with 0.5 % glucose (GM17) at 37 °C, under anaerobic conditions.

Molecular characterisation of the gad *gene*

The total DNA of lactobacilli strain was extracted using the QIA DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The PCR amplification of complete *gad* gene was performed by *gadBBF* (5'-GACCGGTCATGAGGCG-3') and *gadBBR* (5'-CATTACTCGCCGTTCGGC--3') primers (this work). Briefly, 25 μl of reaction mixture contained 500 ng/μl of DNA, 0.5 U of Q5 high-fidelity DNA polymerase (New England BioLabs, Inc.), 200 μM of each deoxynucleoside triphosphate, 0.5 μM of both primers and 1×Q5 reaction buffer. Conditions for DNA amplification were: denaturation at 98 °C for 30 s; 30 cycles of polymerization at 98 °C for 10 s, 38 °C for 30 s and 72 °C for 1 min 30 s; and elongation at 72 °C for 5 min. The obtained PCR amplicons were purified (Qiagen, GmbH, Hilden, Germany) and sequenced (Macrogen, Amsterdam, the Netherlands). Sequence annotation and the database searches for sequence similarities were performed with the BLAST tool available online (http:// //www.ncbi.nlm.nih.gov/BLAST).

Database search and in silico *phylogenetic analysis*

Database search for protein sequence similarities was done using the BLAST tool (http://blast.ncbi.nlm.nih.gov). To build the GAD protein profile the PSI-BLAST[15] homology searches were restricted to *Lactobacillaceae* and e-value cut-off of 1e–4. All hits that exhi-

bited at least 40 % identity and over 70 % of the query fragment length were maintained for further analysis. GAD protein from *L. brevis* BGZLS10-17 was used as a GAD prototype.

The phylogenetic interpretations were obtained by MEGA version 7.0.[16] Multiple protein sequence alignments were performed using Clustal W with default parameters. The construction of a GAD phylogenetic tree was conducted by the maximum-likelihood (ML) method using a Jones–Taylor–Thornton (JTT) model. Bootstrapping of 1000 replicates was used to infer confidence levels of ML trees. An online tool (iTOL) v3 was used for the tree display.[17]

pH evolution in MRS with and without MSG

L. brevis BGZLS10-17 was inoculated (2 %) in MRS medium (Merck) and MRS medium with addition of 1 % MSG (Acros Organics) and the pH was measured with Thermo scientific Orion Versa Star advanced electrochemistry meter using Orion™ Versa Star Pro™ meter software every 15 min for 48 h. Growth was monitored by measuring the optical density at 600 nm, OD_{600} (Eppendorf Bio Photometer Plus, Hamburg, Germany), and bacterial cells were counted by plating serial dilutions on MRS agar plates.

Experimental animals, induction of experimental autoimmune encephalomyelitis (EAE), treatment and evaluation

Female DA rats (8–10 weeks of age) were maintained in the animal facility of the Institute for Biological Research "Siniša Stanković". Animal experiments were approved by the local ethics committee (Institute for Biological Research "Siniša Stanković", No. 03-1/15). Housing of the rats was performed under conventional conditions. Five rats were kept in the same cage. EAE was induced with rat spinal cord homogenate (SCH) in PBS (50 %) mixed with equal volume of CFA (Complete Freund Adjuvant, Difco, Detroit, MI, USA). The animals were injected subcutaneously into the hock of one hind limb. The rats were monitored daily for clinical symptoms (c.s.) of EAE and scored according to the following scale: 0 – no c.s.; 1 – flaccid tail; 2 – hind limb paresis; 3 – hind limb paralysis; 4 – moribund state or death. DA rats had EAE onset on 9–11 d.p.i. (c.s. 1), peak on 12–16 d.p.i. (c.s. 2–4) and recovery on 18–22 d.p.i. (c.s. 1 or less).

Animals, 5 per group, were separated in four groups: group 1 was treated with 50 ml/day MRS broth – control group; group 2 treated with 50 ml/day of BGZLS10-17 strain for 16 h in MRS. (colony forming units [CFU]/ml ~10^9); group 3 treated with 50 ml/day of cell free supernatant obtained from BGZLS10-17 cultivated for 48 h in MRS without MSG (no GABA production); group 4 treated with 50 ml/day of cell-free supernatant from BGZLS10-17 cultivated for 48 h in MRS with MSG (stimulation of GABA production). Each treatment lasted 30 days. Differences between the control and experimental groups were compared using Student's *t*-test. Values of $p < 0.05$ or less were considered statistically significant.

Proteolytic activity

Proteolytic activities of the BGZLS10-17 strain were essentially assayed as described previously.[18] For this purpose, BGZLS10-17 was grown on MCA plates for 48 h, at optimal growth conditions prior to cell collection. Collected fresh cells (10 mg) were resuspended in 50 µl of 0.1 M sodium phosphate buffer, pH 6.8, to approximate density of 10^{10} cells/ml. The cell suspension was mixed with substrate dissolved in the same buffer at a 1:1 volume ratio and incubated for 3 h and 24 h at 37 °C. The substrates used for proteolytic assay were either *α*-, *β*-, *κ*-casein fractions or total casein (5 mg/ml, Sigma, St. Louis, MO, USA). After that, cells were pelleted by centrifugation (5 min at 12.000 *g*), and the supernatant was then used for preparation of samples for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared by heating of obtained supernatant (100 °C for 2 min)

with an equal volume of 0.125 M Tris–HCl buffer (pH 6.8) containing 4 % SDS, 20 % glycerol, 10 vol. % β-mercaptoethanol, and 0.07 % bromophenol blue. Analysis of casein hydrolysis was carried out on SDS-PAGE by loading 12.5 % polyacrylamide gel with prepared samples. Gels were run on vertical electrophoresis cells for 20 h at 10 mA constant current. Gels were stained with Coomassie brilliant blue R250 (SERVA, Heidelberg, Germany) and destained in a mixture of methanol (20 %) and acetic acid (7 %) in distilled water.

Fermented milk beverage preparation

To produce fermented milk beverage strains *L. brevis* BGZLS10-17 and *S. thermophilus* BGKMJ1-36 were used. BGZLS10-17 was cultivated for 16 h in 100 ml of MRS broth at 37 °C anaerobically while BGKMJ1-36 was grown in 100 ml of GM17 broth for 16 h at 37 °C anaerobically.

The acidification ability of the strain BGZLS10-17 in skim milk was tested in following way: *i*) 4 % of BGZLS10-17 of 16 h old culture was inoculated in 100 ml of pasteurized milk (Imlek, Belgrade, Serbia) with or without yeast extract (YE), 4 g/l (Torlak, Belgrade, Serbia) or MRS broth 20 % (Merck). Fermentation process was monitored for 24 h; *ii*) 1 % of BGKMJ1-36 of 16 h old culture was inoculated in 100 ml of pasteurized milk (Imlek) and the fermentation process was monitored for 6 h or until the pH reached 4.9; *iii*) mixed culture: 4 % of BGZLS10-17 and 1 % of BGKMJ1-36 were inoculated in 100 ml of pasteurized milk (Imlek) and the fermentation process was monitored for 6 h or until the pH reached 4.9. All fermentation processes were performed at 42 °C. Then the cultures were cooled to 5 °C for 2 h. After this cooling time, the storage time was considered equal to 0 day (d). During storage (at 5 °C) every 7 d cultures were taken to determine pH and viability of bacteria (CFU/ml) until 21 d. All experiments were done in triplicates.

RESULTS AND DISCUSSION

Molecular characterization and phylogenetic analysis of the L. brevis gadB *gene*

L. brevis BGZLS10-17, the high GABA-producing strain[13] was screened for the presence of the *gad* gene. The PCR amplification with primers complementary to the complete *gad* genes gave a PCR product of 1440 bp. The predicted amino acid sequences of *L. brevis* BGZLS10-17 showed 98 % identity to the *gadB* of *L. brevis* (WP_011666890.1).

The maximum likelihood (ML) phylogenetic analysis of GAD proteins from the entire *Lactobacillacae* family separated GAD proteins into three groups (Fig. 1). Group I consists of the GAD proteins from *L. aviarius*, *L. antri*, *L. oris* and *L. reuteri.* Group II includes BGZLS10-17 isolate (Fig. 1). Although the group II mainly consists of the *gadB* gene from *L. brevis* (55 %) there were also *L. aviarius*, *L. coleohominis*, *L. curvatus*, *L. farraginis*, *L. parakefiri*, *L. plantarum*, *Pediococcus acidilactici*, *L. paracasei*, *L. buchneri*, *L. rennini*, *L. rossiae*, *L. senmaizukei*, *L. spicheri* and *L. sakei*. Dominant GAD protein of the group III belongs to *L. plantarum* (60.4 %). The second abundant species is *L. brevis* (20.8 %), while the rest of the group consists of the GAD protein from *L. gastricus*, *L. fermentum*, *L. suebicus*, *L. collinoides*, *L. paracollinoides*, *L. herbarum*, *L. paraplanatrum*, *L. reuteri* and *L. delbruecki*. Multiple sequence alignment comparisons revealed that 60 % of the *gadB* alleles are polymorphic. There are 13 %

polymorphic *gadB* alleles in group I (60 out of 459 amino acids positions), 61 % polymorphic alleles in group II (297 out of 490 amino acids positions) and group III has 68 % polymorphic alleles (310 out of 457 amino acids positions).

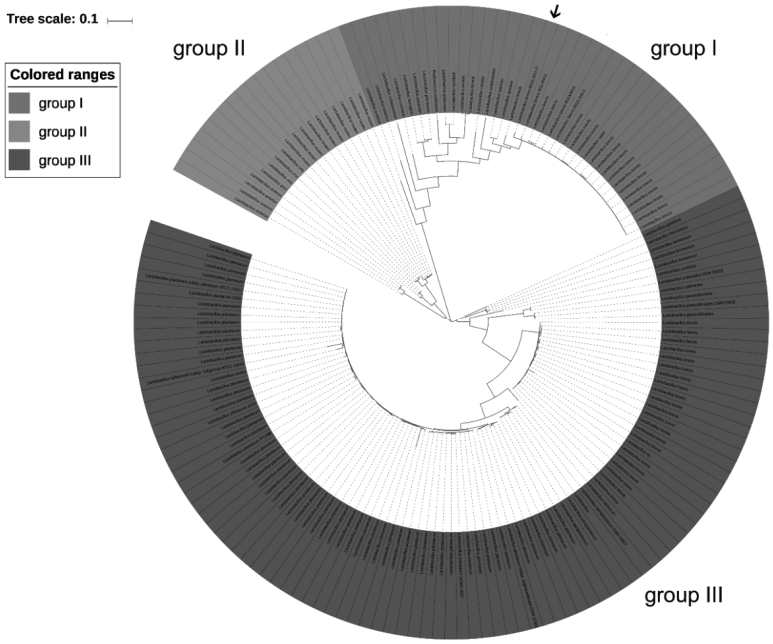

Fig. 1. Phylogenetic inferences of GAD protein among *Lactobacilaceae*. A phylogenetic tree of GAD proteins was constructed with the maximum likelihood (ML) method using a Jones–Taylor–Thornton (JTT) model distance matrix. The confidence levels were computed from 1000 bootstrap resamples of alignment used for phylogenetic inferences by ML method. The shades of gray represent the three different groups (I-III) consisting of *Lactobacillus* spp. from the database and the BGZLS10-17 isolate (arrow).

Treatment of EAE

In order to evaluate the possible beneficial effect of GABA on autoimmune diseases, in this study were treated DA rats, prone to EAE induction, with the GABA-producing strain BGZLS10-17, as well as with GABA-enriched cell-free supernatant of BGZLS17-10 grown for 48 h. The results of this *in vivo* study showed that the strain *L. brevis* BGZLS10-17 decreased the disease severity in DA rats. Treatment with BGZLS10-17 delayed disease manifestation and shortened the duration of the disease, while the peak of the disease was entirely reduced, and the animals were completely recovered after forty days, that was not observed in control animals (Fig. 2).

The amelioration of the EAE symptoms also occurred when GABA-enriched cell-free supernatant of the *L. brevis* BGZLS10-17 grown for 48 h was used,

clearly indicating the role of GABA produced by BGZLS10-17 in improvement of EAE prognosis. However, slight improvement of the EAE symptoms was scored when the cell-free supernatant of the BGZLS10-17, grown for 48 h in MRS without MSG, was used, suggesting that other beneficial metabolites (molecules) in cell-free supernatant of the BGZLS10-17 could act as a possible postbiotic (Fig. 3a and b). Comparing the effects of live strain and its supernatants, it can be observed that treatment with both live BGZLS10-17 and its GABA-containing supernatant alleviate the development of the second peak of EAE (relapse), while application of the live strain shows more significant effect in alleviating EAE symptoms in this phase of the disease. At the same time only the treatments with supernatants statistically delay the onset of the disease probably due to the time needed for the live strain to colonize the GIT, while bioactive molecules contained in the supernatants can achieve its effect immediately after implementation.

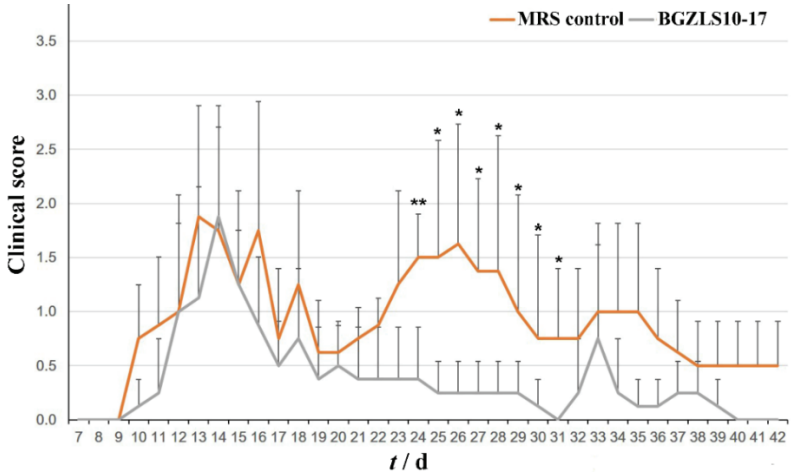

Fig. 2. The effect of the BGZLS10-17 on the clinical score of EAE in female DA rats. Female DA rats were immunized with SCH mixed with CFA, which led to the manifestation of symptoms similar to multiple sclerosis. The animal control group consumed MRS medium, while the experimental group consumed BGZLS10-17 grown in MRS medium for 16 h (10^9 cells/rat daily). *x*-Axis represents clinical score of EAE symptoms, while *y*-axis represents days after immunization. Clinical picture of the disease was monitored from 7 up to 42 days. The results are presented as mean values (n = 5 rats per group) ± standard deviation. *p < 0.05, **p < 0.01; d – days.

GABA-mediated pH resistance of BGZLS10-17

Hence, in order to use the strain *L. brevis* BGZLS10-17 as probiotic for prevention and/or treatment of autoimmune diseases, successful colonization of the host is needed. In that order, orally acquired bacteria must survive the extreme acidic stress in the host stomach. Given that GABA production from glutamate (MSG)

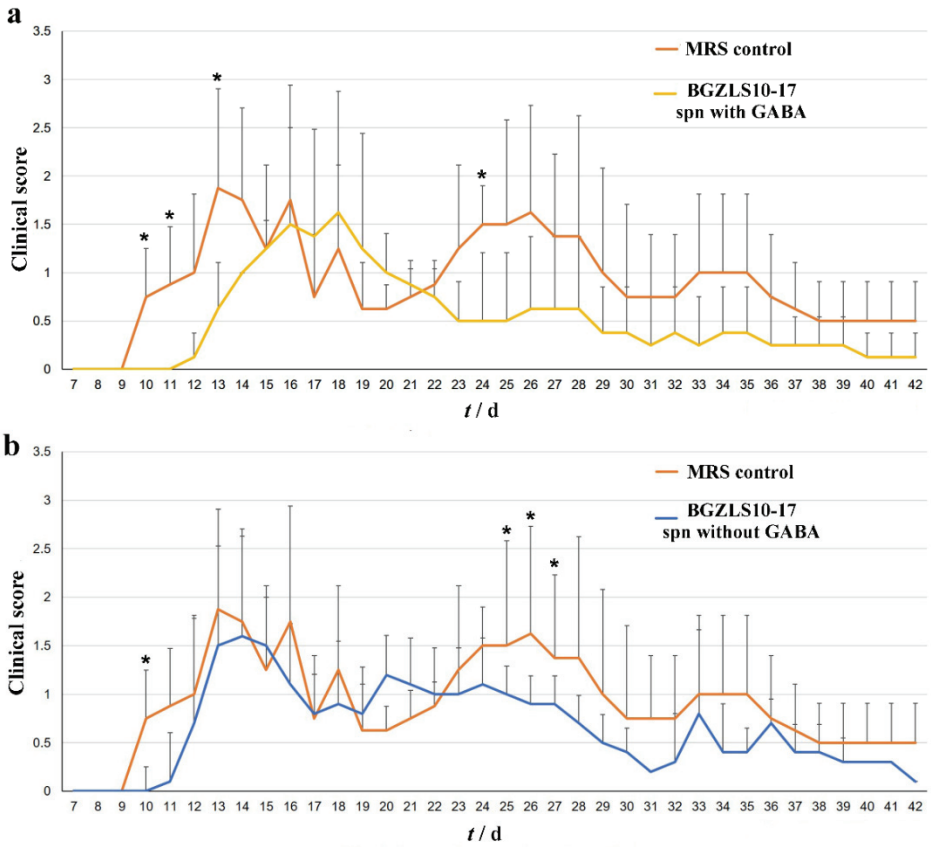

Fig. 3. The effect of the BGZLS10-17 cell-free supernatant with GABA (a) and without GABA (b) on the clinical score of EAE in female DA rats. Female DA rats were immunized with SCH mixed with CFA, which led to the manifestation of symptoms similar to multiple sclerosis. The animal control group consumed MRS medium, while the experimental groups consumed BGZLS10-17 cell-free supernatant from 48 h grown culture in MRS medium or MRS medium with the addition of MSG. *x*-Axis represents clinical score of EAE symptoms, while *y*-axis represents days after immunization. Clinical picture of the disease was monitored from 7 up to 42 days. The results are presented as mean values (n = 5 rats per group) ± standard deviation. * $p < 0.05$; d – days.

is by far the most potent acid resistance system in bacteria[12,19] our aim was to investigate the pH changes in GABA-producing strain *L. brevis* BGZLS10--17 culture during 24 h and 48 h of incubation (Fig. 4).

The pH evolution was dependent on cultivation conditions. Monitoring of the pH of BGZLS10-17 culture grown in MRS with and without MSG revealed that after 48 h of incubation significantly higher pH was observed in the BGZLS10-17 culture grown in medium supplemented with MSG (Fig. 4). These results were consistent with our previous HPLC analysis that showed the high

GABA concentration in supernatant of BGZLS10-17 grown in MRS with MSG for 48 h.[13] Although the OD_{600} was similar for both BGZLS10-17 cultures grown in MRS and MRS with MSG for 24 and 48 h (Fig. 5a) the number of viable bacteria was remarkably higher if they were grown in medium with MSG after 48 h (Fig. 5b). Our results obtained for the pH evolution in *L. brevis* BGZLS10-17 culture confirm that GABA might be involved in the pH regulation.

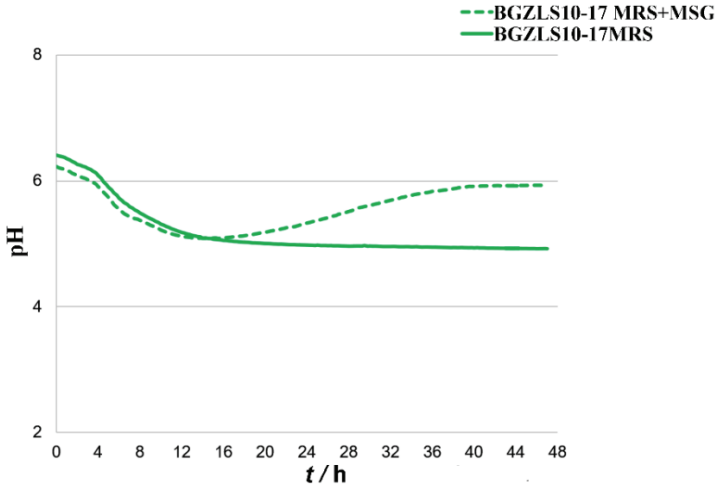

Fig. 4. pH evolution in MRS and MRS with MSG. BGZLS10-17 was grown in MRS medium and MRS medium with the addition of MSG. The change of the pH was monitored every 15 min for 48 h.

According to Papadimitriou *et al.*,[12] the extracellular pH increases during the conversion of glutamate to the more alkaline GABA. The presence of glutamate and/or environmental stress, in this case stationary phase, characterized by depletion of the essential nutrients and acidification of the growth medium caused by formation of lactic acid, induced the GAD pathway in the strain *L. brevis* BGZLS10-17 after the growth in MRS with MSG for 48 h, enabling the pH resistance and higher viability compared to the strain BGZLS10-17 grown in MRS without MSG.

Proteolytic activity

Another important characteristic of probiotic strains used in functional dairy food is their ability to degrade casein, the major milk protein. Lactic acid bacteria are multiple amino acid auxotrophs, hence to grow in milk they depend on the expression of a complex proteolytic system encompassing a cell-wall bound extracellular serine proteinase breaking down casein, oligopeptide transport systems and the intracellular peptidases that hydrolyse oligopeptides to free amino acids.[20]

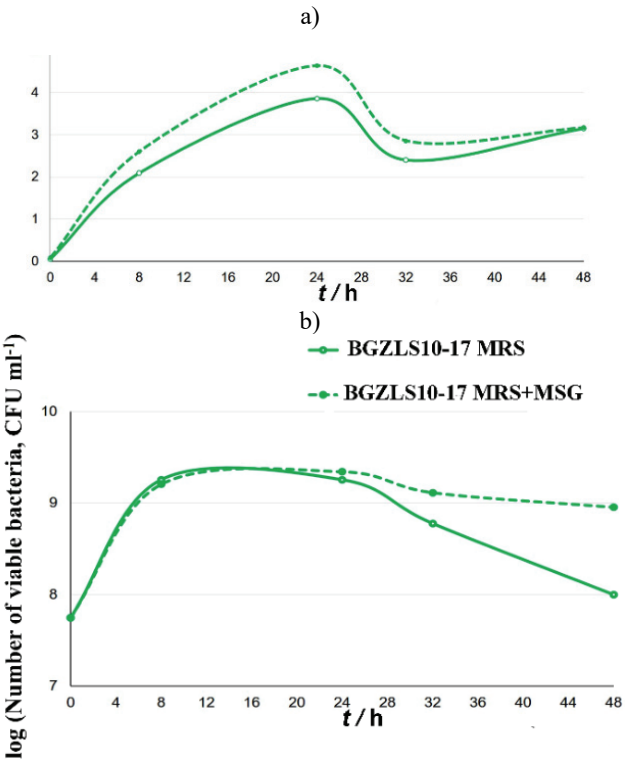

Fig. 5. Monitoring the growth of BGZLS10-17 for 48 h in MRS medium and MRS medium with MSG. Optical density (on y-axis, *OD* / a.u.) measured at 600 nm (a) and logarithmic value of CFU ml^{-1} for BGZLS10-17 (b).

Moreover, during milk fermentation biologically active peptides could be released as a result of proteolytic activity giving the additional health-promoting feature to the functional dairy food.[21,22]

Hence, the proteolytic activity of the strain *L. brevis* BGZLS10-17 was analyzed and the results revealed that after 3 h only partial degradation of *α*-casein was observed (Fig. 6), while the complete casein degradation (*α*-, *β*- and *κ*-casein) was achieved after 24 h (Fig. 6), pointing to the weak proteolytic activity.

The fermented milk beverage preparation

Additionaly, the fermentation time during the preparation of fermented milk beverage with strain *L. brevis* BGZLS10-17 was followed. The effect of fermentation time of BGZLS10-17 on the prepared fermented milk beverage (pH measurement during 24 h) are presented in Table I.

The results indicated very slow milk fermentation process, both milk with YE and milk with MRS, inoculated with BGZLS10-17 after 24 h. Moreover, the rheological properties of the fermented beverage were also unsatisfactory, with

lot of lumps, indicating that due to weak proteolytic activity strain BGZLS10--17 has poor technological characteristics. Hence, in order to be used in functional dairy food, the strain *L. brevis* BGZAL10-17 should be combined with dairy starter culture bacteria. Therefore, in order to prepare fermented beverage with desirable attributes the strain BGZLS10-17 were put together with the strain *S. thermophilus* BGKMJ1-36 in pasteurized milk. The fermentation process was monitored and the number of viable bacteria was observed during 7 h. Results are presented in Table II.

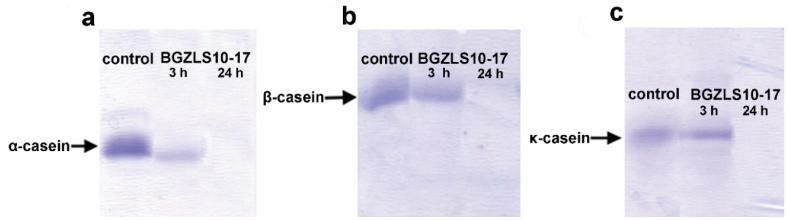

Fig. 6. Proteolitic activity of BGZLS10-17 after 3 and 24 h. BGZLS10-17 was grown on milk citrate agar (MCA) plates for 48 h, at optimal growth conditions. Degradation of α-casein (a), β-casein (b), and κ-casein (c).

TABLE I. pH values (means ± *SD*) of fermented milk beverage manufactured with BGZLS10-17

Name	Time, h					
	Initial	3	4	5	6	24
BGZLA10-17 in milk	6.57±0.01	6.3±0.01	6.3±0.03	6.20±0.01	6.18±0.02	5.39±0.01
BGZLS10-17 in milk with YE	6.58±0.01	6.21±0.03	6.20±0.03	6.20±0.02	6.13±0.02	4.44±0.03
BGZLS10-17 in milk with MRS	6.41±0.02	6.13±0.03	6.10±0.01	6.05±0.01	6.0±0.03	4.28±0.03

TABLE II. pH (means ± *SD*) during seven hours of milk fermentation achieved by mixed culture containing BGZLS10-17 and BGKMJ1-36, as well as by single culture containing only BGKMJ1-36

Name	Time, h				
	3	4	5	6	7
BGZLS10-17 + BGKMJ1-36	5.84±0.01	5.46±0.04	5.43±0.03	5.09±0.02	4.9±0.02
BGKMJ1-36	6.07±0.02	5.59±0.05	5.57±0.03	5.15±0.04	5.0±0.03

The number of bacteria for both strains was determined at the end of the fermentation process and it was 8×10^8 for BGZLS10-17 and 5.5×10^8 for BGKMJ1--36. The results clearly indicated that the mixed culture of BGZLS10-17 and BGKMJ1-36 had much better technological properties and could be suitable for preparing of fermented milk beverage.

Moreover, fermented beverage prepared by mixed culture containing BGZLS10-17 and BGKMJ1-36 was stored at 5 °C and the stability of pH and

bacterial viability were monitored each 7 d until 21 d. The results are presented in Table III.

TABLE III. Stability of fermented beverage during 21 days of storage at 5 °C

Day	pH	Number of viable bacteria, CFU/ml	
		BGZLS10-17	BGKMJ1-36
1	4.9±0.02	8×10^8	5.5×10^8
7	4.84±0.02	7.8×10^8	5.4×10^8
14	4.82±0.01	7.7×10^8	5.5×10^8
21	4.82±0.04	1.5×10^8	8.5×10^7

The obtained results indicate that the manufactured fermented beverage was stable during 21 days, since the pH and the bacterial viability were not significantly changed over time.

CONCLUSION

The results obtained in this study highly indicate the positive role of GABA-producing strain BGZLS10-17 in amelioration of the EAE symptoms in DA rats that could be accredited to GABA present in the cell-free supernatant. Besides, technological characterization suggests promising use of the strain *L. brevis* BGZLS10-17 in combination with the autochthonous strain *S. thermophilus* BGKMJ1-36 as functional starter culture for production of GABA-enriched fermented beverage under controlled conditions, although the beneficial effects of the strain BGZLS10-17 on autoimmune diseases should be confirmed in clinical trials, according to EFSA regulations.[23]

Acknowledgment. This work was financially supported by The Ministry of Education, Science and Technological Development of The Republic of Serbia (project No. 173019, No. 173035).

ИЗВОД

КАРАКТЕРИЗАЦИЈА РЕЗИСТЕНЦИЈЕ НА pH И ПРОТЕОЛИТИЧКЕ АКТИВНОСТИ GABA-ПРОДУКУЈУЋЕГ СОЈА *Lactobacillus brevis* BGZLS10-17 У ПРИПРЕМИ ФЕРМЕНТИСАНОГ МЛЕЧНОГ НАПИТКА И ЕФЕКТИ НА УБЛАЖАВАЊЕ СИМПТОМА ЕКСПЕРИМЕНТАЛНОГ АУТОИМУНСКОГ ЕНЦЕФАЛОМИЈЕЛИТИСА

СВЕТЛАНА С. СОКОВИЋ БАЈИЋ[1,3], САЊА Б. МИХАЈЛОВИЋ[1], ДУШАН Д. РАДОЈЕВИЋ[1], ДУШАНКА Д. ПОПОВИЋ[1], ЈЕЛЕНА М. ЂОКИЋ[1], СУЗАНА М. СТАНИСАВЉЕВИЋ[2], МИЛИЦА Н. ЛАЗАРЕВИЋ[2], ЂОРЂЕ М. МИЉКОВИЋ[2], PATRICIA RUAS-MADIEDO[3], НАТАША Е. ГОЛИЋ[1] и МАЈА С. ТОЛИНАЧКИ[1]

[1]Лабораторија за молекуларну микробиологију, Институт за молекуларну генетику и генетичко инжењерство (ИМГГИ), Универзитет у Београду, [2]Одељење за имунологију, Институт за биолошка истраживања „Синиша Станковић“ (ИБИСС), Универзитет у Београду и [3]Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias – Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Asturias, Spain

Мултипла склероза (MS) је хронично аутоимунско обољење централног нервног система. Циљ овог рада је био изучавање пробиотичког ефекта произвођача γ-аминобутерне киселине (GABA), соја *Lactobacillus brevis* BGZLS10-17, на Dark Agouti (DA) пацовима осет-

љивим на индукцију експерименталног аутоимунског енцефаломијелитиса (EAE), експерименталног модела MS. Пацовима су свакодневно праћени клинички симптоми EAE. Резултати студије су показали да орална примена *L. brevis* BGZLS10-17 ублажава симптоме EAE код DA пацова, одложен је почетак болести, скраћено трајање болести, смањен интензитет болести у периоду када су симптоми код контрола најјаче изражени, а занимљиво је и да се животиње након четрдесет дана потпуно опорављају, што код контролних животиња које нису биле третиране пробиотиком *L. brevis* BGZLS10-17 није био случај. Побољшање EAE симптома је забележено и након примене супернатанта соја *L. brevis* BGZLS10-17 гајеног 48 сати у медијуму за раст бактерија са додатком прекурсора GABA, мононатријум-глутамата (MSG), и без њега. Добијени резултати указују на то да би GABA коју производи сој *L. brevis* BGZLS10-17 могла бити одговорна за ублажавање симптома EAE. Пробиотички ефекат соја *L. brevis* BGZLS10-17 ће бити даље тестиран у клиничким студијама на људима, како би сој могао да се користи као пробиотик у функционалној храни за пацијенте оболеле од аутоимунских болести.

(Примљено 16. јула, ревидирано 22. августа, прихваћено 3. септембра 2019)

REFERENCES

1. N. Ghasemi, S. Razavi, E. Nikzad, *Cell J*. **19** (2017) 1 (https://doi.org/10.22074/cellj.2016.4867)
2. R. L. Terry, I. Ifergan, S. D. Miller, *Methods Mol. Biol.* **1304** (2016) 144 (https://doi:10.1007/7651_2014_88)
3. M. M. Goldenberg, *Pharm. Ther*. **37** (2012) 175 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3351877/)
4. J. Chen, N. Chia, K. R. Kalari, J.Z. Yao, M. Novotna, M. M. Paz Soldan, D. H. Luckey, E. V. Marietta, P. R. Jeraldo, X. Chen, B.G. Weinshenker, M. Rodriguez, O. H., Kantarci, H. Nelson, J. A. Murray, A. K. Mangalam, *Sci. Rep*. (2016), Article number: 28484 (https://doi:10.1038/srep28484)
5. S. Stanisavljević, A. Čepić, S. Bojić, K. Veljović, S. Mihajlović, N. Đedović, B. Jevtić, M. Momčilović, M. Lazarević, M. Mostarica Stojković, Đ. Miljković, N. Golić, *Sci. Rep*. (2019), Article number: 918 (https://doi:10.1038/s41598-018-37505-7)
6. E. V. Demarkova, V. P. Korobov, L. M. Lemkina. *Klin. Lab. Diagn*. **4** (2003) 15 (https://www.ncbi.nlm.nih.gov/pubmed/12774663)
7. M. Carabotti, A. Scirocco, A. A. Maselli, C. Severi. *Ann. Gastroenterol.* **28** (2015) 203 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4367209/)
8. J. C. Nantes, S. Proulx, J. Zhong, S. A. Holmes, S. Narayanan, R. A. Brown, R. D. Hoge, L. Koski, *NeuroImage* **157** (2017) 705 (https://doi.org/10.1016/j.neuroimage.2017.01.033)
9. F. Gao, X. Yin, R. A. E. Edden, A. C. Evans, J. Xu, G. Cao, H. Li, M. Li, B. Zhao, J. Wang, G. Wang, *Hippocampus* **28** (2018) 813 (https://doi:10.1002/hipo.23001)
10. R. Dhakal, V. K. Bajpai, K. H. Baek, *Braz. J. Microbiol*. **43** (2012) 1230 (https://doi:10.1590/S1517-83822012000400001)
11. C. H. Wu, Y. H. Hsueh, J. M. Kuo, S. J. Liu, *Int. J. Mol. Sci*. **19** (2018) 143 (https://doi:10.3390/ijms19010143)
12. K. Papadimitriou, Á. Alegría, P. A. Bron, M. de Angelis, M. Gobbetti, M. Kleerebezem, J. A. Lemos, D. M. Linares, P. Ross, C. Stanton, F. Turroni, D. van Sinderen, P. Varmanen, M. Ventura, M. Zúñiga, E. Tsakalidou, J. Kok, *Microbiol. Mol. Biol. Rev*. **80** (2016) 837 (https://doi:10.1128/MMBR.00076-15)
13. S. Sokovic Bajic, J. Djokic, M. Dinic, K. Veljovic, N. Golic, S. Mihajlovic, M. Tolinacki, *Front. Microbiol*. **10** (2019) 527 (https://doi:10.3389/fmicb.2019.00527)

14. J.A. Valenzuela, A.B. Flórez, L. Vázquez, O.M. Vasek, B. Mayo, *Benefic. Microbes* **10** (2019) 579 (https://doi:10.3920/BM2018.0176)
15. S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res*. **25** (1997) 3389 (https://doi:10.1093/nar/25.17.3389)
16. S. Kumar, G. Stecher, K. Tamura, *Mol. Biol. Evol.* **33** (2016) 1870 (https://doi:10.1093/molbev/msw054)
17. I. Letunic, P. Bork, *Nucleic Acids Res*. **44** (2016) 242 (https://doi:10.1093/nar/gkw290)
18. M. Kojic, D. Fira, B. Bojovic, A. Banina, L. Topisirovic, *Appl. Environ. Microbiol*. **57** (1991) 1753 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC183463/)
19. D. De Biase, E. Pennacchietti, *Mol. Microbiol*. **86** (2012) 770 (https://doi:10.1111/mmi.12020)
20. E. R. S. Kunji, I. Mierau, A. Hagting, B. Poolman, W. N. Konings, *Antonie van Leeuwenhoek* **70** (1996) 187 (https://www.ncbi.nlm.nih.gov/pubmed/8879407)
21. M. Gobbetti, L. Stepaniak, M. De Angelis, A. Corsetti, R. Di Cagno, *Crit. Rev. Food Sci. Nutr.* **42** (2002) 223 (https://doi.10.1080/10408690290825538)
22. Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, T. Takano, *J. Dairy Sci*. **78** (1995) 777 (https://doi:10.3168/jds.S0022-0302(95)76689-9)
23. European Food Safety Authority (EFSA), *EFSA J.* **10** (2012) 2740 (https://doi:10.2903/j.efsa.2012.2740).