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Assessment of stability and bioactive compounds in yogurt containing novel natural starter cultures with the ability to promote longevity in *Caenorhabditis elegans*

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ABSTRACT

Yogurt represent one of the oldest fermented foods containing viable lactic acid bacteria and many bioactive compounds that could exhibit beneficial effects on human health and train our immune system to better respond to invading pathogens. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are commonly used for yogurt preparation under controlled temperature and environmental conditions. In this study, we investigated probiotic features of *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 strains isolated from artisanal sour milk and yogurt by using *Caenorhabditis elegans* as an in vivo model system. Further, we evaluated content of total fat, saturated fatty acids, proteins, and lactose, as well as vitamins and AA of yogurt prepared from above-mentioned starter cultures during 21 d of storage at 4°C to get insights of final product stability. We showed that *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 strains applied in combination upregulated the expression of autophagy-related genes in *C. elegans*. Beside autophagy, we observed activation of TIR-1-dependent transcription of lysozyme-like antimicrobial genes involved in the immune defense of *C. elegans*. Upregulation of these genes strongly correlates with an increase in the longevity of the worms fed with yogurt culture bacteria. Further, we showed that yogurt prepared with *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21, as a final product, is rich with vitamin B_2 and dominant AA known by their prolongevity properties. Taken together, our study pointed to the beneficial features of the tested starter cultures and yogurt and highlighted their potential to be used as a fermented food with added-value properties.

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INTRODUCTION

Over a century ago, Elie Metchnikoff pointed out the importance of gut microbiota in human health and healthy aging (Gordon, 2016). Guided by his observations that people with high yogurt consumption also showed increased longevity and better health, he advocated the consumption of fermented foods to manipulate the gut microbiome and promote the growth of beneficial bacteria (Bischoff, 2016). Lactic acid bacteria (**LAB**) used for yogurt preparation inhabit the gastrointestinal tract and exert various beneficial effects on human health (Aslam et al., 2020). To date, many studies have confirmed the prolongevity role of LAB by using *Caenorhabditis elegans* as a model organism suitable for aging research and host-microbe interaction studies (Heintz and Mair, 2014). The *C. elegans* genome possesses homologues of about two-thirds of all human genes, making it an excellent model system for studying various cellular pathways linked with human aging (Zhang et al., 2020). Experiments conducted on this soil bacterivore nematode with a short lifespan showed that different strains of lactobacilli used for fermented foods preparation can stimulate evolutionary-conserved longevity-promoting mechanisms important for the aging process in humans. These mechanisms, such as HLH-30 dependent autophagy, SKN-1 mediated antioxidative response, p38 MAPK immune pathway, and serotonin signaling, have been shown to be activated by LAB to delay somatic aging (Nakagawa et al., 2016; Dinić et al., 2021a; Kumar et al., 2022). Moreover, dietary lactobacilli could improve lipid metabolism and mitochondrial function in the worms and increase resistance to different pathogens (Dinić et al., 2021a,b).

Yogurt is a fermented milk product produced for several millennia, which is evidenced by Indian Ayurvedic scripts from about 6000 BC (Fisberg and Machado, 2015). According to the Codex Standard for fermented milks (243–2003; CXS, 2003) yogurt contains viable, live, and abundant starter microorganisms. By joint metabolic action in milk, yogurt starter cultures ferment lactose and produce lactic acid, decrease pH value, and at the same time, coagulate milk proteins, creating a specific yogurt texture, taste, and flavor (Hekmat and Reid, 2006). Yogurt production requires the use of a yogurt culture composed of 2 homofermentative LAB species, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, under controlled conditions during the fermentation process (Das et al., 2019). *Streptococcus thermophilus* begins to ferment lactose faster than *L. bulgaricus*, producing lactic acid and other organic acids and lowering the pH value to a level that corresponds to *L. bulgaricus* growth. However, *L. bulgaricus* has better developed proteolytic activity than *S. thermophilus* and hydrolyzes milk proteins, which supplies *S. thermophilus* with peptides, EAA, and putrescine necessary for its growth (Hutkins, 2006; Barros et al., 2020).

Yogurt contains many bioactive compounds that enhance the host immune system, such as bacteriocins, vitamins, AA and peptides, metabolic enzymes, short-chain fatty acids, antioxidants, anti-inflammatory and immune-modulating agents, and exopolysaccharides (**EPS**) (Chugh and Kamal-Eldin, 2020). Regular consumption of yogurt boosts immunity and protects the host against pathogens, lowers blood pressure, reduces obesity and the possibility of colon cancer, and improves the general health status of the host (Balcells et al., 2017; Buendia et al., 2018; Górska et al., 2019). In addition, the low lactose content in yogurt caused by the conversion of lactose to lactic acid by starter cultures is better tolerated by individuals with lactose intolerance. (Facioni et al., 2020). And finally, the low pH of yogurt inhibits the growth of different foodborne pathogenic bacteria (Lund et al., 2020).

In our previous study, we formulated yogurt containing natural starter cultures with health-promoting properties: *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21. Both strains showed the high capability to adhere to intestinal Caco-2 cells without triggering proinflammatory cytokines and significantly upregulated the expression of autophagy, tight-junction proteins, and antimicrobial-peptide–related genes and thus improved the gut epithelial barrier (Popović et al., 2020).

As we previously demonstrated excellent probiotic features of selected starter cultures in in vitro settings, the objective of this work was studying their beneficial effect and evaluation of their properties on the whole organism by using *C. elegans* as an in vivo model. Further, we analyzed the stability and changes in the chemical composition, as well as the composition of vitamins and free AA in yogurt, as a final product, during its storage for 21 d at a refrigeration temperature of 4°C.

MATERIALS AND METHODS

Bacteria Cultivation and Treatment

Probiotic strains *S. thermophilus* BGKMJ1-36 with the accession number LMG P-31742 and *L. bulgaricus* BGVLJ1-21 under the accession number LMG P-28578, both deposited in the Belgian Coordinated Collections of Microorganisms, Laboratory for Microbiology, University of Gent (BCCM/LMG), were isolated from artisanal sour milk and yogurt manufactured in households settled in the villages Jabuka and Mlečiške Mehane, Serbia, respectively (Popović et al., 2020). Cultivation of *S. thermophilus* BGKMJ1-36 was performed in M17 medium (Merck GmbH) with an addition of 0.5% (wt/vol) of glucose (GM17), and *L. bulgaricus* BGVLJ1-21 was cultured in deMan-Rogosa-Sharpe medium (Merck GmbH). Both strains were incubated anaerobically in a CO_2 incubator (HERAcell 150, Thermo Electron LED GmbH) with 5% of CO_2 at 37°C (Popović et al., 2020). The *Escherichia coli* OP50 (**OP50**) for *C. elegans* maintenance was cultivated aerobically overnight in Luria-Bertani medium at 37°C with shaking. Overnight grown cultures of *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 were centrifuged at 5,000 × *g* for 10 min at room temperature, and the obtained pellet was washed twice in PBS and resuspended in the Luria-Bertani medium like OP50, to exclude different medium influence on worms. The nematode growth medium (**NGM**) treatment plates were prepared by spreading the single bacterial suspension (OP50, BGKMJ1-36, or BGVLJ1-21) or yogurt bacterial mixture (BGKMJ1-36: BGVLJ1-21 = 1:3) on plates and dried at room temperature.

C. elegans Maintenance

Caenorhabditis elegans wild-type N_2 (Bristol) strain was maintained on NGM plates seeded with OP50 strain at 20°C by following standard protocols. Worms' synchronization from mix population of egg-bearing worms was done by extracting eggs with the cleaning solution containing 0.5 *M* NaOH with 1% of Na-hypochlorite and then washing them with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 mL of 1 *M* $MgSO_4$, all from Sigma-Aldrich) at least 3 times. Eggs were plated on OP50-seeded NGM plates and incubated overnight at 20°C to obtain synchronized animals. After 2 d of hatching, age-synchronous worms in the fourth larval (**L4**) stage, which is the last devel-

Table 1. List of primers used in this study for evaluation of the expression of autophagy and immune-related genes in *Caenorhabditis elegans*

Primer name	Primer sequence 5′–3′	Reference
act-1 forward	TGCAGAAGGAAATCACCGCT	Dinić et al., 2021a
act-1 reverse	CGGACTCGTCGTATTCTTGC	
unc-51 forward	GCTTTTTGGAAACCCCCAGC	Dinić et al., 2021a
unc-51 reverse	CAGCGACTTTGTACCTCGTGA	
hlh-30 forward	GTCCTGGCTCCCAAATCAGA	Dinić et al., 2021a
hlh-30 reverse	GATGCGTCTGCTGCATCTTC	
atg-7 forward	ACTCACAGCTGAAGGTTCTCA	Dinić et al., 2021a
atg-7 reverse	CCAGGCGTGCATCTTCAAAT	
atg-18 forward	TTGAATTCCGACGTGGCGTA	Dinić et al., 2021a
atg-18 reverse	GGTAGACGCTTCTGGCTTGT	
lgg-2 forward	AAGAAGGCCATTCCATGAGCG	Current study
lgg-2 reverse	ACAATTGACATGAGCTCGGCA	
sqstm-1 forward	TCAACGACCTCTGCAACTCC	Current study
sqstm-1 reverse	GGTGGAAGTGGTGGAACGAT	
tir-1 forward	CCGACCACCAAAGAAATGCC	Dinić et al., 2021b
tir-1 reverse	CTTGGTCCACCGATGCTTCT	
lys-3 forward	TGCGAAGAATCTGGGCTTGT	Dinić et al., 2021b
lys-3 reverse	GTGGCCTGTCTCCATGGTC	
lys-5 forward	TGCAGTGTTGTAAGTGCAGC	Dinić et al., 2021b
lys-5 reverse	CATCGACATCAGTGAGGCCA	
abf-2 forward	TTCCTTGCACTTCTCCTGGC	Dinić et al., 2021b
abf-2 reverse	GACGACCGCTTCGTTTCTTG	
spp-1 forward	TCGTCGAGGGTGGAGAGAAG	Dinić et al., 2021b
spp-1 reverse	ACGCCTTGTCTGGAGAATCC	

opmental stage before adulthood, were transferred to plates containing the appropriate bacterial treatment.

Lifespan Analysis

Worms in the L4 developmental stage were transferred to OP50, BGKMJ1-36, or BGVLJ1-21 treatment plates (3.5-cm plates) containing 20 μ*M* 5-fluorodeoxyuridine (Sigma-Aldrich) to avoid progeny hatching. In total, 75 worms per condition (25 worms per plate) were used in all treatments. The first day of adulthood was calculated as d 1 in lifespan measurement. Animals were scored every other day by prodding with a silver wire, and live worms were transferred to new plates with fresh treatment. The worms that escaped, or died due to internal hatching or protrusions, were not included in the analysis.

RNA Isolation and Quantitative Real-Time PCR

For gene expression analysis, L4 stage worms were incubated overnight on the 9-cm plates seeded with appropriate treatment and collected with M9 buffer. Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific). Isolated RNA was subsequently treated with DNase I (DNA-free DNA Removal Kit) according to the manufacturer's protocol (Thermo Fisher Scientific). Reverse-transcription was done using RevertAid Reverse Transcriptase (Thermo Fisher Scientific) with random hexamers (Thermo Fisher Scientific) and RiboLock RNase inhibitor (Thermo Fisher Scientific) in the reactions. As a template for cDNA synthesis, 0.5 μg of isolated RNA was used. Synthesized cDNA was then subjected to quantitative real-time PCR analysis using FastGene IC Green 2× PCR Universal Mix (Nippon Genetics Europe GmbH) in a 7,500 real-time PCR machine (Applied Biosystems) under the following conditions: 2 min at 95°C activation, 40 cycles of 5 s at 95°C, and 30 s at 60°C. Normalization of the expression was done against the *act-1* gene by using the $2^{-\Delta\Delta Ct}$ method. Primers used in the study are listed in Table 1 and were purchased from Thermo Fisher Scientific. For each condition, 3 independent replicates were used.

Yogurt Manufacturing

Yogurt was manufactured from commercial pasteurized cow milk with 2% fat (dairy in Subotica, Serbia). Overnight cultivation of 2% inoculum of BGKMJ1-36 and BGVLJ1-21 starter cultures, previously grown anaerobically at 37°C for 16 h in GM17 and deMan-Rogosa-Sharpe broth, respectively, was further inoculated in autoclaved reconstituted skimmed milk at 37°C. After incubation, starter cultures were added in 4 L of pasteurized milk, in an amount of 3% of the total milk amount in a 1:3 ratio of BGKMJ1-36:BGVLJ1-21. Glass bottles with inoculated milk were incubated at 42°C for 5 to 5.5 h until the pH value was decreased to about 4.8 and then rapidly cooled in containers with ice to avoid whey extraction. When the temperature of fermented milk dropped to about 15°C, bottles were shaken to obtain a homogenized, uniform viscous, liquid dairy product—yogurt. Further, the yogurt was cooled to 4°C in the refrigerator and stored for stability and bioactive compounds assessment.

Analysis of Chemical Composition and Content of Vitamins and AA of Pasteurized Milk and Yogurts During Storage

The pH values of pasteurized milk used for yogurt preparation, yogurt immediately after production, and yogurts after 7, 14, and 21 d of storage at 4°C were measured by pH Meter pH-2005 (Selecta). Analysis of the chemical composition and content of vitamins and AA of pasteurized milk and yogurts during the storage period at 4°C was done by accredited SP Laboratory (accreditation number 01-018; Bečej, Serbia) registered under number GMP049738. SP Laboratory fulfills the requirements of ISO/IEC17025:2017 standard (SRPS EN ISO/IEC, 2017) and is competent to perform testing activities. Valid scope of accreditation can be found at www.ats.rs.

The determination of fat according to Gerber (32/83, Official Gazette of the SFRY, 1983) is based on dissolving all ingredients of milk and yogurt, except fat, in sulfuric acid. Droplets of milk fat are separated by centrifugal force on the surface by adding amyl alcohol. The amount of fat is read directly on the butyrometer scale and is expressed as the number of grams of fat in 100 g of sample. The method of determination of SFA from C6:0 to C22:6 (ISO method 12966-1, SRPS EN, 2014f) is based on the method of GC with a flame ionization detector and according to the fast transmethylation model with methanol in the presence of potassium hydroxide (**KOH**) as an alkaline catalyst. The method is applied to samples that have a minimum fatty acid content of 0.02%. Determination of protein content was done according to ISO method 14891 (SRPS EN ISO, 2002). The method is based on the determination of the total nitrogen content by burning the sample in a combustion tube at a temperature of at least 960°C in a CO_2 atmosphere enriched with oxygen. At the same time, gaseous decomposition products remain in a closed system. Gases produced during combustion are introduced into columns with catalysts, where they are quantitatively transformed. Inorganic and organic nitrogen compounds are oxidized or evaporated. Combustion products are nitrogen oxides or molecular nitrogen N_2. After the conversion of all forms of nitrogen into N_2, the total nitrogen content is measured using a thermal conductivity detector, in relation to the reference gas. Ion chromatography with an electrochemical detector with reference silver (AgCl/Ag) electrode and a working gold (Au) electrode was used for determination of lactose content (method 11292; ISO, 1995). The method is applied in food samples at a concentration of 0.01%.

Vitamins A and E are released from the samples by alkaline hydrolysis with a KOH solution in the presence of ascorbic acid as an oxidant. After saponification, vitamin A is extracted with n-hexane and determined by normal-phase chromatography using a HPLC with a photodiode array detector (method 12822, SRPS EN, 2014a; method 12823–1, SRPS EN, 2014b). The method is applied in food at a concentration of 0.1 mg/100 g or 0.1 mg/100 mL. Vitamin D_3 is determined by extraction with a branched solvent after saponification of a food sample using a KOH in an alcoholic medium (method 12821, SRPS EN, 2012). Purification of the extract containing vitamin D_3 is performed on a system for normal-phase chromatography by collecting the eluate fraction containing vitamin D_3. Determination of vitamin D_3 from the eluate is performed on a system for reverse-phase chromatography with a diode array detector. Quantification of vitamin D_3 is performed using the internal standard method, wherein the internal standard is vitamin D_2. The method is applied in food at a concentration of 0.75 μg/100 g or 0.75 μg/100 mL of vitamin D_3 or 30 IU/100 g or 30 IU/100 mL of vitamin D_3. An HPLC with a fluorescent detector was used to determine content of vitamin B_1 (method 14122, SRPS EN, 2014c) and vitamin B_2 (method 14152, SRPS EN, 2014d). Both vitamins B_1 and B_2 are extracted from the samples by acid hydrolysis followed by dephosphorylation by the action of enzymes. Vitamin B_1 is oxidized to thiochrome in the derivatization process with potassium hexacyanoferrate III solution and quantified as such. The methods are applied in food at a concentration of 0.1 mg/100 g or 0.1 mg/100 mL. The method of determination of content of vitamin B_6 or pyridoxin (method 14164, SRPS EN, 2014e) is based on HPLC with a fluorescent detector. This method does not include the β-glycoside forms of vitamin B_6. Pyridoxal, pyridoxamine, and pyridoxine are extracted from samples by acid hydrolysis with HCl solution, which is followed by enzymatic dephosphorylation using acid phosphatase enzymes. By reacting with glyoxal acid in the presence of Fe^{2+} as a catalyst, pyridoxamine is converted into pyridoxal, which is then reduced to pyridoxine in a reaction with sodium borohydride in a basic medium and is quantified in that form. The method is applied in food at a concentration of 0.1 mg/100 g or 0.1 mg/100 mL.

Amino acids such as L-lysine, L-alanine, L-threonine, glycine, L-valine, L-serine, L-proline, L-isoleucine, L-leucine, L-methionine, L-histidine, L-phenylalanine, L-glutamate, L-aspartate, L-cystine, L-tyrosine, and

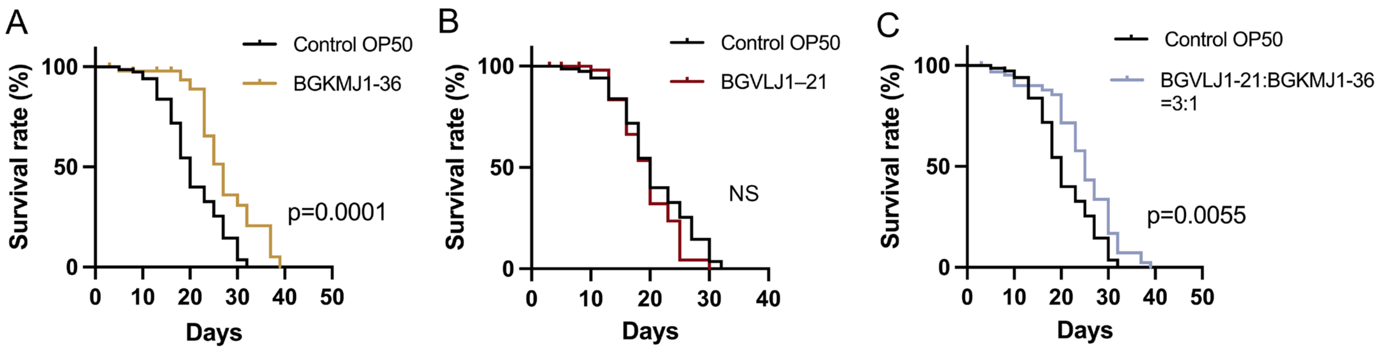

Figure 1. Yogurt starter cultures affect longevity of *Caenorhabditis elegans*. Lifespan curve of N_2 worms fed with control *Escherichia coli* OP50 and *Streptococcus thermophilus* BGKMJ1-36 (A), *Lactobacillus delbrueckii* ssp. *bulgaricus* BGVLJ1-21 (B), and *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 in a ratio of 1:3 (C) from the fourth larval (L4) stage, which is the last developmental stage before adulthood, maintained at 20°C (n = 75 worms per group). All values are presented as mean ± SD. The differences between survival curves were analyzed by using the log-rank (Mantel-Cox) test.

L-arginine were determined using ISO method 13903 standard (SRPS EN ISO, 2005). This method defines the method of sample preparation (acid hydrolysis with HCl) for the determination of AA using ion chromatography with an electrochemical detector, silver reference electrode (Ag/AgCl) and a gold working electrode (Au). The method is applied to food in a concentration of min 0.01%.

Statistical Analysis

All results are presented as mean values ± standard deviation. Student's *t*-test was used to compare the differences between control and treatment groups. The differences between survival curves in lifespan measurement were analyzed by using the log-rank (Mantel-Cox) test. A *P*-value less than 0.05 was considered statistically significant. The statistical analysis was performed and graphs were drawn in GraphPad Prism 9 software (https://www.graphpad.com).

RESULTS

Yogurt Starter Cultures Increased Longevity of C. elegans

To examine the beneficial effect of *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21, we first assessed their effect on worms aging, as a main visible indicator of dietary beneficial effects. By measuring the worm's lifespan, we were able to show that the *S. thermophilus* BGKMJ1-36 strain exhibited a significant increase (P = 0.0001) in both median and maximal lifespan, compared with OP50 treated control worms (Figure 1A). However, worms fed *L. bulgaricus* BGVLJ1-21 had the same lifespan as OP50 treated worms, but without causing some negative effects on the worms' health (Figure 1B). More importantly, the final combination of both strains, BGKMJ1-36/BGVLJ1-21 in ratio design for yogurt preparation, also showed a significant increase (P = 0.0055) in median and maximal lifespan (Figure 1C). Overall, these results indicate that the yogurt bacteria in combination are able to delay aging of the host and imply that both strains are compatible for yogurt formulation and could trigger strain-specific beneficial effects.

Yogurt Mixed Starter Culture BGKMJ1-36/BGVLJ1-21 Upregulated Expression of Autophagy-Related Genes in C. elegans

To gain further insights into the mechanisms behind observed prolongevity effect, we first evaluated autophagy, previously shown to be triggered by the *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 combination in epithelial Caco-2 cells (Popović et al., 2020). Autophagy is one of the major cellular catabolic processes that regulate epithelial barrier homeostasis and promotes a healthy gut, and reports showed that it could be modulated by LAB in both in vitro and in vivo conditions (Inaba et al., 2016; Haq et al., 2019; Soković Bajić et al., 2020). Therefore, to confirm our in vitro data and translate our research, we performed a quantitative real-time PCR analysis of autophagy-relevant genes in the N_2 strain of *C. elegans*. The results revealed that worms fed overnight with yogurt mixed starter culture BGKMJ1-36/BGVLJ1-21 exhibited upregulated levels of genes involved in all steps of the autophagy process, including autophagy induction (*unc-51*; P = 0.0406), autophagosome expansion (*atg-7*, P = 0.03; *lgg-2*, P = 0.0073), and retrieval of proteins for autophagy degradation (*sqstm-1*; P = 0.0088), compared

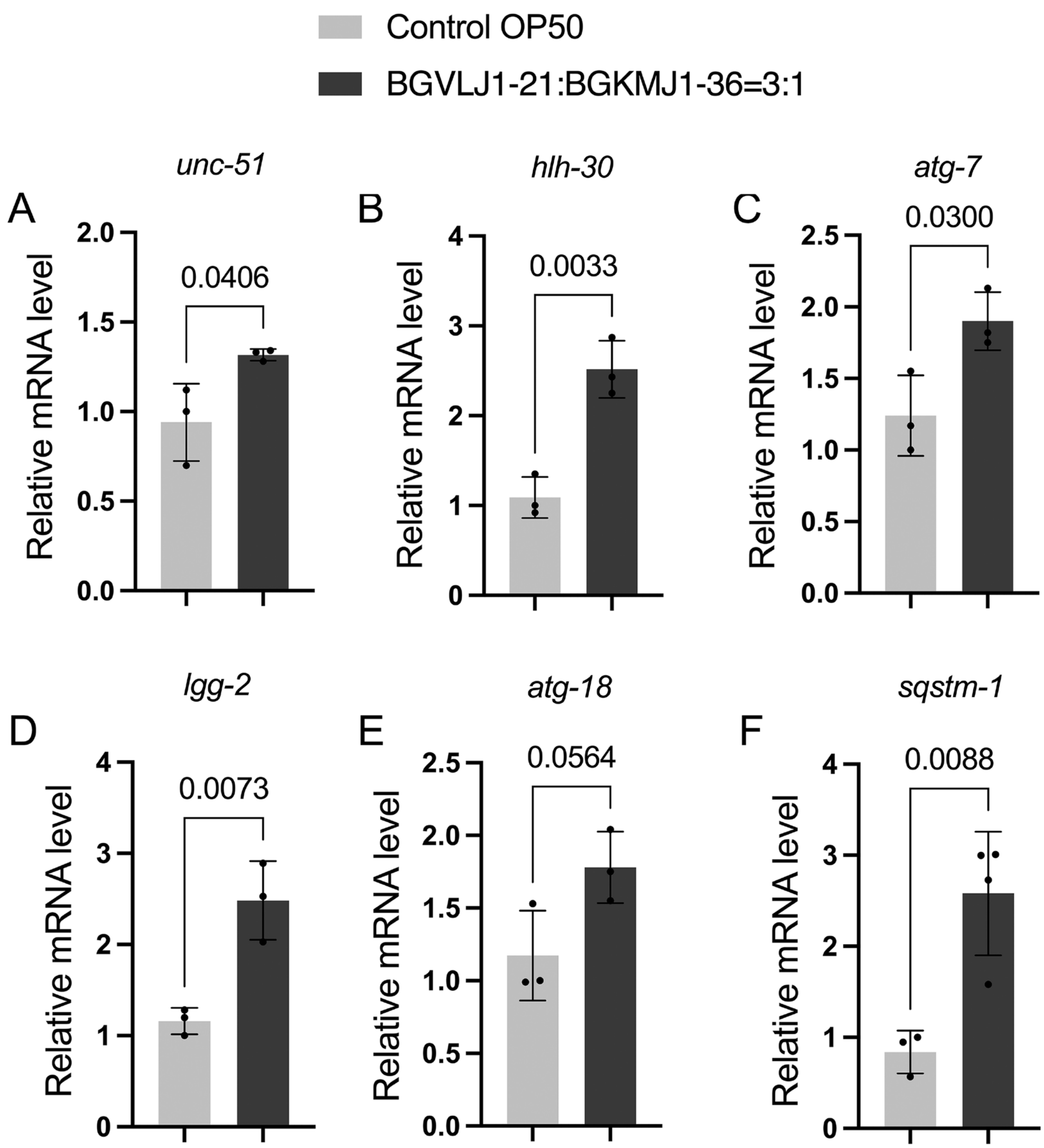

Figure 2. Yogurt starter cultures trigger autophagy in *Caenorhabditis elegans*. Expression of autophagy-related genes, including *unc-51* (A), *hlh-30* (B), *atg-7* (C), *lgg-2* (D), *atg-18* (E), and *sqstm-1* (F), was measured by quantitative real-time PCR in the fourth larval (L4) stage N_2 worms after overnight feeding with control *Escherichia coli* OP50 and combination of *Streptococcus thermophilus* BGKMJ1-36 and *Lactobacillus delbrueckii* ssp. *bulgaricus* BGVLJ1-21 in a ratio of 1:3 (n = 3; 3 independent experiments). All values are presented as mean ± SD. Student's *t*-test was used for 2-group comparison.

with OP50 treated worms (Figure 2). The *atg-18* gene, also responsible for the increased longevity of *C. elegans* (Lapierre et al., 2011), showed slight upregulation and almost reached statistical significance with a *P*-value of $P = 0.0564$. Finally, significantly increased expression of the *hlh-30* gene ($P = 0.0033$), that codes an ortholog of mammalian transcription factor EB (TFEB) was observed (Figure 2). Taken together, our data pointed out that autophagy stimulation correlates with lifespan extension triggered by the *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 combination.

Yogurt Mixed Starter Culture BGKMJ1-36/BGVLJ1-21 Triggered TIR-1 Mediated Immune Response and Expression of Lysozyme-Like Antimicrobial Proteins in C. elegans

The most common effect of yogurt cultures on human health is the ability to increase the body's resistance to pathogens mainly via 2 mechanisms: (1) inhibition of pathogenic bacteria by bacteriocins production and by reduction of the pH and free oxygen levels, and (2) by reinforcement of the epithelial barrier and mucosal immunity (Hori et al., 2020). We showed that the *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 combination could stimulate the expression of tight-junctions encoding genes together with human β-defensin 1 in vitro (Popović et al., 2020). Here, we evaluated the induction of conserved immune signaling in worms after overnight treatment of the worms with a yogurt bacteria combination. In addition to the negligible role of toll-like protein TOL-1 in worms' defense, another gene product containing toll/IL-1R (TIR) protein-protein interaction domain (TIR-1) emerged as a major defense factor in *C. elegans*, acting as an upstream activator of the PMK-1 pathway that corresponds to mammalian p38 MAPK (Ermolaeva and Schumacher, 2014). We started with measuring the *tir-1* expression and noticed that the yogurt mixed starter culture BGKMJ1-36/BGVLJ1-21 significantly stimulated the expression of this gene (P = 0.0354). Further, from a variety of *C. elegans* antimicrobials, we detected that worms treated with the yogurt mixed starter culture BGKMJ1-36/BGVLJ1-21 had higher mRNA levels of lysozyme-like antimicrobial genes (*lys-3*, P = 0.0350; and *lys-5*, P = 0.0006), but transcription of antimicrobial peptides *abf-2* and *spp-1* were at the same level as in the control (Figure 3). Therefore, in addition to the already demonstrated direct antimicrobial effects of *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 (Popović et al., 2020), we confirmed the ability of the mixed starter culture BGKMJ1-36/BGVLJ1-21 to increase immune surveillance at the organism level.

Characterization of the Yogurt Starter Cultures

Physiological, biochemical, technological, and probiotic characterization of *L. bulgaricus* BGVLJ1-21 and *S. thermophilus* BGKMJ1-36 were done previously (Popović et al., 2020). Both strains curdled the milk for about 5 h at 42°C. The strain *L. bulgaricus* BGVLJ1-21 showed better proteolytic activity than *S. thermophilus* BGKMJ1-36. Also, *L. bulgaricus* BGVLJ1-21 and *S. thermophilus* BGKMJ1-36 inhibited the growth of *Listeria monocytogenes* ATCC19111. In addition, *S. thermophilus* BGKMJ1-36 is an EPS producer. Growth kinetics of *L. bulgaricus* BGVLJ1-21 and *S. thermophilus* BGKMJ1-36 strains showed that the total number of viable bacteria in yogurt immediately after production was 10^8 cfu/mL, and after a 21-d storage period at 4°C, that number was slightly lower and was 10^7 cfu/mL of yogurt (Popović et al., 2020).

Yogurt cultures are usually added to milk in a ratio of 1:1, and it's best to add them separately in milk when preparing a new amount of yogurt. Otherwise, the ratio between cultures could be changed and yogurt would not be of appropriate quality (Hutkins, 2006). In our study, formulation of final product was done by mixing starter cultures *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 in a ratio of 1:3 instead of the previously used ratio of 1:2 (Popović et al., 2020) for 2 reasons: (1) to reduce the density of yogurt due to the extreme EPS production by BGKMJ1-36 strain, and (2) to reduce the pH drop-value of the final product during storage. Literature data showed that *S. thermophilus* is primarily responsible for the creation of lactic acid, thus lowering the pH value of the fermented product (Sabbah et al., 2012). Initial measurement after yogurt production showed that the pH value was 4.82 and slowly decreased to 4.61 after 21 d of storage at 4°C (Table 2). The 1:2 ratio of BGKMJ1-36:BGVLJ1-21 decreased pH value from 4.74 initially to 4.13 after 21 d of storage at 4°C (Popović et al., 2020), which made it a little bit sour to consumers. Therefore, in this study, we changed the ratio of BGKMJ1-36 and BGVLJ1-21 to 1:3, to improve the flavor of the final product.

Changes in the Chemical Composition and Vitamins Content During Yogurt Storage at 4°C

Further, in Table 2 the results of the content of basic chemical components including total fat, SFA, proteins, and lactose in pasteurized cow milk and yogurt immediately after production and during the 3-wk storage at 4°C are presented. The results of measuring of the content of basic chemical parameters showed that the content of fat, SFA, and proteins in pasteurized cow milk, yogurt immediately after production, and yogurt after 21 d of storage at 4°C were unchanged and stable (Table 2). However, lactose concentration was measured to be 4.5% in pasteurized milk and decreased to 2.88% in yogurt immediately after production, and it remained at that level (2.8%) after 21 d of storage at 4°C (Table 2).

Although in very low concentrations, vitamins are essential bioactive compounds necessary in human nutrition. Milk and dairy products are an important source of various vitamins, especially group B vitamins, except biotin (Graulet and Girard, 2017). In our study, we identified vitamin B_2 (riboflavin) as the most abundant of all vitamins. The concentration of vitamin B_2 in pasteurized milk was 0.17 mg/100 g, and this value slightly increased to 0.18 mg/100 g in 21-d stored yogurt (Table 3). However, contents of vitamins A, D_3, E, B_1, and B_6 were detected as less than 0.1 mg/100 g (for vitamin D_3, μg/100 g) in pasteurized milk and in yogurts during a storage period at 4°C (Table 3).

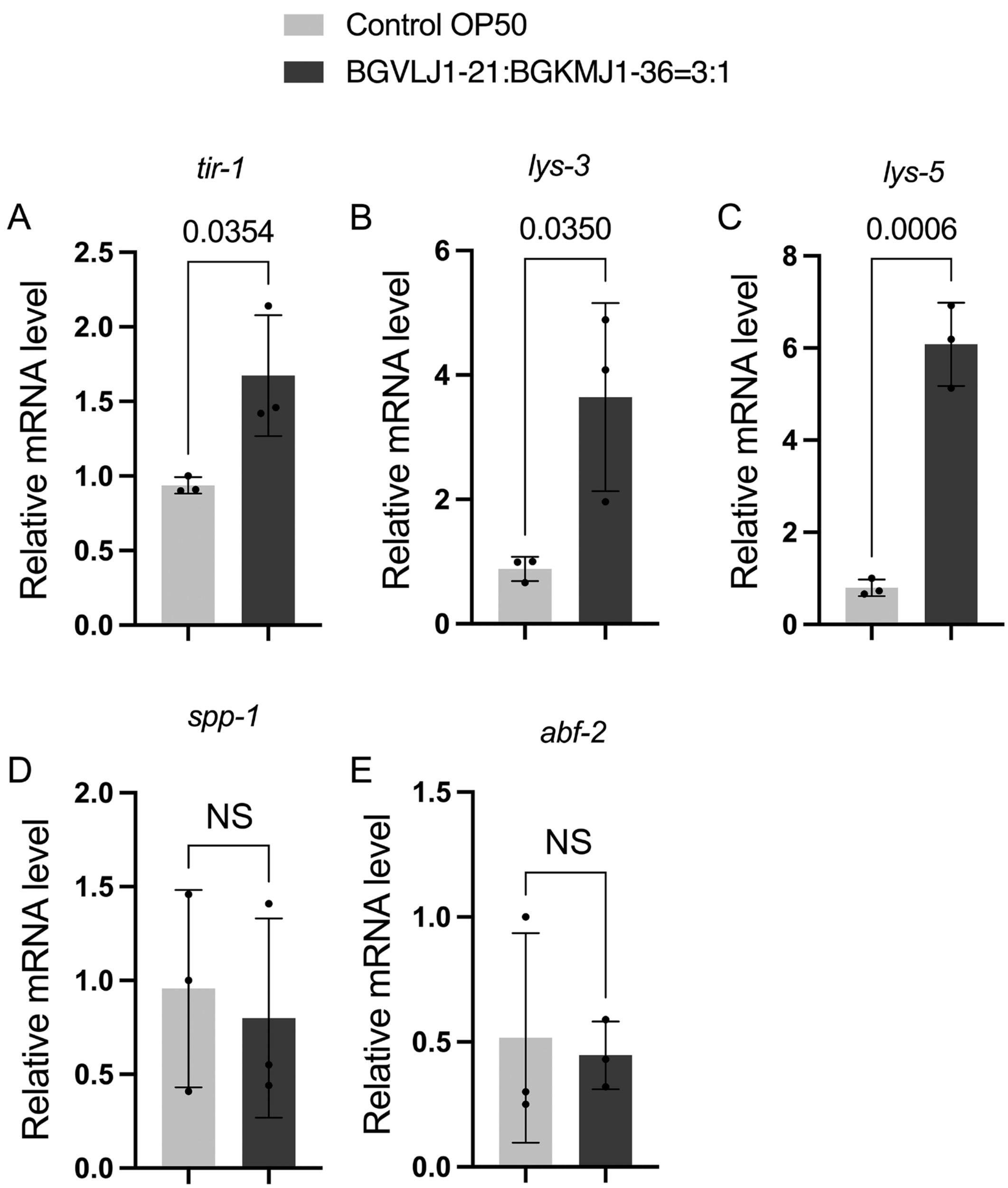

Figure 3. Yogurt starter cultures upregulate the immune defense of *Caenorhabditis elegans*. Expression of the *tir-1* (A) and antimicrobial genes including *lys-3* (B), *lys-5* (C), *spp-1* (D), and *abf-2* (E) was measured by quantitative real-time PCR in the fourth larval (L4) stage N_2 worms after overnight feeding with control *Escherichia coli* OP50 and a combination of *Streptococcus thermophilus* BGKMJ1-36 and *Lactobacillus delbrueckii* ssp. *bulgaricus* BGVLJ1-21 in a ratio of 1:3 (n = 3; 3 independent experiments). All values are presented as mean ± SD. Student's *t*-test was used for statistical comparison between 2 groups.

AA Composition During Yogurt Storage at 4°C

During lactic acid fermentation, the proteins are partially degraded into peptides and free AA by the action of bacterial proteolytic enzymes, which contribute directly to the taste and flavor of the final product (Lim et al., 2009). Results obtained after analyzing the AA composition of commercial pasteurized cow milk and yogurt immediately after production and during a storage period of 21 d at 4°C are listed in Table 4. The content of 17 AA was monitored, including EAA such as lysine, threonine, valine, isoleucine, leucine, methionine, histidine, phenylalanine, and arginine. It can be noticed that leucine, a branched-chain AA that regulates metabolic health, health span, and aging (Babygirija and Lamming, 2021), is detected in the highest

Table 2. Physicochemical composition (± SD) of pasteurized milk and yogurt of different periods of storage at 4°C

	Measured value				
Physicochemical parameter	Pasteurized milk	1-d yogurt	7-d yogurt	14-d yogurt	21-d yogurt
pH	6.62 ± 0.01	4.82 ± 0.026	4.77 ± 0.025	4.71 ± 0.023	4.63 ± 0.025
Fat (g/100 g)	2 ± 0.096	1.98 ± 0.099	1.87 ± 0.094	1.98 ± 0.099	1.98 ± 0.099
SFA (g/100 g)	1.3 ± 0.247	1.28 ± 0.256	1.21 ± 0.242	1.29 ± 0.258	1.31 ± 0.126
Proteins, N × 6.25 (%)	3.18 ± 0.127	3.38 ± 0.135	3.32 ± 0.133	3.43 ± 0.137	3.15 ± 0.126
Lactose (g/100 g)	4.45 ± 1.113	2.88 ± 0.720	3 ± 0.8	3.07 ± 0.768	2.80 ± 0.70

concentration (0.36%). In contrast, the concentration of methionine and histidine was the lowest (0.09% and 0.05%, respectively). All 17 AA detected in pasteurized milk were detected also in yogurt after 21 d of storage at 4°C. Contents of lysine, alanine, glutamate, and arginine in 21-d-old yogurt were higher than in pasteurized milk. Higher contents of histidine and aspartate were in yogurt immediately after production, with lysine and glutamate in yogurt stored for 7 d, and the highest content of arginine was recorded in 14- and 21-d-old yogurt, compared with the content in pasteurized milk and yogurt immediately after production (Table 4).

DISCUSSION

In the present study, we reported the potential of yogurt mixed starter cultures containing *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 strains to stimulate expression of autophagy and immune-related genes in *C. elegans*, which contributed to an increase in lifespan of the worms studied. Lifespan extension achieved with yogurt mixed starter cultures BGKMJ1-36/BHVLJ1-21 was comparable to that induced by the *S. thermophilus* BGKMJ1-36 strain when applied alone, suggesting that prolongevity signals probably come from *S. thermophilus* BGKMJ1-36. A recent study already showed that *S. thermophilus* could extend the lifespan of *C. elegans* through the activation of DAF-16-mediated upregulation of superoxide dismutase and catalase antioxidative genes (Desaka et al., 2022). The probiotic effects of LAB are generally reflected in the interaction between bacterial biomolecules, either cell wall bound or secreted, and the host (Lebeer et al., 2010). The strain *S. thermophilus* BGKMJ1-36 has the capability of producing a viscous extracellular polysaccharide biomolecule, EPS, which covers all bacterial surface and shields other bacterial biomolecules and antigens (Nikolic et al., 2012; Popović et al., 2020). Exopolysaccharides are responsible for rheological properties of the yogurt formulation, giving it a fine viscous texture (Folkenberg et al., 2006). In addition to technological properties, EPS produced by various LAB possess immunomodulatory activity but also can promote autophagy and protect the host from enteropathogens invasion (Zivkovic et al., 2015; Dinić et al., 2018; Yuan et al., 2021).

In addition, bacterial-produced polysaccharides, such as colanic acid, could extend host longevity (Han et al., 2017). Therefore, all these data pointed out that EPS could contribute to the observed prolongevity ef-

Table 3. Vitamin content (± SD) of pasteurized milk and yogurt of different periods of storage at 4°C

	Measured value in[1]				
Vitamin	Pasteurized milk	1-d yogurt	7-d yogurt	14-d yogurt	21-d yogurt
Vitamin A (retinol; mg/100 g)	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%
Vitamin D_3 (cholecalciferol; μg/100 g)	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%
Vitamin E (α tocopherol; mg/100 g)	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%
Vitamin B_1 (thiamine; mg/100 g)	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%
Vitamin B_2 (riboflavin; mg/100 g)	0.17 ± 0.034	0.14 ± 0.028	0.18 ± 0.036	0.17 ± 0.034	0.18 ± 0.036
Vitamin B_6 (pyridoxine; mg/100 g)	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%	<0.1 ± 20%

[1]Limit of quantification is <0.1 for vitamins A, D_3, E, B_1, and B_6. Extended measurement uncertainty of 20% is expressed as a combined standard measurement uncertainty increased by the coverage factor k = 2 for a confidence level of approximately 95%.

Table 4. Amino acid content (± SD) of pasteurized milk and yogurt of different periods of storage at 4°C

	Measured value in[1]				
AA (%)	Pasteurized milk	1-d yogurt	7-d yogurt	14-d yogurt	21-d yogurt
L-Lysine	0.19 ± 0.038	0.25 ± 0.050	0.28 ± 0.056	0.25 ± 0.050	0.21 ± 0.042
L-Alanine	0.11 ± 0.022	0.1 ± 0.02	0.16 ± 0.032	0.17 ± 0.034	0.12 ± 0.024
L-Threonine	0.19 ± 0.038	0.17 ± 0.034	0.15 ± 0.030	0.14 ± 0.028	0.13 ± 0.026
Glycine	0.12 ± 0.024	0.11 ± 0.022	0.06 ± 0.012	0.07 ± 0.014	0.08 ± 0.016
L-Valine	0.19 ± 0.038	0.19 ± 0.038	0.18 ± 0.036	0.1 ± 0.02	0.16 ± 0.032
L-Serine	0.23 ± 0.046	0.21 ± 0.042	0.22 ± 0.044	0.2 ± 0.04	0.15 ± 0.030
L-Proline	0.32 ± 0.064	0.31 ± 0.062	0.31 ± 0.062	0.27 ± 0.054	0.24 ± 0.048
L-Isoleucine	0.17 ± 0.034	0.16 ± 0.032	0.13 ± 0.026	0.18 ± 0.036	0.15 ± 0.030
L-Leucine	0.36 ± 0.072	0.34 ± 0.068	0.24 ± 0.048	0.4 ± 0.08	0.34 ± 0.068
L-Methionine	0.09 ± 0.018	0.09 ± 0.018	0.06 ± 0.012	0.09 ± 0.018	0.07 ± 0.014
L-Histidine	0.05 ± 0.010	0.09 ± 0.018	0.08 ± 0.016	0.08 ± 0.016	0.07 ± 0.014
L-Phenylalanine	0.1 ± 0.02	0.12 ± 0.024	0.13 ± 0.026	0.11 ± 0.022	0.12 ± 0.024
L-Glutamate	0.54 ± 0.108	0.6 ± 0.12	0.69 ± 0.138	0.65 ± 0.130	0.6 ± 0.12
L-Aspartate	0.24 ± 0.048	0.26 ± 0.052	0.24 ± 0.048	0.22 ± 0.044	0.22 ± 0.044
L-Cystine	0.04 ± 0.008	0.03 ± 0.006	0.024 ± 0.004	0.01 ± 0.002	0.01 ± 0.002
L-Tyrosine	0.19 ± 0.038	0.18 ± 0.036	0.13 ± 0.026	0.16 ± 0.032	0.15 ± 0.030
L-Arginine	0.06 ± 0.012	0.07 ± 0.0142	0.1 ± 0.02	0.16 ± 0.032	0.14 ± 0.028

[1]Extended measurement uncertainty is expressed as a combined standard measurement uncertainty increased by the coverage factor k = 2 for a confidence level of approximately 95%.

fects of *S. thermophilus* BGKMJ1-36. In contrast, other reports showed that commercial and foodborne isolates of *L. bulgaricus* could also increase worms' longevity, but the mechanism remained unknown (Zanni et al., 2017). However, we did not notice this effect with the *L. bulgaricus* BGVLJ1-21 strain, which suggests that the longevity-induced potential of the LAB are strain-specific. Even though *L. bulgaricus* BGVLJ1-21 did not alter the worms' lifespan, we previously showed that *L. bulgaricus* BGVLJ1-21 possesses other beneficial qualities, such as antimicrobial activity toward foodborne pathogen *Listeria monocytogenes*, and has an important role in the yogurt fermentation process (Popović et al., 2020). Moreover, some probiotic strains could exhibit aging-delay effects in *C. elegans*, even when they are added in a small amount (1:2 or 1:4 ratio), as a supplement to the normal *E. coli* food (Dinić et al., 2021a; Zhang et al., 2022). However, a combination of LAB in different ratios and their effects on worms' lifespan has not been tested before. Therefore, we chose to focus our research on the evaluation of these effects of both strains applied in combination.

Our previous study showed that HLH-30 is essential for the extended lifespan observed in *C. elegans* fed with probiotic *L. fermentum* BGHV110 strain, which is in accordance with the data presented here (Dinić et al., 2021a). HLH-30 have a major role in autophagy regulation in worms, but also in lifespan determination (Lapierre et al., 2013). Our results listed autophagy as an additional mechanism that could be triggered by *S. thermophilus* BGKMJ1-36, in line with already reported DAF-16 mediated antioxidative response (Desaka et al., 2022). Further, interplay of *S. thermophilus* and *L. bulgaricus* with autophagy in in vivo conditions is only reported in the study where these 2 species were part of a formulation made of 9 live bacterial strains that showed restoration of impaired neuronal autophagy in mice (Bonfili et al., 2017). This treatment involved application of 9 LAB strains; it is not clear what was the contribution of individual strains in autophagy modulation. Biomolecules and metabolites that are LAB-derived or *C. elegans* bioactive molecules that are produced in response to its interaction with these 2 strains could be responsible for the upregulation of autophagy-related genes, especially considering that EPS molecule has been already linked with autophagy signaling (Yuan et al., 2021).

Besides autophagy, we demonstrated that the combination of *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 strains stimulated expression of the genes involved in immune defense of *C. elegans*. Similar results were obtained for the *Lactobacillus curvatus* BGMK2-41 probiotic strain, which showed the capability to induce PMK-1/p38 MAPK dependent transcription of lysozymes and mediated higher worm resistance toward *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dinić et al., 2021b). In addition to its immune function, PMK-1, similar to HLH-30, also contributes to longevity by activating the transcription of different sets of genes related mainly to oxidative stress (Ermolaeva and Schumacher, 2014). Even though no data exists regarding the effects of these 2 bacterial species on worms' immunity, a recent report showed that *L. bulgaricus* ME-552 (ME552) and *S. thermophilus*

ME-553 could modulate T-cell effector functions and enhance mucosal immunity and production of IFN-γ and IL-17 in mice (Kamiya et al., 2016).

According to literature data, the minimum therapeutic level of viable probiotic bacteria during the product shelf life should be at least 10^6 cfu/g of food labeled as a probiotic functional food (Gibson et al., 2017). They suggested that ~100 g/d of probiotic foods should be consumed, which is 10^8 to 10^9 cfu/g bacteria at the same time, and that that amount would be sufficient to cause positive physiological functions in humans. Colony-forming unit counts of both tested strains detected in the newly formulated yogurt were in accordance with these recommendations. However, in addition to probiotic starter cultures from the yogurt, milk nutrients contained in yogurt are of crucial importance in the human diet and enable normal function of the body. They can be divided into element builders (proteins, carbohydrates, and lipids) and functional elements, which include various vitamins and minerals (Pereira, 2014). Even though content of fat, SFA, and proteins were unchanged and stable in prepared yogurt, the observed decrease in lactose concentration was expected considering the activity of the starter cultures, which fermented lactose to lactic acid by their metabolic mechanism of action (Leeuwendaal et al., 2022). This is of great importance for consumers suffering from lactose intolerance who could replace regular milk with fermented products such as yogurt.

Vitamins from the yogurt represent important health-promoting nutrients, and their lack in the daily diet would have a negative effect on the overall functioning of the organism (Graulet et al., 2013). Riboflavin was the most abundant of all tested vitamins, its concentration was higher than the values of the vitamin B_2 usually reported for yogurt collected from local stores in Serbia (0.13 mg/100 g; Sunarić et al., 2012). Riboflavin is a water-soluble vitamin exhibiting antioxidative, anti-inflammatory, antinociceptive, and antiaging properties (Suwannasom et al., 2020). It has been reported that the application of 120 μg/mL of vitamin B_2 is sufficient to increase the longevity of fruit fly *Drosophila melanogaster* via an antioxidative mechanism (Zou et al., 2017). Therefore, the combination of starter cultures with longevity-promoting effects and vitamin B_2 could exhibit a synergistic antiaging effect on the host. In addition, the absence of vitamins A, E, B_1, B_2, and B_6 was in accordance with a previous study reporting that their concentration detected in cow milk were less than 0.1 mg/100 g (Temerbayeva et al., 2018). Brodziak et al. (2021) stated that the vitamin content depends on many factors, such as the milk used for yogurt production (raw milk from farm or commercial milk), along with production season, type of pasture, lactation period, and fat content. We supposed that it was the reason for lower content of certain vitamins compared with the vitamin content found in natural yogurts with 2% fat (Mojka, 2013; Brodziak et al., 2021).

Finally, the importance of AA for the human body is immeasurable. Tryptophan, tyrosine, histidine, and proline are strong antioxidants, whereas valine and leucine inhibit lipid peroxidation (Tonolo et al., 2019; Bielecka et al., 2022). In addition, Kepert et al. (2017) showed that tryptophan has an immunomodulatory effect against chronic immune diseases, especially allergic reactions. Methionine and cysteine, as AA with sulfhydryl group, alleviate oxidative stress, protecting tissue against damage (Bin et al., 2017). But most importantly, arginine, lysine, glutamine, and alanine have antiaging properties and can extend the lifespan of *C. elegans* (Canfield and Bradshaw, 2019). We identified that with storage, the concentrations of arginine, lysine, glutamate, and alanine increase in the yogurt, suggesting that the proteinases activity of *L. bulgaricus* BGVLJ1-21 is directed toward releasing antiaging AA from casein more than the other AA. Moreover, glutamate represents the substrate for different LAB with the capability to synthesize γ-aminobutyric acid (**GABA**), an inhibitory neurotransmitter important for the immunomodulatory properties of the LAB. Our previous work showed that LAB grown with glutamate can increase the expression of tight-junction proteins of the gut epithelium by producing GABA (Soković Bajić et al., 2019). Overall, according to the obtained results, the AA concentration is mainly dictated by the milk used for yogurt production, but the proteolytic activity of *L. bulgaricus* BGVLJ1-21 could further shift AA content by favoring the production of AA associated with increased longevity.

CONCLUSIONS

Industrial starter cultures lack the necessary characteristics for the product diversification. Because the biodiversity of commercial starter cultures is limited, they have exhausted their technological and probiotic potential. Natural LAB from artisanal dairy products could be excellent candidates for designing innovative starter cultures for the production of functional dairy foods with improved probiotic and sensory properties, comparing to those in grocery stores. Novel natural starter cultures *S. thermophilus* BGKMJ1-36 and *L. bulgaricus* BGVLJ1-21 upregulate transcription of autophagy-related genes and genes involved in immune defense necessary for longevity assurance in *C. elegans*. Further, our results indicate the prevalence of riboflavin and AA with prolongevity properties in the yogurt, which altogether could exhibit a synergistic effect in terms of host longevity. Therefore, better understanding of beneficial effects of LAB enables the application of a new generation of functional LAB starters and offer technological and health benefits for the dairy community and consumers.

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