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Original Research

Traditional Bulgarian Fermented Foods as a Source of Beneficial Lactic Acid Bacteria

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Abstract

Background: Traditional Bulgarian fermented foods are prominent for their uniqueness of local ingredients, production methods, and endemic microbial species. The present research investigated the diversity and beneficial biological potential of lactic acid bacteria (LAB) isolated from various types of unique Bulgarian fermented foods. **Methods**: Species identification was performed via 16S rDNA sequencing. Biological activity was evaluated by determining antibacterial activity (via agar well diffusion assay), H_2O_2 production, spectrophotometrically determined auto- and co-aggregation, microbial adhesion to hydrocarbon, and biofilm formation. The biosafety of the isolated lactic acid bacteria was established based on hemolytic activity and phenotypic and genotypic antibiotic susceptibility. **Results**: Forty-five strains were isolated from fermented foods (sauerkraut, fermented green tomatoes, fermented cucumbers, kefir, white cheese, and Izvara (curdled milk)). Five species were detected: *Lactiplantibacillus plantarum*, *Levilactobacillus koreensis*, *Levilactobacillus brevis*, *Lactobacillus helveticus*, and *Levilactobacillus yonginensis*. The most prominent species was *L. plantarum*, at 47%. For the first time, *L. koreensis* and *L. yonginensis*, isolated from unique Bulgarian fermented foods, are reported in this study. The antibacterial effect of the cell-free supernatants was evaluated. An antagonistic effect was observed against *Escherichia coli* (57%) and *Salmonella enterica* subsp. *enterica* serotype Enteritidis (19%) for several *L. plantarum* strains. Only one *L. brevis* (Sauerkraut, S15) strain showed activity against *E. coli*. The best autoaggregation ability at hour 4 was observed for *L. koreensis* (fermented cucumbers, FC4) (48%) and *L. brevis* S2 (44%). The highest percentage of co-aggregation with *Candida albicans,* at hou 4 in the experiments, was observed for strains *L. koreensis* (fermented green tomato, FGT1) (70%), *L. plantarum* strains S2 (54%), S13 (51%), and S6 (50%), while at hour 24 for strains *L. koreensis* FGT1 (95%), *L. brevis* (Kefir, K7) (89%), *L. plantarum* S2 (72%), and *L. koreensis* FC2 (70%). Seven of the isolated LAB strains showed hydrophobicity above 40%. Our results showed that the ability of biofilm formation is strain–dependent. No hemolytic activity was detected. The antibiotic resistance to 10 antibiotics from different groups was tested phenotypically and genotypically. No amplification products were observed in any strains, confirming that the isolates did not carry antibiotic-resistance genes. **Conclusions**: Traditional fermented Bulgarian foods can be considered functional foods and beneficial LAB sources.

Keywords: lactic acid bacteria; functional foods; bio-protection; beneficial bacteria; antibacterial potential; acquired-antibiotic resistance

1. Introduction

For centuries, fermented products derived from plant or animal materials have played an essential role in human nutrition worldwide. Food fermentation is considered one of the most ancient ways of processing and preserving food. Fermentation enhances the flavor and nutritional quality of food and increases its shelf life. Fermented foods are considered a good source of natural probiotics because their intake has been reported to improve intestinal function, help boost immunity, and prevent allergies [1]. These factors contribute to the growing interest in researching the health benefits of consuming fermented foods [2]. Among the most widespread and traditionally prepared fermented foods in Bulgaria are sauerkraut (whole sour cabbage), fermented green tomatoes, fermented cucumbers, kefir, boza, yogurt, etc. Their distinctive feature is their diverse and unique taste qualities. Moreover, their microbiota can vary depending on the raw substrate used in fermentation. Thus, exploring the beneficial microbial diversity in naturally fermented foods could be considered valuable with a strict focus on isolation, selection, and biotechnological application of bacterial strains, with the potential to act as probiotics [3].The term probiotics, however, refers to live microbes that, administered in sufficient amounts, lead to the improved health of the host [1].

Natural fermentation is a process that is inherent and carried out by a diverse microbiota, including bacteria, yeast, and molds [4,5]. The final products of the different fermentation processes depend on the microbes, starting substrates, and fermentation conditions [6]. Microorganisms involved in food fermentation should not be pathogenic, and the enzymes produced during these processes (proteases, amylases, and lipases) help break down the initial substrates into final products with sought-after qualities [7].

Lactic acid bacteria (LAB) are the most prevalent microorganisms found in vegetable- or milk-fermented foods, and lactic acid fermentation is regarded as the primary contributor to the beneficial properties these foods possess [4]. The reported dominant microbiota in vegetable-fermented products (sauerkraut) consists of *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, and *Leuconostoc mesenteroides* [8]. Conversely, the prevalence of LAB microorganisms in traditional fermented milk products (kumis, jiaoke, cheese, cottage cheese, shubat, and yogurt) analyzed in Mongolia, Kazakhstan, and Russia showed significant diversity, including *Lactobacillus helveticus*, *Lactococcus lactis*, *Streptococcus parauberis*, *Lactobacillus kefiranofaciens*, *Lactobacillus delbrueckii*, *Streptococcus salivarius*, *Lactococcus raffinolactis*, *Lactococcus piscium*, and *Streptococcus parasuis* [9]. The significance of LAB is mostly related to their safe metabolic activity, which provides numerous functional and health benefits to fermented foods [10]. The LAB strains, which have the so-called Generally Recognized as Safe (GRAS) status and fulfill the requirements of qualified presumption of safety (QPS) in Europe, are considered suitable to be used in various products in food industries (dairy products, meat products, fermented cereals, etc.) [11]. Moreover, part of the LAB in fermented foods meets the criteria for probiotics [12]. Such valuable criteria could overcome the enzymatic activity in the oral cavity and survival through the harsh gastrointestinal tract (GIT) environment. Upon reaching the lower part of the small intestine and colon (their site of action), the LAB colonizes, multiplies, and adheres to the host cells, where they exert their beneficial effects [12].

Among the reported health benefits from the consumption of probiotic LAB are balancing the intestinal microbial community, stimulating the immune system, reducing the risk of bacterial and viral-associated diarrhea, and lowering serum cholesterol [13]. It has also been proposed that the mechanisms of probiotic activity include the production of antimicrobial substances, thereby preventing the pathogen from adhesion to the epithelial layer of the intestine and competing for nutrients [14]. The ability of probiotic bacteria to colonize in the host GIT epithelial cells is considered to be determined by their cell surface hydrophobicity [15]. This property prevents the adhesion of pathogens to mucosal layers and increases the persistence time of LAB in the host [12].

The synthesis of organic acids [16,17], reuterin [18], proteinaceous substances [19], and cyclic dipeptides [20] has been linked to the antibacterial action of LAB. The bacteriocins synthesized by LAB have also been reported to be involved in two fundamental processes: (1) target cell membrane depolarization and/or suppression of the formation of the bacterial cell wall and (2) degradation of the murein layer [21]. The broad-spectrum antimicrobial substance reuterin has been thought to inhibit the ribonucleotide reductase enzyme competitively. It is also believed to degrade proteins and smaller molecules, which inhibits microorganisms from proliferating [22,23]. Undissociated and hydrophobic organic acid molecules permeate the pathogens' cell membranes, neutralizing their electrochemical properties and increasing permeation, ultimately leading to bacteriostasis and death [24,25].

The food in each geographical region is distinguished by the specificity of local ingredients and production methods. This uniqueness is deeply anchored in the Bulgarian folk tradition and is related to the production of unique foods obtained with the participation of endemic microbial species. In light of this, the present research aimed to study the diversity and beneficial biological activity of the lactic acid microflora isolated from various types of unique Bulgarian fermented foods. The objects of the investigation are products that have not been subjected to such extensive examination previously (sauerkraut, fermented green tomatoes, fermented cucumbers). The isolated strains were identified and screened for their bioprotective attributes, alongside exploration of their beneficial potential in biotechnological applications.

2. Materials and Methods

2.1 Sample Collection and Isolation of LAB

Specimens of different types of fermented food products (sauerkraut, fermented green tomatoes, fermented cucumbers, kefir, white cheese, and Izvara (curdled milk)) were prepared according to traditional Bulgarian recipes and used as sources of LAB isolation. Among these products were homemade and artisanal markets. The samples were collected in sterile containers from different regions in Bulgaria. The potential LAB in the samples were enriched via the cultivation of 1 g (mL) of each product in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany), separately. The resulting enriched cultures were diluted ten-fold, and aliquots of 100 µL were spread onto the MRS agar surface. The Petri dishes were incubated at 30–37 °C in an anaerobic jar (utilizing the BBL GasPack anaerobic system) for 48 hours. Colonies with different morphological characteristics were selected and re-streaked to obtain pure bacterial cultures. Preliminary selection of potential LAB isolates was performed according to results obtained for Gram-staining (Gram-staining Kit, Sigma Aldrich, St. Louis, MO, USA) to determine oxidase (Oxidase strips, Sigma-Aldrich) and catalase activities [26].

2.2 DNA Isolation, Polymerase Chain Reaction (PCR) Amplification, and 16S rDNA Sequencing

The total DNA of all isolates was extracted from overnight MRS broth cultures using a Tissue and Bacterial DNA Purification kit (EURx Ltd., Gdansk, Poland), following the manufacturer's instructions. The DNA Purification kit purified the isolated DNA (PCR/DNA Clean-up, GeneMatrix, EURx Ltd., Gdansk, Poland). The quantity of purified DNA was measured by BioDrop µLITE+ (100 ng/µL ± 20 ng/µL). The extracted DNA samples were stored at –20 °C and used for all PCR analyses in this study. PCR am-

plification of the entire length of 16S rDNA was performed using universal primers 27F and 1492R [27] and ready-to-go PCR mixtures iProof HF MasterMix (BioRad, Laboratories Inc., Hercules, CA, USA). The reactions were carried out using a PCR thermal cycler (Techne® Prime, VWR International, Radnor, PE, USA) following the protocol: 94 °C, 5 min; 35 cycles of 94 °C, 45 s; 56 °C, 45 s; 72 °C, 45 s. The final elongation steps were at 72 °C (10 min). The PCR amplification products were analyzed by 1.5% gel agarose electrophoresis. Purified PCR products were sequenced by Macrogen Europe (Meibergdreef 57 1105 BA, Amsterdam, The Netherlands). The obtained sequences were subjected to comparative analyses using BLASTN (NCBI).

2.3 Determination of Antimicrobial Activity

The antimicrobial activity of the isolated LAB was tested against the following pathogenic test microorganisms: *Listeria innocua* F 4078, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* NBIMCC 3548, *Salmonella enterica* subsp. *enterica* serotype Enteritidis NBIMCC 8691, and *Candida albicans* ATCC 10231 using the agar–well diffusion method [28,29]. The pathogenic bacteria and yeast were cultivated overnight on Brain Heart Infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) and Yeast Peptone Dextrose (YPD) broth (Merck KGaA, Darmstadt, Germany), respectively, to obtain log cultures. The LAB isolates were cultivated in MRS broth at 37 °C for 24 hours, and after that, cell-free supernatants (CFS) were obtained by centrifugation (at 6000 ×g and 5 °C for 15 min). The experiments were conducted using three CFS variants—obtained by following the methodology described before [30,31]: (1) CFS, (2) neutralized cell-free supernatants (NtCFS), and (3) heat-treated (100 °C for 10 min) neutralized cell-free supernatant (htNtCFS). Then, these supernatant variants were introduced into the prepared agar wells for diffusion. The plates were incubated at 37 °C for 24 hours. The presence of clear zones surrounding the wells confirmed antibacterial activity, and the diameters of the inhibition zones were measured and expressed in millimeters. All experiments were performed in triplicate, and mean values were calculated.

2.4 Production of H_2O_2

The ability of the LAB isolates to produce H_2O_2 was examined by the agar plate method, described by Maldonado *et al*. [32]. Horseradish peroxidase (Sigma Chemical Co, St. Louise, MO, USA) and tetramethyl–benzidine (TMB) (Sigma Chemical Co, St. Louise, MO, USA) were added to MRS agar medium. The LAB isolates were surface spot-inoculated in the TMB–MRS plates and anaerobically cultivated at 37 °C in an anaerostat for 48 hours. The results were interpreted after storing at room temperature outside the anaerostat for 2 hours, according to the intensity of the blue color of the LAB cultures in the plates as negative (–), weakly positive (±), moderately positive (+), and strongly positive (++).

2.5 Autoaggregation and Co-Aggregation Assays

Autoaggregation (AA) and co-aggregation (CA) assays were carried out according to Tuo *et al*. [33]. The LAB isolates were grown on MRS broth at 37 °C overnight. For AA, the bacterial cells were harvested by centrifugation at 10,000 ×g and 4 °C for 10 min, washed twice with phosphate-buffered saline (PBS) (pH 7.2), and then resuspended in PBS until the required cell concentration (1×10^8 CFU/mL, optical density, OD_{600nm} = 0.25) was obtained. The AA degree was measured at 600 nm ($A_{600\ nm}$) at three time points: 0, 4, and 24 hours. The autoaggregation percentage was calculated using the formula: $[1 - A_t/A_0] \times 100$, where A_t represents the absorbance at time t = 4 or 24 hours, and A_0 the absorbance at t = 0. The CA was carried out with mixtures of LAB and suspensions of the pathogenic microorganism *C. albicans* (1×10^8 CFU/mL) in equal volumes (5 mL), incubated at 37 °C without agitation. The co-aggregation percentage was measured at 600 nm ($A_{600\ nm}$) at three time points: 0, 4, and 24 hours and calculated according to the following formula $[(A_0 - A_t)/A_0] \times 100$, where A_0 represents the absorbance of the mix immediately after mixing, and A_t represents the absorbance of the mix at 4 or 24 hours [2].

2.6 Microbial Adhesion to Hexane (MAH) Test

The degree of hydrophobicity on the surface of bacteria was evaluated by measuring their adherence to hexane using the modified protocol of Collado *et al*. [34]. LAB isolates were cultivated in MRS at 37 °C for 24 hours, washed twice in PBS, and resuspended in 3 mL of PBS buffer to achieve approximately 1×10^8 CFU/mL ($OD_{600\ nm}$ = 0.25). The absorbance of each suspension was measured at 600 nm (A_0). Three milliliters of hexane were added to each cell suspension to form two-phase systems, and after incubating at room temperature for 10 min, the tubes were mixed by vortexing for 3 min. After incubating at room temperature (approximately 23 °C) for 1 hour (t = 1), the aqueous phase was carefully removed, and its absorbance was measured at 600 nm (A). The percentage of cell surface hydrophobicity (H, %) was expressed as adhesion percentage according to the following formula: $H\ (\%) = [(A_0 - A)/A_0] \times 100$, where A represents the absorbance at t = 1 and A_0, the absorbance at t = 0 [34].

2.7 Biofilm Formation Assay

With a few minor adjustments, an evaluation of the capacity of each LAB isolate to produce a biofilm was conducted in accordance with earlier protocols by Bujnakova *et al*. [35] and Gómez *et al*. [36]. The isolated LAB strains were cultivated in 5 mL MRS broth and incubated at 37 °C for 24 hours. The resulting cultures were centrifuged (10,000 ×g, 10 min), and the pellets were resuspended in PBS (McFarland 1.0, corresponding to 3×10^8 CFU/mL). A mixture of 180 µL MRS and 20 µL cell suspension was added dropwise into 96-well polystyrene microtitre plates (Nunc, Roskilde, Sjælland) and incubated at 37 °C for 24

hours. The analyses were conducted separately in triplicate for each LAB strain. The wells in which only MRS broth was added were used as the negative control. After incubation, the wells were carefully rinsed three times with 2 mL of sterile deionized water. Two milliliters of methanol (Romyl, Leics, UK) were used to fix the attached bacteria for 15 min. The methanol was extracted, and the plates were left to dry at room temperature. Subsequently, the cells in the wells were stained with 2 mL of a 0.1% (v/v) crystal violet solution for 5 min. After that, the excess staining was eliminated by running a gentle stream of tap water over the plate. The addition of 50 µL of 33% (v/v) glacial acetic acid removed the stain from the adhering cells. The OD of each well was measured at 595 nm using a plate reader ELIZA (Multiskan EX, Cat. no. 51118170 (200–240 V), Thermo Fisher Scientific, Vantaa, Finland). The ODc was defined as the mean OD value of the negative control. Based on the OD, strains can be classified as non-biofilm producers ($OD \leq OD_C$), weak biofilm producers ($OD_C < OD \leq 2 \times OD_C$), moderate ($2 \times OD_C < OD \leq 4 \times OD_C$), or strong biofilm producers ($4 \times OD_C < OD$) [37].

2.8 Hemolytic Activity

The hemolytic activity was evaluated following the methodology reported by Carrillo *et al*. [38]. Pure LAB cultures were surface spot inoculated on blood agar plates supplemented with 5% (v/v) horse blood and incubated at a temperature of 37 °C for a duration of 24 to 48 hours. The hemolytic activity of the isolated LAB strains was evaluated after the incubation period. The strains were classified according to the changes in blood agar medium around the spot culture as follows: (1) green zones around spot culture (α-hemolysis), (2) clear zones around spot culture (β-hemolysis), and (3) no zones around spot culture (γ-hemolysis). Only strains with γ-hemolysis were considered safe [39].

2.9 Antibiotic Susceptibility Testing

The susceptibility of all isolates to different antibiotics was tested according to the agar disc diffusion procedure described before by Bauer *et al.* [40] and Yasmin *et al*. [1], with modifications. Various antibiotics (AB), belonging to different groups were used (ampicillin/sulbactam (A/S)—10 µg/disc, vancomycin (VA)—5 µg/disc, chloramphenicol (C)—30 µg/disc, erythromycin (E)—15 µg/disc, tetracycline (T)—10 µg/disc, ciprofloxacin (CP)—10 µg/disc, cephalothin (CF)—10 µg/disc, gentamicin (G)—10 µg/disc, streptomycin (S) — 10 µg/disc, and neomycin (N)—5 µg/disc). A total of 1 mL of each strain (approximately 1×10^8 CFU/mL) was inoculated and mixed with 20 mL melted MRS agar and left to solidify at room temperature. After that, discs containing different antibiotics were placed on the agar surfaces and stored at 4 °C for 2 hours for AB diffusion. Then, the Petri dishes were cultivated at 37 °C in an anaerobic environment (utilizing the BBL GasPack anaerobic system) for 24 hours. The diameters of the inhibition zones surrounding the antibiotic discs were measured in millimeters and categorized as <8 mm—resistant (R), between 8 and 10 mm—moderately susceptible (MS), and >10 mm—strongly susceptible (SS) [1].

2.10 PCR Detection of Genes Encoding Antibiotic Resistance

The genes related to antibiotic resistance to gentamicin (*aac(6')-aph(2'')*), chloramphenicol (*cat*), tetracycline (*tet*(M)), β-lactamase (*bla*Z), a macrolide (*mefA*), kanamycin (*aph (3')*-III), erythromycin (*ermA, ermB*), ciprofloxacin (*gyr*A), and vancomycin (*vanA*) were examined by PCR using specific primers, according to the protocol of Guo *et al*. [41] and Liu *et al*. [42]. The PCR products were visualized using 1.5% agarose gel electrophoresis.

3. Results and Discussion

3.1 Isolation of LAB Strains from Fermented Foods with Different Origins

A total of 45 potential LAB strains (Table 1) were obtained from various traditional Bulgarian fermented foods (sauerkraut, fermented green tomatoes, fermented cucumbers, kefir, white cheese, and Izvara (curdled milk)). Of these, 25 strains were from fermented vegetable products, and 20 were from fermented milk products. Phenotypic characterization of the isolates showed that all strains formed white to creamy-colored colonies with smooth or jagged edges, predominantly with a convex profile and a uniform to slightly granular structure. Micromorphologically, the cells were rod-shaped with different lengths. All isolates were Gram-positive bacteria, catalase, and oxidase-negative. These phenotypic data are typical characteristics of the LAB group.

3.2 Molecular Identification of Isolates

All 45 isolated strains from traditional homemade and artisanal Bulgarian fermented products were subjected to molecular identification. The genetic material of all potential LAB isolates was extracted, and the 16S rDNA gene was amplified using universal primers 27F/1492R [27]. The obtained PCR products (approximately 1500 bp) were purified and sequenced by Macrogen in The Netherlands. The results were processed and compared with the NCBI database. All LAB isolates with varying similarity percentages were identified at genus and species levels. Among them, representatives of three genera, according to the new reclassification within the family *Lactobacillaceae* proposed by Zheng *et al*. [43], were found (Fig. 1A). The most prevalent genera were found to be *Lactiplantibacillus* (47%) and *Levilactobacillus* (40%). Less represented was the genus *Lactobacillus* (13%). The LAB diversity established after species identification revealed the predominance of five bacterial species: *L. plantarum*, *Levilactobacillus koreensis, L. brevis, L. helveticus, and Levilactobacillus yonginensis* (Fig. 1B). *L. plantarum* was the most prominent (47%) species, as it was found in most tested

Table 1. Characterization of LAB strains isolated from traditional Bulgarian fermented vegetable and milk products.

Number	Strains	Origin of isolation	Year of isolation	Media	Cell morphology	Gram stain	Oxidase	Catalase	Production of H_2O_2	Species identification by 16S rDNA gene sequence
1	S1	Sauerkraut*	2019	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
2	S2		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus brevis*
3	S3		2019	MRS	Rod-shaped	+	–	–	+	*Lactiplantibacillus plantarum*
4	S4		2019	MRS	Rod-shaped	+	–	–	+	*Lactiplantibacillus plantarum*
5	S5		2019	MRS	Rod-shaped	+	–	–	+	*Lactiplantibacillus plantarum*
6	S6	Sauerkraut**	2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
7	S7		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
8	S7’		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
9	S8		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
10	S9		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
11	S10		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
12	S11		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
13	S12		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
14	S13		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
15	S1		2018	MRS	Rod-shaped	+	–	–	+	*Lactiplantibacillus plantarum*
16	S15		2018	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
17	S16		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus yonginensis*
18	FGT1	Fermented green tomatoes**	2019	MRS	Rod-shaped	+	–	–	–	*Levilactobacillus koreensis*
19	FGT2		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus koreensis*
20	FGT3		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus koreensis*
21	FGT4		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus koreensis*
22	FC1	Fermented cucumber**	2019	MRS	Rod-shaped	+	–	–	–	*Lactiplantibacillus plantarum*
23	FC2		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus koreensis*
24	FC3		2019	MRS	Rod-shaped	+	–	–	+	*Levilactobacillus koreensis*
25	FC4		2019	MRS	Rod-shaped	+	–	–	–	*Levilactobacillus koreensis*
26	K1	Kefir*	2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
27	K2		2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
28	K3		2019	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
29	K4		2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
30	K5		2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
31	K6		2019	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
32	K7		2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
33	K8		2019	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*

Table 1. Continued.

Number	Strains	Origin of isolation	Year of isolation	Media	Cell morphology	Gram stain	Oxidase	Catalase	Production of H_2O_2	Species identification by 16S rDNA gene sequence
34	WC1	White cheese*	2018	MRS	Rod-shaped	+	–	–	±	*Lactobacillus helveticus*
35	WC2		2018	MRS	Rod-shaped	+	–	–	–	*Lactobacillus helveticus*
36	WC3		2018	MRS	Rod-shaped	+	–	–	±	*Lactobacillus helveticus*
37	WC4		2018	MRS	Rod-shaped	+	–	–	±	*Lactobacillus helveticus*
38	WC5		2018	MRS	Rod-shaped	+	–	–	±	*Lactobacillus helveticus*
39	WC6		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
40	I1	Izvara* (curdled milk)	2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
41	I2		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
42	I3		2018	MRS	Rod-shaped	+	–	–	±	*Levilactobacillus brevis*
43	I4		2018	MRS	Rod-shaped	+	–	–	±	*Lactobacillus helveticus*
44	I5		2018	MRS	Rod-shaped	+	–	–	±	*Lactiplantibacillus plantarum*
45	I6		2018	MRS	Rod-shaped	+	–	–	–	*Levilactobacillus brevis*

*homemade products; **artisanal (market) products. LAB, Lactic acid bacteria; FGT, Fermented green tomatoes; FC, Fermented cucumber; K, Kefir; WC, White cheese; I, Izvara; MRS, de Man, Rogosa, and Sharpe; +, moderately positive reaction; ±, weakly positive reaction; –, negative reaction.

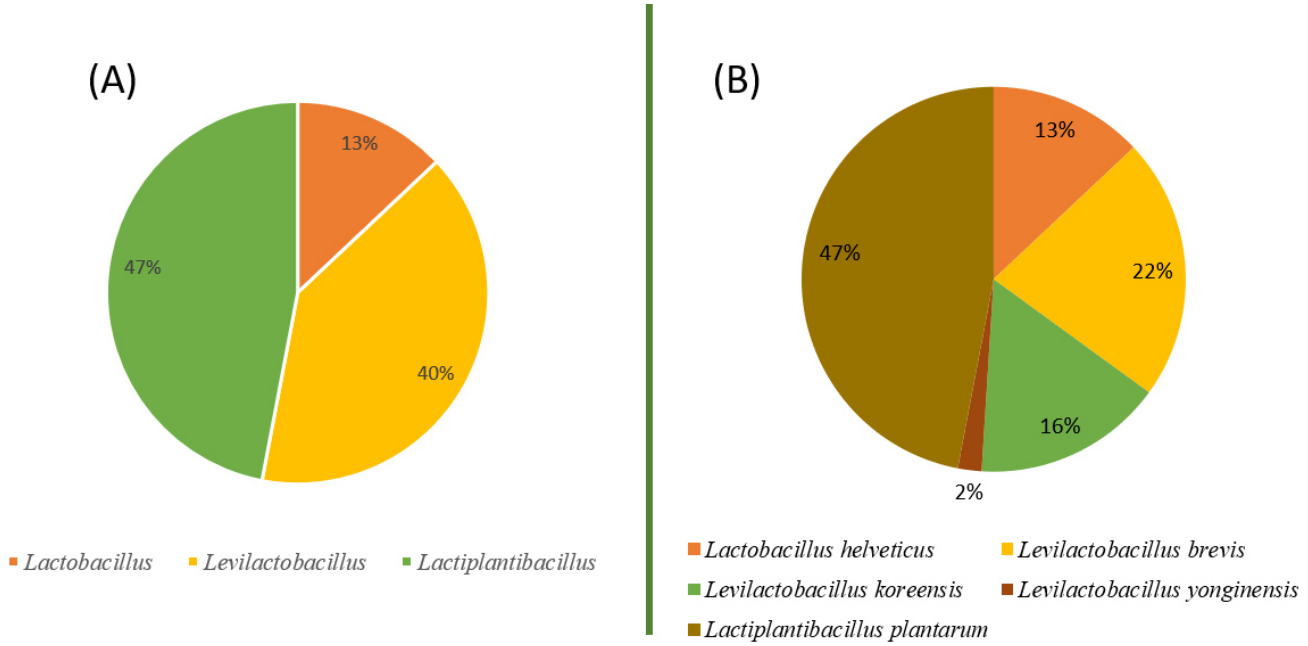

Fig. 1. Diversity of LAB in the studied foods. (A) Genus diversity of LAB strains isolated from traditional homemade and artisanal Bulgarian fermented products. (B) Species diversity of LAB isolated from traditional homemade and artisanal Bulgarian fermented products. LAB, lactic acid bacteria.

products. It is interesting to note that this species represents 83% and 80% of all isolated LAB from the sauerkraut products: artisanal market and homemade, respectively. Similar results for the distribution of *L. plantarum* in traditional fermented foods have also been reported globally [3,44]. Other authors have reported the presence of *Leuconostoc, Weissela,* and *lactobacilli* in sauerkraut fermentation, expressing the opinion that the composition of LAB microflora depends on the cultivar of the cabbage used in the fermentation process [45]. The only product where *L. plantarum* was not found was fermented green tomato (FGT), where the dominant LAB species was *L. koreensis*. Interestingly, *L. koreensis* was found only in two products (fermented green tomatoes, FGT, and fermented cucumbers, FC). Surprisingly, only one strain of *L. yonginensis* was isolated solely from artisanal sauerkraut. Moreover, both species (*L. koreensis* and *L. yonginensis*) are more commonly found in Asian fermented foods such as kimchi [46]. To our knowledge, this study is the first report of *L. koreensis* and *L. yonginensis* isolated from fermented vegetable foods in Bulgaria. *L. helveticus* was the predominant species in the white cheese and Izvara products. It seems that *L. brevis* is an irrevocable part of fermentative microflora, as it was found in 57% of the tested products in this study (Table 1).

3.3 Determination of Antimicrobial Activity and H_2O_2 Production

The fermentation process, which turns sugars into organic acids (lactic and acetic acids), resulting in the formation of acidic environments, is mostly responsible for the preservation behavior of LAB. However, these bacteria can generate and release inhibitory compounds in addition to lactic and acetic acids. Such substances have been reported to have antagonistic effects on various microorganisms. They include a variety of less well-defined or entirely unknown inhibitory substances, such as hydrogen peroxide, diacetyl, ammonia, ethanol, bacteriocins, antibiotics, etc. They are produced in much smaller amounts than lactic and acetic acids. Several of these compounds have demonstrated antagonistic effects against a variety of harmful bacteria found in food, such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, and *S. aureus* [47,48].

Our LAB isolates were evaluated for their antimicrobial potential and ability to produce hydrogen peroxide. We found that only 11 strains showed moderate ability to produce H_2O_2 (S2, S3, S4, S5, S14, S16, FGT2, FGT3, FGT4, FC2, FC3). The remaining isolates were negative or weak producers. The antimicrobial potential of the isolated lactic acid bacteria was studied against two Gram-positive species (*L. innocua* F, *S. aureus*), two Gram-negative species (*Sal.* Enteritidis, *E. coli*), and one yeast species (*C. albicans*). Antibacterial activity was evaluated using the agar well diffusion method. The size of the inhibition zones was expressed in mm. The inhibitory properties of the supernatants separated after centrifugation were studied in three variants: 1/CFS, 2/NtCFS, and 3/htNtCFS.

The obtained results showed that antibacterial activity was observed only for CFS (Table 2). No inhibitory activity was detected for NtCFS and htNtCFS. The lack of such activity means the isolated LAB does not produce bacteri-

Table 2. Antimicrobial activity of CFS, derived from the isolated LAB strains.

Strains	Test microorganisms (inhibition zones, mm)				
	Sal. Enteritidis	*L. innocua*	*E. coli*	*S. aureus*	*C. albicans*
L. plantarum S1	–	–	–	–	–
L. brevis S2	–	–	–	–	–
L. plantarum S3	–	–	±*	–	–
L. plantarum S4	–	–	14.0 ± 0.50	±	–
L. plantarum S5	–	–	16.0 ± 0.25	±	–
L. plantarum S6	–	–	14.0 ± 0.75	±	–
L. plantarum S7	12.0 ± 0.01	–	12.0 ± 0.04	±	–
L. plantarum S7'	–	–	11.0 ± 0.05	–	–
L. plantarum S8	–	±	11.0 ± 0.04	–	–
L. plantarum S9	11.0 ± 0.05	–	13.0 ± 0.75	–	–
L. plantarum S10	11.0 ± 0.01	±	12.0 ± 0.05	–	–
L. plantarum S11	11.0 ± 0.25	±	13.0 ± 0.50	–	–
L. plantarum S12	±	±	11.0 ± 0.04	–	–
L. plantarum S13	–	–	16.0 ± 0.75	–	–
L. plantarum S14	–	–	12.0 ± 0.05	–	–
L. brevis S15	–	–	11.0 ± 0.50	–	–
L. yonginensis S16	–	–	–	–	–
L.koreensis FGT1	–	–	–	–	–
L. koreensis FGT2	±	–	±	–	–
L.koreensis FGT3	–	–	–	–	–
L. koreensis FGT4	–	–	–	–	–
L. plantarum FC1	–	–	–	–	–
L. koreensis FC2	±	–	±	±	–
L. koreensis FC3	–	–	–	–	–
L. koreensis FC4	–	–	–	–	–
L. brevis K1	–	–	–	–	–
L. brevis K2	–	–	–	–	–
L. plantarum K3	–	±	–	±	–
L. brevis K4	–	–	–	–	–
L.brevis K5	–	–	–	–	–
L. plantarum K6	–	–	–	–	–
L. brevis K7	–	–	–	–	–
L. brevis K8	–	–	–	–	–
L. helveticus WC1	–	–	–	±	–
L. helveticus WC2	–	–	–	–	–
L. helveticus WC3	–	–	–	–	–
L. helveticus WC4	–	–	–	–	–
L. helveticus WC5	–	–	–	–	–
L. plantarum WC6	–	–	–	–	–
L. plantarum I1	–	–	–	–	–
L. plantarum I2	–	–	–	–	–
L. brevis I3	–	–	–	–	–
L. helveticus I4	–	–	–	–	–
L. plantarum I5	±	–	±	±	–
L. brevis I6	–	–	–	–	–

*±, very small inhibition zone (1–2 mm). CFS, cell-free supernatants.

ocins or bacteriocin-like substances. The observed inhibition activity of CFS against some of the test microorganisms is probably due to the production of lactic acid, hydrogen peroxide, or other substances with an inhibitory effect. Different percentages of the strains of *L. plantarum* showed apparent antagonistic effects against *E. coli* and *Sal.* Enteritidis (57% and 19%, respectively). All these strains were isolated from artisanal markets and homemade sauerkraut. Very weak inhibitory activity (inhibition zones 1–2 mm) against *Listeria innocua* F and *S. aureus* was detected in

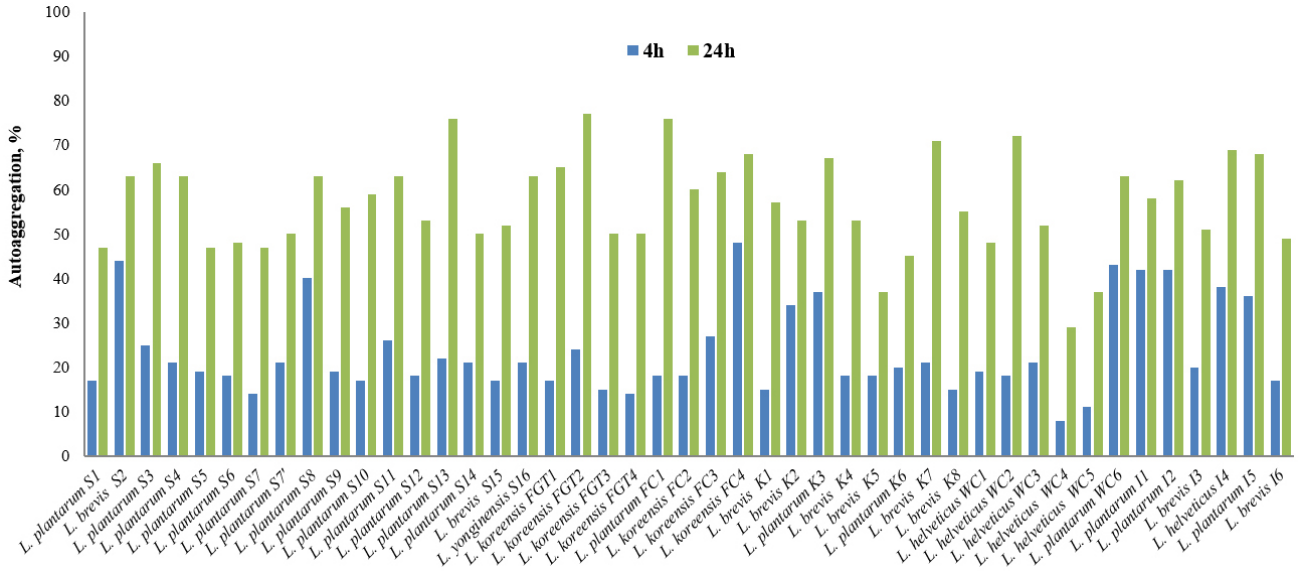

Fig. 2. Autoaggregation ability (in percentage, %) of the isolated LAB strains, measured at 4 and 24 hours.

24% and 29% CFS of all *L. plantarum* isolates, respectively. Similar weak, barely detectable activity against *Sal.* Enteritidis, *E. coli*, and *S. aureus* were also established for two strains of *L. koreensis* (FGT2 and FC2). Very weak antimicrobial activity by CFS, obtained from lactobacilli isolated from sauerkraut, was previously reported against *Listeria monocytogenes* [45]. Only one of our strains *L. brevis* (S15—isolated from artisanal sauerkraut) showed activity against *E. coli* (Table 2). None of our isolates showed inhibitory properties against *C. albicans*. According to our results and those reported by other authors, we can conclude that no correlation between antibacterial activity and H_2O_2 production was found [49]. However, three strains isolated from sauerkraut (S4, S5, and S14) were the exceptions since they demonstrated antibacterial activity against *E. coli* and are moderate producers of H_2O_2.

3.4 Autoaggregation and Co-Aggregation Assays

The ability to autoaggregate is an essential property of LAB to form a barrier on the GIT mucosa, which prevents pathogenic bacteria from attaching to it. Some authors claim that lactobacilli have a modest to moderate capability for autoaggregation [50]. To verify the autoaggregation potential of our strains, all isolates were tested for their ability to autoaggregate, and the results were analyzed at 4 and 24 hours. From the group of isolates identified as *L. plantarum*, four strains (S8, WC6, I1, and I2) showed very good autoaggregation properties after 4 hours—more than 40% of the cells autoaggregated (Fig. 2). However, the best autoaggregation ability after 4 hours was observed for *L. koreensis* FC4 (48%) and *L. brevis* S2 (44%). The lowest AA activity was observed for two strains of *L. helveticus* isolated from white cheese (WC4, 8%, and WC5, 11%). The percent of AA in the rest of the LAB isolates varied from approximately 15% to 25%.

The autoaggregation activity increased alongside the incubation time, and after 24 hours, it reached between 29 and 77%. The highest AA ability was detected for *L. koreensis* FGT2 (77%), *L. plantarum* S13 and FC1 (both 76%), *L. helveticus* WC2 (72%), and *L. brevis* K7 (71%). However, for FC1 and WC2, the AA ability detected after 4 hours was relatively low, at 18%. Interestingly, the two strains with the highest autoaggregation ability after 4 hours did not possess the maximum AA activity after 24 hours (S2: 63%; FC4: 68%). Fifteen of the isolates (S2, S3, S4, S8, S11, S16, FGT1, FC2, FC3, FC4, K3, WC6, I2, I4, and I5) showed over 60% autoaggregation after 24 hours. Recently, numerous authors have investigated the autoaggregation abilities of lactobacilli as part of their probiotic potential. There is still no single scale for determining the degree of autoaggregation. However, most authors accept 35–40% as sufficient for lactobacilli to be defined as strains with a high aggregation potential. Notably, the strains of the *L. plantarum* species show different degrees of autoaggregation. Ramos *et al*. [51] reported that one *L. plantarum* and one *L. fermentum* strain reached autoaggregation values of 61.83% and 55.61%, respectively. *L. plantarum* strain 557, isolated from vegetables, exhibited 34% autoaggregation [49]. Comparing these reported results with those obtained by our study, we can conclude that our *L. plantarum* isolates showed similar AA abilities. Seven strains of the *L. fermentum* species isolated from fermented Chinese products presented highly varying autoaggregation limits ranging from 0.86% to 65.15% [52]. In a study of nine strains isolated from milk and milk products (cheese, yogurt), vegetables, and the intestinal tract, Ren *et al*. [49] described a high rate of autoaggregation only in *Lactobacillus salivarius* subsp. *salicinius* CICC 23174 (46%), and *Lactobacillus acidophilus* CGMCC 1.1854 (45%), compared to the reference strain *L. rhamnosus* LGG (33%). Our results are

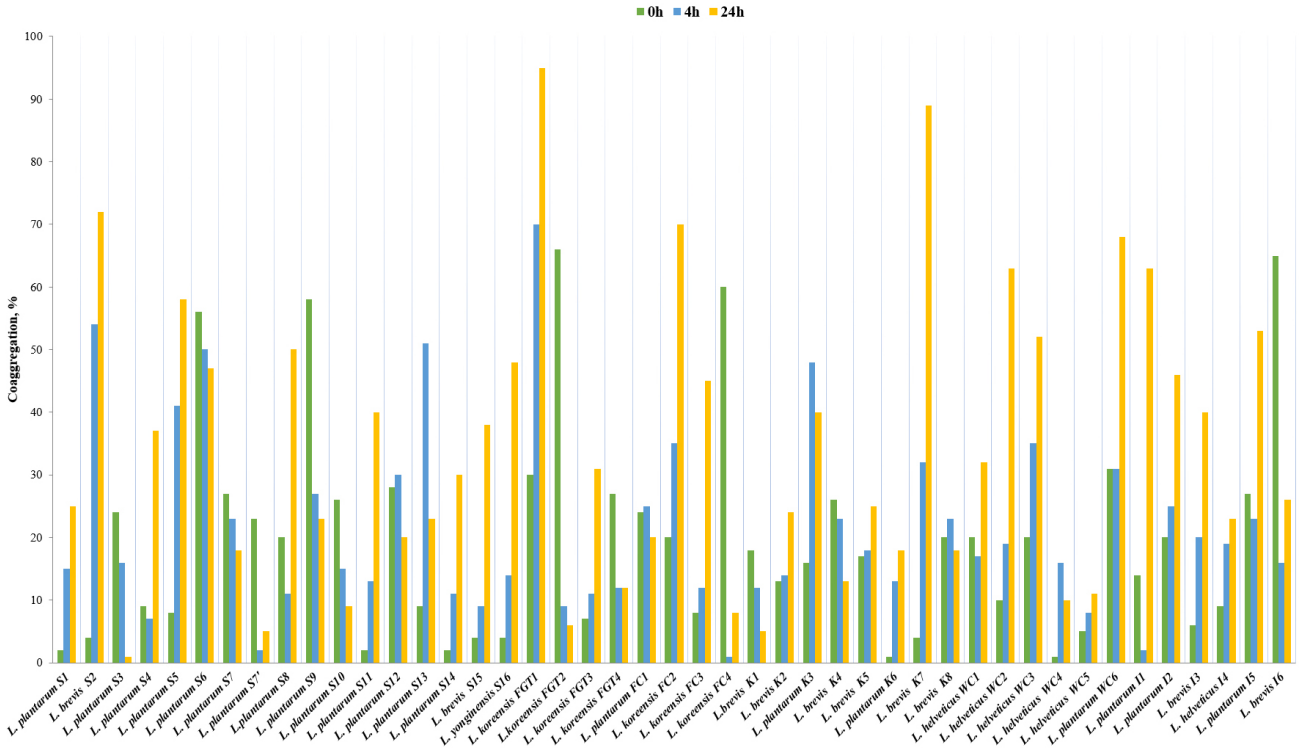

Fig. 3. Co-aggregation ability (in percentage, %) of the isolated LAB strains, measured at three time points: 0, 4, and 24 hours.

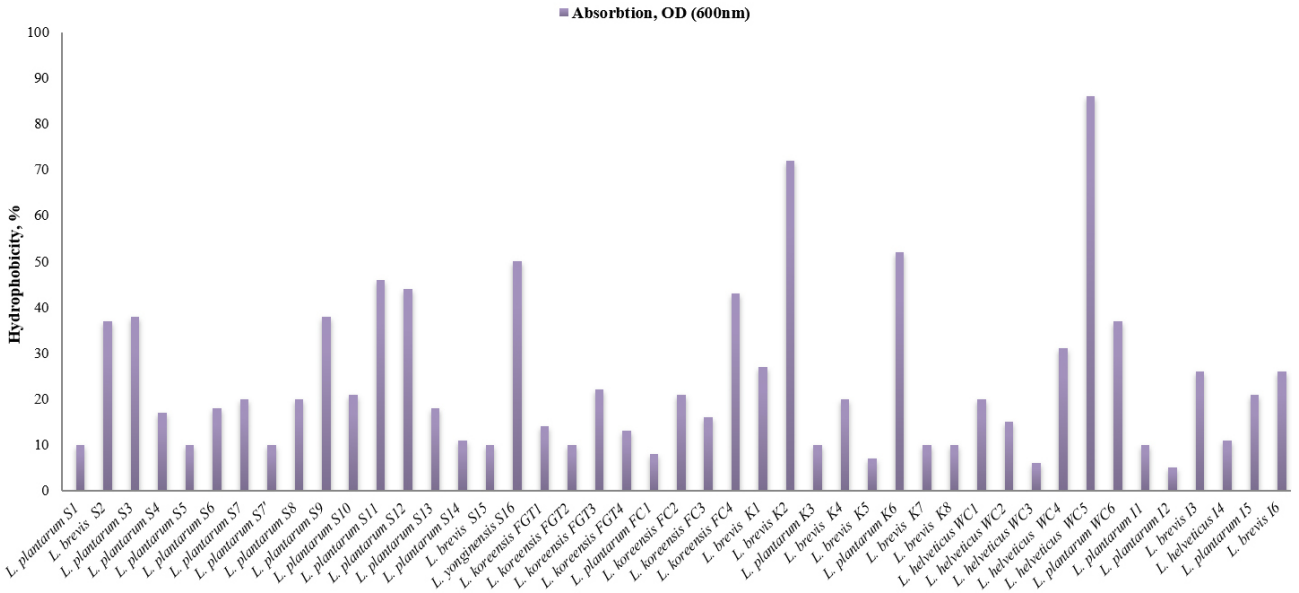

Fig. 4. Cell surface hydrophobicity (in percentage, %) of the isolated LAB strains.

similar to those for the strains isolated from milk and milk products, as three *L. plantarum* strains isolated from white cheese and Izvara (WC6, I1, and I2) showed high autoaggregation potential.

The co-aggregation between probiotic microorganisms and harmful microorganisms creates an unsuitable environment for developing pathogens. This reduces their growth, eliminates the pathogen, and restores the normal gastric microbiota [53]. In the present study, the pathogenic yeast species *C. albicans* and the studied lactic acid isolates were co-inoculated to monitor their co-aggregation.

In the studied population of LAB isolates, four strains showed the highest percentage of CA with the pathogen after 4 hours: FGT1 (70%), S2 (54%), S13 (51%), and S6 (50%). The FGT1, K7, S2, and FC2 strains reached the greatest CA after 24 hours: 95%, 89%, 72%, and 70%, respectively (Fig. 3). Thus, based on our results, we can conclude that *L. koreensis* FGT1 could be considered a can-

didate for potential probiotic application, according to this criterion. Interestingly, another group of strains, including FGT2, I6, FC4, S9, and S6, showed great CA activity at the moment of contact between the LAB and the pathogen cells (t_0): 66%, 65%, 60%, 58%, and 56%, respectively. The high CA level at t_0 of *L. koreensis* FC4 with *C. albicans* may be due to its high AA percentage. The rest of the strains had relatively low AA activities. The observed high CA activity of these isolates may be due to the affinity of the surface structures of the LAB with those of the pathogen. Mastromarino *et al*. [54] observed a high level of co-aggregation of *L. salivarius* and *L. gasseri* with *Gardnerella vaginalis*. In other studies, L. fermentum and L. plantarum strains efficiently co-aggregate with *E.coli*, *Shigella flexneri*, *Sal.* Enteritidis, *Listeria monocytogenes,* and *S. aureus* [51,55].

3.5 Microbial Adhesion to Hexane (MAH) Test

Bacterial attachment and colonization to the inner side of the GIT depend on cell surface hydrophobicity and AA. The LAB strain must possess a minimum value of 40% hydrophobicity to be called a probiotic strain [56]. Based on this criterion, 84% of our isolates could not be considered presumptive probiotic strains (Fig. 4). However, seven of the isolated LAB strains showed hydrophobicity above 40%. The great ability to adhere to hexane was detected in three LAB strains isolated from milk—fermented products (kefir and white cheese): *L. helveticus* WC5, 86%; *L. brevis* K2, 72%, and *L. plantarum* K6, 52%. The other four LAB strains were isolated from fermented vegetable products (sauerkraut and fermented cucumbers): *L. yonginensis* S16, 50%; *L. plantarum* S11, 46%; *L. plantarum* S12, 44%, and *L. koreensis* FC4, 43%. The last four stains, except strain S12, also showed good AA ability and were detected after 24 hours. Microbial cell adhesion is affected by a combination of electrostatic and van der Waals forces, as well as the hydrophobicity of the surfaces [57]. Hydrophobicity is important in the initial interaction between bacterial cells and mucosal or epithelial cells [58]. Lactobacilli adhesion is also connected with particular interactions involving the receptor on the bacterial surface recognizing a particular area or ligand [59]. It turns out that the ability of LAB to attach to hydrocarbons is encountered relatively rarely among LAB. For example, Ren *et al*. [49] investigated nine strains, of which only two strains (22%) showed relatively high levels of hydrophobicity (59% and 43%, respectively). Hoxha *et al*. [3] reported that none of the twelve LAB strains tested had hydrophobicity above 40%.

3.6 Biofilm Formation Assay

Bacterial biofilms are crucial in understanding how bacteria adapt to environmental stress and colonize diverse habitats. To investigate the ability of the LAB strains isolated from traditional Bulgarian fermented food to form biofilms, they were cultivated in a 96-well plate for 24 hours. Based on the obtained results, the strains were grouped as follows: Non-biofilm producers, 18% of the strains (S2, S3, S4, S6, S7, S7', S8, S14); weak biofilm producers, 13% of the strains (S11, S12, S15, K2, K4, K8); moderate biofilm producers, 53% (S5, S9, S10, S13, K1, K6, WC1, WC5, I1, I2, I4, I5, I6, WC3, WC4, FC3, FGT1, FC1, FC4, FGT2, FGT3, FGT4, S16, FC2); strong biofilm producers, 16% of the strains (S1, K3, K5, K7, WC6, I3, WC2). Based on these results, we can conclude that the ability to form a biofilm is rather strain-dependent.

3.7 Hemolytic Activity

An important requirement for probiotic strains is their safety. An important initial step in developing or selecting new beneficial microorganisms is to evaluate the lack of hemolytic activity or cytotoxicity [60]. Although hemolytic activity is not inherent to lactobacilli and is relatively rarely found in enterococci, evaluating each case is necessary. Even though many LAB strains have been used for a long time due to their technological properties for food preparation or as probiotics in humans and animals, they are not directly granted GRAS status [61,62]. This makes verifying that the isolated LAB meets this criterion mandatory. For this reason, the hemolytic activity of all isolated 45 strains was evaluated on blood agar. Hemolytic activity was not detected in any of the tested strains, as evidenced by the absence of hydrolysis zones around the colonies of the isolates. Thus, the tested strains cover one of the safety criteria, such as the absence of hemolytic activity.

3.8 Antibiotic Susceptibility Testing

Natural resistance to ciprofloxacin, bacitracin, streptomycin, cefoxitin, fusidic acid, kanamycin, nitrofurantoin, gentamicin, metronidazole, norfloxacin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin has been widely reported for some LAB. [63]. A significantly elevated rate of spontaneous mutation to nitrofurazone (10^{-5}), kanamycin, and streptomycin has been documented for various lactobacilli [64]. Some authors suggested that genes encoding antibiotic resistance can be transferred between bacteria of diverse origins [65]. Concerning the potential consequences for human health, probiotic bacteria should not be able to transfer antibiotic resistance genes [66]. This ability must be regarded as a crucial factor in selecting beneficial strains.

The antibiotic susceptibility of our LAB isolates to 10 antibiotics (ampicillin, tetracycline, erythromycin, gentamicin, streptomycin, vancomycin, neomycin, cephalothin, ciprofloxacin, and chloramphenicol) was evaluated using the agar disc diffusion method. The obtained results are summarized in Table 3, where the isolates were categorized according to Yasmin *et al*. [67]. Variable susceptibility to the tested antibiotics was observed even at the intraspecies level. All tested strains showed resistance to vancomycin and streptomycin (except *L. plantarum* S13, which showed strong sensitivity). Our results differ from those reported by Ren *et al*. [49], showing that all LAB isolated from fermented food were susceptible to streptomycin. A

Table 3. Antibiotic susceptibility testing of the isolated LAB strains.

Strains	Test antibiotics (inhibition zone, mm)									
	A/S	T	VA	CP	CF	C	G	E	S	N
L. plantarum S1	35/SS	21/SS	0/R	0/R	0/R	32/SS	10/MS	26/SS	0/R	11/SS
L. brevis S2	30/SS	16/SS	0/R	0/R	0/R	31/SS	13/SS	25/SS	0/R	15/SS
L. plantarum S3	31/SS	21/SS	0/R	0/R	0/R	33/SS	10/MS	26/SS	0/R	12/SS
L. plantarum S4	34/SS	21/SS	0/R	0/R	0/R	34/SS	11/SS	25/SS	0/R	11/SS
L. plantarum S5	24/SS	22/SS	0/R	0/R	0/R	32/SS	10/MS	26/SS	0/R	8/MS
L. plantarum S6	40/SS	22/SS	0/R	0/R	17/SS	35/SS	13/SS	27/SS	0/R	15/SS
L. plantarum S7	30/SS	22/SS	0/R	0/R	17/SS	33/SS	12/SS	27/SS	0/R	13/SS
L. plantarum S7'	36/SS	21/SS	0/R	0/R	13/SS	32/SS	15/SS	29/SS	0/R	9/MS
L. plantarum S8	27/SS	25/SS	0/R	0/R	14/SS	36/SS	9/MS	27/SS	0/R	0/R
L. plantarum S9	40/SS	25/SS	0/R	0/R	16/SS	35/SS	11/SS	27/SS	0/R	9/MS
L. plantarum S10	40/SS	24/SS	0/R	0/R	0/R	35/SS	14/SS	29/SS	0/R	0/R
L. plantarum S11	35/SS	23/SS	0/R	0/R	19/SS	34/SS	10/MS	30/SS	0/R	15/SS
L. plantarum S12	32/SS	25/SS	0/R	0/R	10/MS	36/SS	10/MS	30/SS	0/R	10/MS
L. plantarum S13	35/SS	20/SS	0/R	0/R	0/R	33/SS	18/SS	27/SS	17/SS	15/SS
L. plantarum S14	35/SS	25/SS	0/R	0/R	0/R	35/SS	8/MS	27/SS	0/R	20/SS
L. brevis S15	26/SS	15/SS	0/R	0/R	0/R	31/SS	12/SS	29/SS	0/R	0/R
L. yonginensis S16	30/SS	0/R	0/R	15/SS	0/R	32/SS	15/SS	35/SS	0/R	18/SS
L. koreensis FGT1	30/SS	16/SS	0/R	0/R	0/R	30/SS	0/R	26/SS	0/R	0/R
L. koreensis FGT2	37/SS	15/SS	0/R	0/R	0/R	30/SS	0/R	25/SS	0/R	0/R
L. koreensis FGT3	26/SS	15/SS	0/R	0/R	0/R	32/SS	0/R	30/SS	0/R	0/R
L. koreensis FGT4	25/SS	18/SS	0/R	0/R	0/R	30/SS	0/R	26/SS	0/R	0/R
L. plantarum FC1	25/SS	18/SS	0/R	0/R	0/R	30/SS	0/R	25/SS	0/R	10/MS
L. koreensis FC2	31/SS	21/SS	0/R	0/R	0/R	36/SS	0/R	30/SS	0/R	10/MS
L. koreensis FC3	30/SS	16/SS	0/R	0/R	0/R	36/SS	0/R	30/SS	0/R	0/R
L. koreensis FC4	25/SS	17/SS	0/R	0/R	0/R	33/SS	0/R	25/SS	0/R	0/R
L.brevis K1	29/SS	20/SS	0/R	10/MS	0/R	32/SS	22/SS	30/SS	0/R	13/SS
L. brevis K2	39/SS	30/SS	0/R	12/SS	0/R	39/SS	9/MS	31/SS	0/R	13/SS
L. plantarum K3	26/SS	15/SS	0/R	0/R	0/R	27/SS	10/MS	29/SS	0/R	12/SS
L. brevis K4	25/SS	12/SS	0/R	0/R	0/R	25/SS	10/MS	15/SS	0/R	11/SS
L. brevis K5	23/SS	15/SS	0/R	0/R	0/R	27/SS	11/MS	25/SS	0/R	11/SS
L. plantarum K6	28/SS	18/SS	0/R	0/R	0/R	26/SS	0/R	24/SS	0/R	8/MS
L. brevis K7	27/SS	20/SS	0/R	10/MS	0/R	30/SS	15/SS	30/SS	0/R	16/SS
L. brevis K8	25/SS	23/SS	0/R	0/R	0/R	33/SS	20/SS	30/SS	0/R	25/SS
L. helveticus WC1	37/SS	20/SS	0/R	11/SS	9/MS	31/SS	13/SS	28/SS	0/R	0/R
L. helveticus WC2	28/SS	18/SS	0/R	10/MS	13/SS	34/SS	13/SS	25/SS	0/R	0/R
L. helveticus WC3	45/SS	19/SS	0/R	0/R	0/R	35/SS	20/SS	30/SS	0/R	14/SS
L. helveticus WC4	36/SS	27/SS	0/R	10/MS	0/R	35/SS	10/MS	29/SS	0/R	13/SS
L. helveticus WC5	40/SS	30/SS	0/R	12/SS	17/SS	37/SS	14/SS	31/SS	0/R	0/R
L. plantarum WC6	33/SS	25/SS	0/R	13/SS	15/SS	34/SS	18/SS	29/SS	0/R	0/R
L. plantarum I1	35/SS	27/SS	0/R	0/R	15/SS	36/SS	14/SS	30/SS	0/R	0/R
L. plantarum I2	35/SS	20/SS	0/R	38/SS	0/R	0/R	19/SS	30/SS	0/R	0/R
L. brevis I3	35/SS	26/SS	0/R	37/SS	15/SS	0/R	14/SS	29/SS	0/R	0/R
L. helveticus I4	30/SS	26/SS	0/R	35/SS	0/R	0/R	19/SS	30/SS	0/R	9/MS
L. plantarum I5	31/SS	26/SS	0/R	11/SS	12/SS	34/SS	14/SS	28/SS	0/R	0/R
L. brevis I 6	40/SS	25/SS	0/R	8/MS	17/SS	36/SS	11/SS	31/SS	0/R	0/R

A/S, ampicillin/sulbactam; T, tetracycline; VA, vancomycin; CP, ciprofloxacin; CF, cephalothin; C, chloramphenicol; G, gentamicin; E, erythromycin; S, streptomycin; N, neomycin.

large proportion of strains showed resistance or moderate susceptibility to cephalothin (71%), neomycin (58%), and ciprofloxacin (80%). Three strains (I2, I3, and I4) showed resistance to chloramphenicol; the remaining strains were strongly sensitive. The group of strains (47%) isolated from all fermented products showed resistance or moderate susceptibility to gentamicin. The strains were strongly susceptible to three of the tested antibiotics: Ampicillin, tetracy-

cline, and erythromycin. *L. yonginensis* S16 was the exception since it showed resistance to tetracycline.

3.9 PCR Detection of Antibiotic Resistance Genes

Distinguishing between intrinsic (non-specific, non-transferable) and acquired resistance by the beneficial strains is also necessary [68]. Molecular techniques that directly screen bacterial strains for the presence of antibiotic resistance genes could be considered an important addition to phenotypic testing. Usually, these include PCR-based methods [69,70] or DNA microarrays that combine various antibiotic resistance genes [71].

Phenotypic analyses showed that all LAB isolates in this study, belonging to the family *Lactobacillaceae*, showed resistance to one or more antibiotics. To clarify the nature of this antibiotic resistance, PCR amplification was performed with specific primers for acquired resistance genes: Gentamicin, *aac(6')-aph(2'')*; chloramphenicol, *cat*; tetracycline, *tet*(M); β-lactamase, *bla*Z; macrolide, *mefA*; kanamycin, *aph* (*3'*)-III; erythromycin, *ermA*, *ermB*; ciprofloxacin, *gyr*A, and vancomycin, *vanA*. No amplification products were observed in any strains, confirming that the isolates did not possess these resistance genes. These experiments conclude that the lactic acid strains isolated from Bulgarian fermented foods possess intrinsic resistance based on different mechanisms for neutralizing the activity of some of the antibiotics studied and are not potential vectors for antibiotic resistance genes.

4. Conclusions

This study analyzed 45 LAB strains originating from traditional Bulgarian fermented foods. Species identification revealed the presence of five species: *L. plantarum*, *L. brevis*, *L. helveticus, L. koreensis,* and *L. yonginensis*. The strains isolated from fermented milk products most commonly belonged to the species *L. plantarum*, *L. brevis,* and *L. helveticus*, as well as in fermented vegetable foods—the dominant species was *L. plantarum*. This study reports the isolation of two species, L. koreensis and L. yonginensis, from Bulgarian fermented vegetable foods for the first time. All strains were tested for a set of abilities regarding their beneficial potential and safety application. We can summarize some significant findings: Among the strains, there were several that showed an apparent antagonistic effect, mainly against Gram-negative pathogenic bacteria; the ability of autoaggregation, co-aggregation, adhesion to hydrocarbons (hexane), and biofilm production were differentially represented among strains; we established that the observed phenotypic antibiotic resistance to different antibiotics is not the result of the presence of resistance genes and none of the strains showed hemolytic activity.

In conclusion, traditional fermented Bulgarian foods can be considered sources of variable beneficial LAB. The main observation in our study was that none of the tested strains simultaneously met all probiotic criteria. However, it can be proposed that the complex action of the LAB members in the microflora of fermented foods prevents the development of pathogenic bacteria and creates safe food. Further, a significant number of strains possessing beneficial properties were isolated from the traditional Bulgarian food, sauerkraut; thus, it can be considered a functional food. Moreover, this type of cabbage preservation is strictly typical in our country; therefore, we can speculate that its natural microbial diversity is some kind of endemic. However, the obtained preliminary results for the beneficial potential of traditional Bulgarian fermented foods, reported in this paper, could serve as a solid basis for further elaboration of complex starter cultures with the potential for biotechnological applications.

Availability of Data and Materials

All used materials and generated data are included in this manuscript.

Author Contributions

Substantial contributions to conception and design: VYMY, PKH and YKK; performed the research: VYMY; analysis and interpretation of data: PKH, YKK and IKR; writing the original draft of the manuscript: VYMY; review the draft version of the manuscript: PKH and YKK; All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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