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Identification of Lactic Acid Bacteria Strains Isolated from Sourdoughs Prepared with Different Flour Types

Zoltan Urshev [1], Dilyana Doynova [1], Ivan Prasev [2], Rositsa Denkova-Kostova [3,*], Anna Koleva [2], Zapryana Denkova [4], Bogdan Goranov [4] and Georgi Kostov [5]

1 LB Bulgaricum Plc., 86 Tintyava Str., 1113 Sofia, Bulgaria; zoltan.urshev@lbbulgaricum.bg (Z.U.); ddoynova@abv.bg (D.D.)
2 Department of Technology of Grain, Fodder, Bakery and Confectionery Products, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria; iv_pr@abv.bg (I.P.); a_koleva@uft-plovdiv.bg (A.K.)
3 Department of Biochemistry and Molecular Biology, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria
4 Department of Microbiology, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria; z_denkova@uft-plovdiv.bg (Z.D.); b_goranov@uft-plovdiv.bg (B.G.)
5 Department of Wine and Beer Technology, University of Food Technologies, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria; g_kostov@uft-plovdiv.bg
* Correspondence: rositsa_denkova@uft-plovdiv.bg

Abstract: Species identification is the first step in the examination of newly isolated microorganism strains, including the cases when they are intended for application in the development of probiotic preparations or starters for different food products. The thorough identification process of newly isolated strains combines the application of different physiological, biochemical, and molecular genetic methods. The aim of the present study was to identify the species-level lactic acid bacteria (LAB) strains isolated from spontaneously fermented sourdoughs prepared from different flour types: Khorosan wheat, wheat, barley, buckwheat, spelled, spelt, and corn. Both classical phenotypic (cellular morphology characterization, and API 50 CHL) and molecular genetic methods (RAPD, ARDRA-analysis, 16S rDNA sequencing, and species-specific PCR) were applied. It was found that cultures with a short-rod morphology predominated among the 30 sourdough isolates. According to the RAPD profiles obtained, the isolates were divided into nine genotypes corresponding to nine genetically distinct strains. It was determined that individual sourdoughs made with different flour types shared cultures with a common genotype. The analysis of the physiological and biochemical profiles of the LAB isolates performed with the API 50 CHL system divided them into two groups according to their identification: *Lactiplantibacillus plantarum* (*Lp. plantarum*) 1 and *Levilactobacillus brevis* (*Lv. brevis*) 3. According to the 16S rDNA restriction profile, the LAB isolates showed two profiles corresponding to the *Lp. plantarum* and *Lv. brevis* groups. 16S rDNA sequencing and a comparison of the partially read 16S rDNA sequences of the studied isolates confirmed that some of them belonged to the *Lv. Brevis* species, but did not provide sufficient evidence that the rest of the cultures belonged to the *Lp. Plantarum* species. The species-specific PCR clearly separated the isolates from the *Lp. plantarum* group into two groups: isolates of the *Lp. plantarum* species and isolates of the *Lp. paraplantarum* species. The summary of the results of the conducted polyphasic taxonomic study determined the investigated LAB strains isolated from spontaneously fermented sourdoughs as representatives of the *Lv. brevis*, *Lp. plantarum* ssp. *paraplantarum*, and *Lp. paraplantarum* species.

Keywords: *Levilactobacillus brevis*; *Lactiplantibacillus plantarum*; RAPD; sourdough; 16S rRNA gene sequencing

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1. Introduction

Lactobacilli are a major group of lactic acid bacteria (LAB) that are Gram-positive, facultatively anaerobic, rod-shaped, non-spore-forming, and motile [1]. They are oxidase-

and catalase-negative; they ferment carbohydrates and hydrolyze esculin [2]. Bacteria of the *Lactobacillus* genus are widespread in the environment, with high biological and functional activity, which determines their practical application as components of probiotics and synbiotics, of starter cultures for the production of lactic acid products, raw dried meat products, bread and bakery products, and fermented fruits and vegetables, and their role in the targeted fermentation process and biological preservation of food emulsions [3–10]. In addition, *Lactobacillus* sp. are resident microorganisms colonizing the gastrointestinal tract and female genital tract, where they contribute to the partial inhibition of pathogens through the organic acids and bacteriocins produced [3,11,12]. Due to the large number of species defined in the genus (>80), taxonomic changes were necessary, and they led to the introduction of 26 genera into the *Lactobacillaceae* family, 23 of which were new [13].

Not all lactobacilli strains can be applied in the food industry, only those that meet certain criteria. New cultures should provide a vigorous acidification and fermentation process, produce metabolites that contribute to food conservation and safety, add to the sensory value of the product, and be in many contemporary applications in order to demonstrate beneficial properties related to human health [14–16]. This, in turn, requires the selection of lactobacilli strains with specific properties for the development of new fermented foods [17]. The characterization and identification of bacteria in the food industry are based on their morphological, physiological, biochemical, and technological properties. The main properties studied are Gram staining; the determination of the optimal and limiting growth temperatures and pH of the medium; growth in NaCl-containing hydrolyzed milk, bile; acid-forming activity; the formation of CO_2, the formation of ammonia from arginine; and the fermentation of carbohydrates and alcohols.

Strains that are included in the composition of probiotics or starter cultures for probiotic functional foods are isolated from healthy people, food matrices, or plant species [18,19]. They are identified to the species level according to their pheno- and genotypic characteristics. Additionally, safety requirements have to be met, unless the applied species has a proven GRAS-status [20,21]. Based on phenotypic traits, different test systems have been developed for the rapid identification of different groups of microorganisms: API-20E, API 50 CH, API 20 C Aux, Enterotube, Mycotube, Patho-Tec, and others. The API 50 CH system (BioMérieux® SA, Marcy-l'Etoile, France) is used to identify species of the *Lactobacillus* genus or *Bacillus* genus based on their ability to utilize 49 carbon sources [22]. The accurate identification of lactobacilli is achieved by applying well-established or novel molecular genetic methods, such as polymerase chain reaction, matrix-activated laser desorption/ionization (mass spectrometer MALDI-TOF MS), gas chromatography-mass spectrometry, and metagenomic studies [23,24]. Methods based on the analysis and comparison of the 16S rDNA sequence have become a reference in identification, as the 16S rDNA sequence is sufficiently conserved in the evolution of microorganisms; therefore, it is used for the species identification of prokaryotic microorganisms, including lactobacilli [25,26]. Such methods include the ribosomal DNA (rDNA) restriction analysis method ARDRA [22,27–31] or the partial or complete sequencing of the 16S rRNA gene (16S rDNA) and comparison with reference sequences in DNA databases such as GenBank. When the 16S rDNA sequence diversity is insufficient for accurate identification, species-specific PCR methods are applied, targeting highly specific genes [32–34]. Unequivocally, genome sequencing and its analysis shows the specific identification of strains.

Molecular methods are also versatile in analyzing the biodiversity of lactobacilli in different environments and food. Strain diversity among *Lactobacillus* isolates is often analyzed with the Random Amplification of Polymorphic DNA (RAPD) method. RAPD involves the random amplification of fragments from the target bacterial genome using single primers under less stringent conditions. In this way, a complex profile is created out of the large number of amplification products from random loci scattered throughout the genome [25,35,36].

The spontaneous fermentation of a flour–water mixture to produce sourdough as a leavening agent in bread and bakery product preparation precedes the widespread applica-

tion of baker's yeast. With the introduction of yeast, the sourdough application was limited to artisan production practices. However, in recent decades, the interest in sourdough as an element in bread production has been renewed due to the metabolic activities of lactobacilli, such as organic acid production, the synthesis of aroma-related volatile components, proteolysis, and the production of metabolites with antifungal activity [36]. Fermentation by lactobacilli contributes to the improved shelf life, sensory, and nutritional quality of a product [37,38].

The microbial composition of sourdoughs is dominated by lactobacilli, yeast, and acetic acid bacteria. De Vuyst et al. (2017) [39] reported more than 90 different species of lactic acid bacteria in sourdoughs. The most prevalent LAB species are *Fructilactobacillus sanfranciscensis*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Companilactobacillus paralimentarius*, and *Limosilactobacillus fermentum* [40,41]. The microbial consortium found in sourdough depends on the production method and flour composition. Flours may differ vastly in their composition, all the more so because cereals (wheat, rye, barley, and maize) and non-cereals (buckwheat, quinoa, and alternative seeds) can be used for flour production [42,43]. The production method divides sourdoughs into two main groups: Type I, with a dough yield (DY) of less than 200 and incubation at temperatures up to 30 °C for 24 h with regular back-slopping, and Type II, with a DY above 200, fermentation at an elevated temperature, and a prolonged fermentation time [44].

The aim of the present study was to isolate and identify the species and examine the strain diversity of the lactic acid bacteria isolated from spontaneously fermented sourdoughs prepared from different flour types, i.e., Khorosan wheat, wheat, barley, buckwheat, spelled, spelt, and corn.

2. Materials and Methods

2.1. Sourdough Preparation

The study was conducted with LAB strains isolated from spontaneously fermented sourdoughs (back-slopped sourdoughs without the addition of starter cultures), prepared with one of seven flour types (Ecosem Ltd., Stambolovo, Bulgaria) (see Table 1) according to the following scheme:

Table 1. List of the tested isolates according to the flour type used for the preparation of the corresponding sourdough.

	Flour Type						
	Khorasan	**Buckwheat**	**Spelled**	**Barley**	**Wheat**	**Spelt**	**Corn**
Isolate designation	X1	El1	Cn1	Ech1	Ph1	L1	Car
	X2	El2	Cn2	Ech2	Ph2	L2	
	X4		Cn3	Ech3	Ph3		
			Cn4	Ech4	Ph4		
			Cn5	Ech6	Ph5		
			Cn6	Ech7			
				Ch42			
				Ch44			
				Ch51			
				Ch53			
				Ch54			

Day 1: Mixing flour and water in a ratio corresponding to the water absorption capacity of each flour type (according to the manufacturer's labeling) and the incubation of the resulting mixture at a temperature of 30 ± 1 °C for 24 h.

Day 2: The preparation of a new mixture of the same flour and water in the same ratio. Mixing the new flour/water mixture with the 24 h sourdough (from Day 1) in a ratio of 75%:25%. Incubation at 30 ± 1 °C for 24 h.

Day 3 and Day 4: Repeating the Day 2 steps.

2.2. Isolation of Bacteria

On Day 5, appropriate tenfold dilutions of the sourdough in saline solution were prepared, plated on MRS-agar medium (Merck, Sofia, Bulgaria) (g/dm^3: peptone from casein—10; yeast extract—4; meat extract—8; glucose—20; K_2HPO_4—2; sodium acetate—5; diammonium citrate—2; $MgSO_4$—0.2; $MnSO_4$—0.04; agar (Oxoid)—20; Tween 80—1 mL; pH 6.5), and cultivated at 30 ± 1 °C for 72 h in anaerobic conditions until the appearance of countable single colonies; selected colonies were isolated in MRS-broth medium.

The isolates were stored at the Department of Microbiology at the University of Food Technologies, Plovdiv. A list of the analyzed cultures is presented in Table 1. Additionally, *Levilactobacillus brevis* LMG 6906^T, *Lacticaseibacillus ramnosus* LMG 6400^T, *Lacticaseibacillus paracassei* LMG 13087^T, *Lactiplantibacillus plantarum* DSM 20174^T, and *Limosilactobacillus fermentum* DSM 20052^T were used as reference cultures.

After a microscope observation of the cell morphology of the isolates, only rod-shaped cultures were used for further analysis, as lactobacilli were the object of primary interest in this study.

2.3. Identification of the Isolates

The identification of the isolates was performed using phenotypic and molecular methods.

2.3.1. Phenotypic Identification

For phenotypic identification, a fresh 24 h culture of each isolate was centrifuged, washed twice with a PBS buffer, resuspended in an API 50 CHL medium, and introduced to the API 50 CHL carbohydrate metabolism test (BioMérieux® SA, Marcy-l'Etoile, France) following the manufacturer's manual. The API 50 CHL system is used to identify species of the *Lactobacillus* genus or *Bacillus* genus based on their ability to utilize 49 carbon sources [22,30]. The results were considered to be positive when the color changed from dark blue (no utilization of the carbon source) to green or bright yellow. The results obtained were processed with the Apiweb® identification software (BioMérieux® SA, France).

2.3.2. Molecular Identification Methods

Isolation of total DNA. The DNA of the resultant cultures was isolated using the E.Z.N.A. Bacterial DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) in accordance with the manufacturer's instructions. A 24 h broth culture obtained in MRS was used.

RAPD. Strain differentiation was performed by a random amplified polymorphic DNA (RAPD) analysis [45] through PCR amplification with the primers rapd-4 (5′-AAGAGCCC GT-3′) or opp-7 (5′-GTCCATGCCA-3′) used separately. The PCR reactions (25 µL) contained diluted VWR Taq 2× Mastermix, 2.0 mM $MgCl_2$ (VWR), 20 pmol of primer, and 50 ng of genomic DNA. The amplification program consisted of 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 34 °C for 1 min, and 72 °C for 2.5 min, and a final elongation at 72 °C for 7 min. The resulting products were visualized on 1.5% agarose gel at 100 V for 90 min and visualized with ethidium bromide.

ARDRA. Isolates with different RAPD profiles were then grouped using Amplified rDNA Restriction Analysis (ARDRA) [46] by the PCR amplification of a 16S rDNA gene region with the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR reactions (25 µL) contained diluted VWR Taq 2× Mastermix, 2.0 mM $MgCl_2$ (VWR), 10 pmol of each primer, and 50 ng of genomic DNA. The amplification program consisted of 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The restriction polymorphism of the amplicons was determined by digesting 10 microliters of the PCR product with 0.5 U of *Hae*III (Invitrogen at 37 °C) for 1 h. The obtained fragments were separated on 2% agarose and visualized with ethidium bromide.

Preparation of the amplified 16S rDNA genes for sequencing. For partial sequencing of the 16S rDNA gene, PCR amplifications were performed as described above for ARDRA,

and the PCR product was purified with Clean-Easy PCR and a Gel Purification kit (Canvas Biotech, Valladolid, Spain) according to the manufacturer's instructions. The sequencing was performed by Macrogen Inc. (Seoul, Republic of Korea) on an automatic sequencer (Applied Biosystems Inc., Foster City, CA, USA) with the di-deoxy termination procedure in both directions using the universal primers 27F and 1492R. The resulting sequences were processed with the *CLC Sequence Viewer* program v.6.6.1 (www.clcbio.com). The resulting sequences were run entries in the GenBank database of the National Center for Biotechnology Information (NCBI) with the BLASTN 2.2.25+ search engine [47]. A phylogenetic tree of the most common *Lactobacillus* species found in sourdough and the isolates obtained in this study was built based on a ca. 1356 bp partial sequence of the 16S rRNA gene. The tree was generated by the CLC Sequence Viewer software ver. 6.6.1 (www.clcbio.com, CLC bio A/S) by the UPGMA algorithm. Bootstrap values were obtained based on 100 replicates. Sequences of type strains were derived from GenBank (NCBI).

Species-specific PCR. Additionally, in order to differentiate between *Lactiplantibacillus plantarum*, *Lp. Pentosus*, and *Lp. paraplantarum* isolates, species-specific PCR targeting the *recA* gene was performed as described by Torriani et al. (2001) [33].

3. Results and Discussion

3.1. Isolation of Cultures

A total of 30 isolates were obtained from sourdough prepared from different types of flour. The number of isolates by flour type was distributed as follows: Khorasan—3; buckwheat—2; spelled—6; barley—11; wheat—5; spelt—2, and corn—1. A complete list of the isolates is presented in Table 1.

3.2. Cell Morphology

Microscope observations of the cell morphology of the isolates grown on MRS-agar showed that the majority of cultures were represented by short rods or rods polymorphic in size with or without volutine, and only a few isolates were categorized as cocci (Supplementary file, Table S1). A large difference in the cellular morphology of one and the same isolate was observed when cells grown on MRS agar and cells from MRS broth were compared (Figure 1). In the MRS broth, short to highly elongated rods rich in volutine were the dominant type. This difference may be attributed to the different culture conditions and, above all, to the presence of oxygen, as the cultivation on MRS agar was conducted under anaerobic conditions.

3.3. Strain Differentiation by RAPD

Strain differentiation was performed by RAPD only for the isolates with rod morphology. A comparison of the obtained profiles grouped the isolates into nine genotypes (Table 2). Grouping into the same genotype implies the identity of isolates down to the strain level or closely related strains. The electrophoretic RAPD profiles of the isolates are presented in Figure 2.

It is noteworthy that the sourdough prepared from barley, spelled, and spelt flour shared cultures with a common genotype. Common cultures were also found originating from barley and Khorasan wheat flour, as well as wheat and maize flour (Table 2).

Thirteen rod-shaped cultures that represented the different genotypes and different sourdough types (isolates Ech1; Ech3; L1; L2; El1; Ph1; Ph2; Ph3; Ph5; X2; X4; Cn1; and Car) were selected for a further analysis of precise species identification.

3.4. Physiological–Biochemical Profile of the Selected Isolates

After characterizing the isolates by applying morphological and molecular–genetic methods, 13 isolates were selected and subjected to biochemical profiling with the API 50 CHL system (BioMérieux® SA, France) for the identification of LAB based on the ability of each isolate to consume the 49 carbon sources included in the kit.

Table 2. Distribution of the obtained rod-shaped isolates by genotype (RAPD) and origin (flour type).

Genotype (RAPD)	Flour Type/Isolate						
	Khorasan	Buckwheat	Spelled	Barley	Wheat	Spelt	Corn
1			Cn1 * Cn2 Cn3 Cn4 Cn5 Cn6	Ech1 * Ech2 Ech4 Ech6 Ech7		L1 *	
2	X4 *			Ech3 *			
3	X2 *						
4						L2 *	
5					Ph1 *		Car *
6		El1 *					
7					Ph2 * Ph4		
8					Ph3 *		
9					Ph5 *		

* Isolates selected for further work on the species identification (isolates were selected so that each genotype was presented and each type of flour had at least one representative culture).

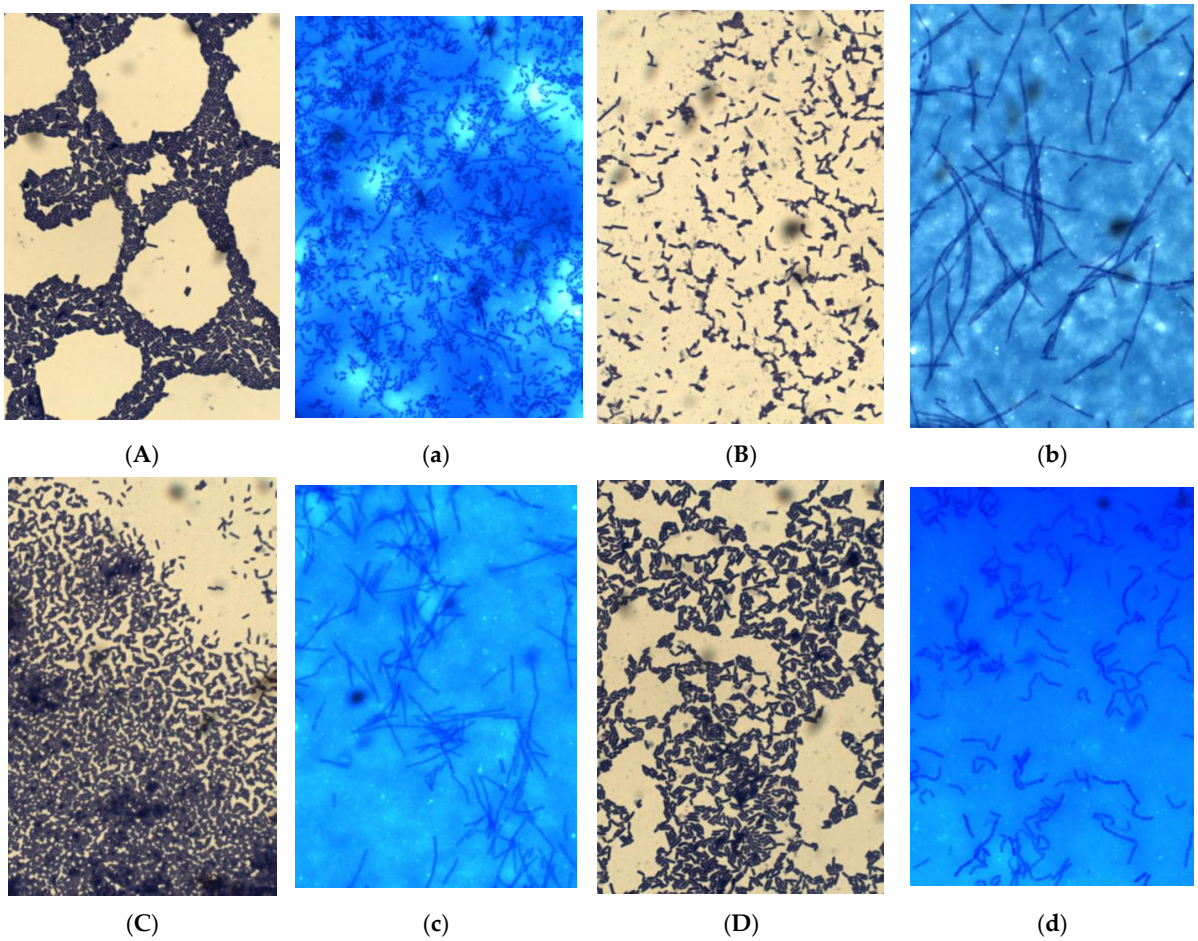

Figure 1. Cell morphology of the isolates grown on MRS-agar (capital letters) vs MRS-broth (small letters). Isolates Ech1 (**A**,**a**), Ech3 (**B**,**b**), Ph1 (**C**,**c**), and Ph3 (**D**,**d**). Methylene blue staining, 1000 × magnification.

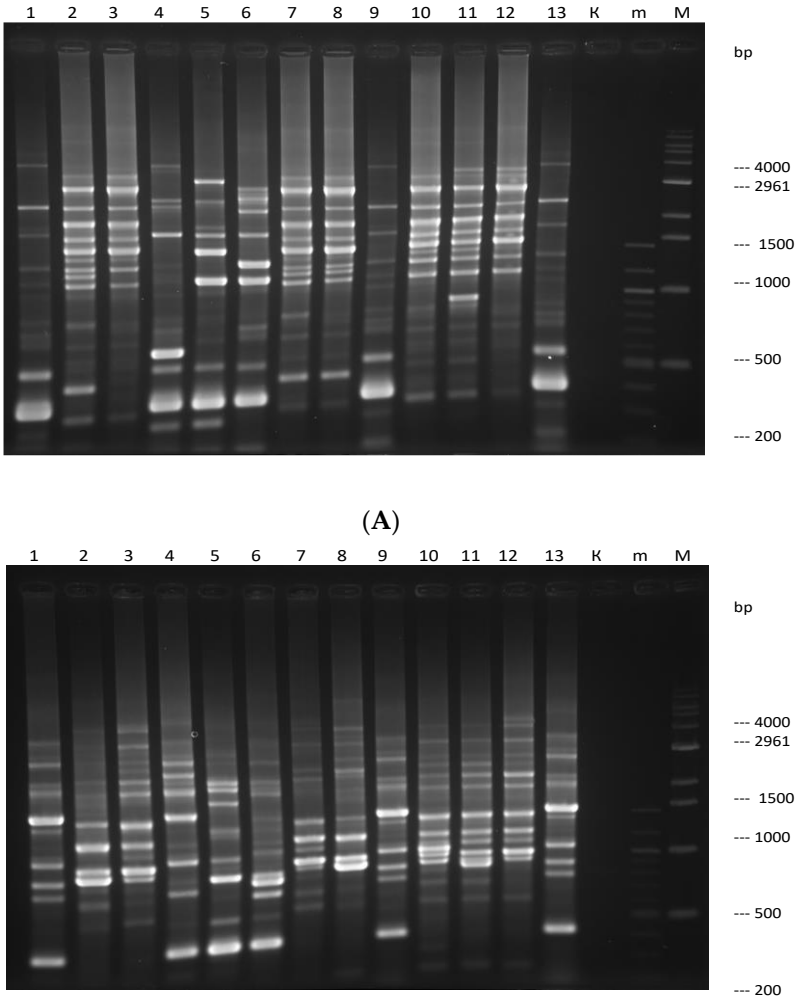

Figure 2. RAPD profiles of sourdough isolates (rods) obtained with primer rapd-4 (**A**) and opp-7 (**B**). Lanes-isolate: 1—Ech1; 2—Ech3; 3—Ph1; 4—Ph2; 5—Ph3; 6—Ph5; 7—X2; 8—X4; 9—L1; 10—L2; 11—El1; 12—Car; 13—Cn1; K—negative control; m—100-bp DNA ladder; and M—1000-bp ladder.

According to the set of digestible carbohydrates, the LAB isolates were divided into two groups: the group of *Lactiplantibacillus plantarum* 1 and that of *Levilactobacillus brevis* 3 (Table 3). The isolates in the *Lactiplantibacillus plantarum* 1 group showed a much wider range of digestible carbohydrates. This somewhat corresponded to the more diverse biochemical pathways for the carbohydrate assimilation characteristic of facultatively heterofermentative LAB bacteria (such as the *Lactiplantibacillus plantarum* species) compared to obligate heterofermentative LAB bacteria (such as the *Levilactobacillus brevis* species) or homofermentative lactobacilli. A distinctive feature of all isolates identified by the system as *Levilactobacillus brevis* 3 was the utilization of D-xylose. The most discriminative carbohydrate sources and the biochemical profile of the studied cultures are presented in the Supplementary file (Table S2).

3.5. Grouping of the Isolates according to the Restriction Profile of Their 16S rDNA

The 16S rDNA of each isolate was amplified by PCR and subjected to restriction with the *Hae*III enzyme. The resulting fragments were electrophoretically unfolded against referent LAB type strains (Supplementary file, Figure S1).

The obtained profiles of the isolates were divided into two groups, one group being identical to the profile of *Lactiplantibacillus plantarum* DSM 20174^{T} and the other group to

the profile of *Levilactobacillus brevis* LMG 6906^T. According to their profiles, the isolates were divided in a manner identical to the division made according to the results of the biochemical tests (Table 3).

No profiles identical to *Lacticaseibacillus rhamnosus* LMG 6400^T, *Lacticaseibacillus paracasei* LMG 13087^T, and *Limosilactobacillus fermentum* DSM 20052^T were found, indicating that representatives of these three species were absent among the analyzed isolates.

Table 3. Identification of sourdough isolates by their carbohydrate profile (processed with the Apiweb® software, BioMérieux® SA, France).

Isolate	Identification	Reliability
Ech1	*Lactiplantibacillus plantarum* 1	99.9%
Ech3	*Levilactobacillus brevis* 3	99.6%
L1	*Lactiplantibacillus plantarum* 1	99.7%
L2	*Levilactobacillus brevis* 3	97.2%
El1	*Levilactobacillus brevis* 3	99.1%
X2	*Levilactobacillus brevis* 3	75.1%
X4	*Levilactobacillus brevis* 3	99.6%
Cn1	*Lactiplantibacillus plantarum* 1	99.9%
Ph1	*Levilactobacillus brevis* 3	98.4%
Ph2	*Lactiplantibacillus plantarum* 1	99.5%
Ph3	*Lactiplantibacillus plantarum* 1	97.3%
Ph5	*Lactiplantibacillus plantarum* 1	72.1%
Car	*Levilactobacillus brevis* 3	98.2%

3.6. Sequencing of 16S rDNA

A comparison of the obtained sequences of the partially sequenced 16S rDNAs of the 13 isolates based on ≥99% similarity of the nucleotide sequence divided them into two groups (Supplementary file, Table S3). The first group was represented by isolates that confirmed their affiliation with the *Lv. brevis* species. The second group of isolates, that of the *Lp. plantarum* group, however, could not be unequivocally identified at the species and subspecies levels by its partial 16S rDNA sequence, as for some cultures, high identity was also established with *Lp. plantarum* ssp. *argentoratensis* and *Lp. pentosus*. In this case, 16S rDNA sequencing alone could not definitively indicate the species identification of the studied isolates, and required the application of additional molecular–genetic analysis [33]. A phylogenetic tree of the most common LAB species found in sourdough and the isolates obtained in this study based on a ca. 1356 bp partial sequence of the 16S rRNA gene is presented in the Supplementary file (Figure S2).

3.7. Species-Specific PCR for the Lactiplantibacillus plantarum Group

Species-specific PCR was used to further clarify the species affiliation of the isolates, which, in the other analyses, were included in the *Lp. Plantarum* group. This method is based on the specific amplification of the *recA* gene, which is sufficiently polymorphic to distinguish species within the *Lp. Plantarum* group. The *Lactiplantibacillus plantarum* group includes *Lp. Plantarum*, *Lp. pentosus*, and *Lp. paraplantarum*. In this analysis, amplificons of different sizes in the three species were obtained: 318 bp in *Lp. plantarum*, 218 bp in *Lp. pentosus*, and 107 bp in *Lp. paraplantarum* [33].

Of the six isolates tested, Ech1, Ph2, L1, and Cn1 gave an amplificon of the characteristic size of *Lp. plantarum* (318 bp), while the isolates Ph3 and Ph5 gave an amplificon of the characteristic size of *Lp. paraplantarum* (107 bp). The electrophoretically separated PCR products are presented in Figure 3. With this test, *Lp. plantarum* ssp. *argentoratensis* produced an unusual pattern with a weak band at 318 bp and a strong band at 120 bp [43,48]. As such a pattern was not observed for any of the isolates from the *Lp. plantarum* group, their possible affiliation with the *Lp. plantarum* ssp. *argentoratensis* subspecies was excluded. The final species identification of the sourdough isolates in this study is presented in Table 4.

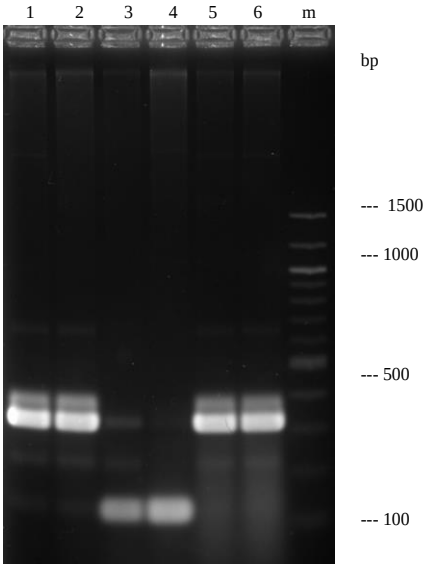

Figure 3. Species-specific PCR amplificons distinguishing the *Lactiplantibacillus plantarum*, *Lp. pentosus*, and *Lp. paraplantarum* species. Lanes-isolate: 1—Ech1; 2—Ph2; 3—Ph3; 4—Ph5; 5—L1; 6—Cn1; and m—100-bp DNA ladder.

Table 4. Species identification of sourdough isolates of lactobacilli based on combining 16S rDNA sequence analysis and species-specific PCR.

Flour Type	Isolate	Identification
Barley flour	Ech1	*Lactiplantibacillus plantarum* ssp. *plantarum*
	Ech3	*Levilactobacillus brevis*
Spelt flour	L1	*Lactiplantibacillus plantarum* ssp. *plantarum*
	L2	*Levilactobacillus brevis*
Buckwheat flour	El1	*Levilactobacillus brevis*
Khorosan	X2	*Levilactobacillus brevis*
	X4	*Levilactobacillus brevis*
Spelled flour	Cn1	*Lactiplantibacillus plantarum* ssp. *plantarum*
Wheat flour	Ph1	*Levilactobacillus brevis*
	Ph2	*Lactiplantibacillus plantarum* ssp. *plantarum*
	Ph3	*Lactiplantibacillus paraplantarum*
	Ph5	*Lactiplantibacillus paraplantarum*
Corn flour	Car	*Levilactobacillus brevis*

4. Discussion

Three LAB species are by far the most dominant representatives of sourdough microbiota: *Fructilactobacillus sanfranciscensis*, *Lv. brevis*, and *Lp. plantarum* [40,41]. This corresponds well with the LAB species composition of the sourdough preparations from the different types of flour in our study, where strains of *Lv. brevis*, *Lp. plantarum* ssp. *plantarum*, and *Lp. paraplantarum* were identified. Corsetti and Settanni (2007) [49] noted that, in sourdough, *Lp. pentosus* and *Lp. paraplantarum* may also be present, but misidentified as *Lp. plantarum* unless species-specific PCR targeting the *recA* gene is performed [33]. Indeed, in our experiments, the identification of *Lp. paraplantarum* isolates was possible only after this additional species-specific PCR analysis had been performed.

In the analyzed sourdough samples, no *F. sanfranciscensis* isolates were identified, but it has been shown that the presence of this species in sourdough may be closely related to the method of sourdough production [50,51]. Van Kerrebroeck et al. (2017) [44] demonstrated that *Fr. sanfranciscensis* was a characteristic species in Type I sourdough fermented

at room temperature for 24 h and regularly back-slopped. Other types of sourdough have been described, type 0 being sponge dough with fast growth of baker's yeast and limited time for LAB development; type II, liquid dough with less dry matter; and type III, dried derivatives of type II sourdough [39]. The spontaneously fermented sourdoughs produced from different types of flour in this study were typical Type I sourdoughs, as they were fermented at 30 °C for 24 h and regularly back-slopped. According to a review by Gänzle and Zheng, 2019 [52], the literature data on 227 type I sourdoughs, which included mainly samples from Germany, Italy, France, Belgium, the U.S., and Canada, since 2015, demonstrated that more than 95% of sourdoughs contain heterofermentative LAB alone or in association with homofermentative lactobacilli. *Fr. sanfranciscensis* was most frequently identified. Other frequent representatives included *Lp. plantarum* and *Lv. brevis*, species in the *Companilactobacillus alimentarius* group (*C. paralimentarius*, *L. crustorum*, *L. mindensis*, and *L. nantensis*), *Leuconostoc* sp., and *Weissella* sp. An extensive review of De Vuyst et al. (2021) [39] pointed out that "in general less than three different LAB species inhabit a single bakery sourdough" and the microbiota included both homo- and heterofermentative lactobacilli [44]. The results of the present study agree with both authors, with *Lv. brevis* being the obligately heterofermentative representative and *Lp. plantarum* the facultatively heterofermentative species. The absence of *F. sanfranciscensis* in all analyzed sourdough samples in this study may be attributed to its smaller metabolic abilities due to its small genome [53], which may result in its elimination by competition with a more versatile species such as *Lv. Brevis*, or, taking into consideration the fact that all flour samples were obtained locally in Bulgaria, the ecological source of *F. sanfranciscensis* might be absent in the region. However, other researchers have found *F. sanfranciscensis*, but not *Lv. brevis* or *Lp. plantarum,* in spontaneously fermented rye sourdough from the region of Stara Zagora in Bulgaria [54]. On the other hand, *Lv. brevis* and *Lp. plantarum* strains have been shown to be the major species in sourdough for Italian varieties of bread [55,56].

It is noteworthy that sourdough, as a rule, contains a tandem of heterofermentative (*F. sanfranciscensis* and *Lv. brevis*) and facultatively heterofermentative (*Lp. plantarum* and *C. paraalimentarius*) lactobacilli. De Vuyst et al. (2021) [43] pointed out two major adaptations of these two groups of lactobacilli to sourdough; the first, that of *F. sanfranciscensis* and *Lv. brevis*, being the ability to ferment maltose, and the second, that of *Lp. plantarum* and *C. paraalimentarius*, the ability to ferment both pentoses and hexoses present in flour. These two traits may well explain the mutual benefit of the fermentation ability of these two groups of lactobacilli found in sourdough [57].

The presented study also includes a description of the strain diversity among the lactic acid bacteria isolates. This is of particular interest, as sourdough prepared from different flour types offers an abundance of the culture variety necessary for the successful development of versatile *Lv. brevis*- and *Lp. plantarum*-based sourdough starters varying in their desired properties.

5. Conclusions

In the present study, the lactobacilli in sourdough obtained from seven different types of flour were identified at the species level. The isolated cultures were affiliated with the *Lv. brevis*, *Lp. plantarum* ssp. *plantarum*, and *Lp. paraplantarum* species. Although the application of some flour types resulted in the isolation of common strains, nine different genotypes were identified, underlining the high strain diversity of lactobacilli in sourdough. The precise species identification of lactobacilli was only possible by combining the results of different phenotypic and molecular–genetic approaches. The next step in the research would involve the examination of the different technological properties of the studied strains, including the characterization of beneficial gene content by the whole-genome sequencing of promising cultures and the development of multi-strain sourdough starters for the preparation of bread from different flour types.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14052093/s1: **Table S1.** Cell morphology of the obtained isolates grown on MRS-agar. **Table S2.** Most discriminative carbohydrate utilization patterns of the tested isolates. **Figure S1.** Restriction profiles of 16S-ribosomal DNA of the studied isolates treated with the restriction enzyme *Hae*III. **Table S3.** Hits from the NCBI database with ≥99% similarity to the partial 16S-rDNA sequences of the studied isolates. **Figure S2.** Phylogenetic tree of the most common *Lactobacillus* species found in sourdough.

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