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Article

Identification of Lactic Acid Bacteria Isolated from Sourdoughs Obtained with Flour of Different Cereals in North-Western Spain Trough MALDI-TOF MS and *pheS* Gene Analyses

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Abstract

Lactic acid bacteria (LAB) are Gram positive cocci or rods from different genera and species which are dominant in cereal sourdoughs. Although the production and consumption of sourdough breads have increased in the last decade there are still few studies about the identity of LAB present in sourdoughs from different countries. Here we identified LAB strains isolated from artisan wheat sourdoughs elaborated in bakeries of North-Western Spain, and in experimental mature sourdoughs elaborated by an expert baker with four different cereals, wheat, rye, spelt and tritordeum. Through a combination of MALDI-TOF and *pheS* gene sequence analyses we identified *Lactiplantibacillus plantarum* (formerly *Lactobacillus*) and *Levilactobacillus brevis* in all analysed sourdoughs. *Lactiplantibacillus paraplantarum*, *Lacticaseibacillus paracasei*, *Weissella paramesenteroides*, *Lactococcus lactis* and *Pediococcus pentosaceus* were also identified in different sourdoughs. These species were also isolated from sourdoughs obtained in other European countries, although we did not isolate strains of the genera *Limosilactobacillus*, *Companilactobacillus*, *Latilactobacillus* or *Leuconostoc* which were found in some of them. Our results showed the need of identify the LAB species and strains present in sourdoughs of different countries before to design suitable starters to develop inoculated sourdoughs made with different cereal matrices. To our knowledge, this is the first report about the identity of LAB present in tritordeum sourdoughs in Spain.

Keywords: lactic acid bacteria; sourdough; cereals; MALDI-TOF MS; *pheS* gene; Spain

1. Introduction

The production and consumption of bread elaborated with sourdoughs (called levain in French, sauersteig in German, pasta madre or lievito madre in Italian, and masa madre in Spanish) have increased considerably in the last decade. The sourdoughs can be elaborated with different cereals being wheat and rye the most traditionally used in Europe [1] and more recently with spelt, an ancient variety of wheat [2] and tritordeum, a hybrid of wheat and barley, which was developed in Spain at the end of the past century [3]. Currently, tritordeum is cultivated in near to 600 ha in European countries being the highest producers Spain, Italy and Greece [4] where this cereal is already used

to produce bread which is a reliable alternative to wheat in the elaboration of gluten-low breads [5–7] improving their digestibility [8].

The sourdoughs are very complex biological systems dominated by lactic acid bacteria (LAB) that synergistically coexist with yeasts well adapted to this acidic environment but present at lower cell concentrations than LAB [9]. LAB isolated from sourdoughs are generally accepted as safe microorganisms that play important roles in food fermentation and preservation and therefore, they could be used to formulate novel starters for the bakery industry [10]. The lactic acid bacteria (LAB) are Gram positive cocci or rods belonging to different families, genera and species included in the order *Lactobacillales* [11]. They are ubiquitous bacteria living in different ecosystems such as cereals whose flours contain LAB being responsible of fermentation processes during the production of foods [12]. The production and consumption of sourdoughs bread have increased considerably in the last decade as well as the studies carried out about the identity and diversity of LAB in sourdoughs in different countries showing the presence of more than 100 species from more than 25 genera [13].

In several works performed in European countries strains of the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Weissella* have been found in wheat sourdoughs from Italy [14–18] and France [19,20], the genera *Lactobacillus*, *Leuconostoc* and *Weissella* in Turkey [21], the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc* in Belgium [21] and the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* in Austria [23] and Bulgaria [24]. In oat sourdoughs the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* were found in Ireland [25] and in rye sourdoughs the genus *Lactobacillus* was identified in Estonia [26] and Bulgaria [27], the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* in Lithuania [28] and the genera *Leuconostoc* and *Weissella* in Spain [29].

All these studies were performed before the split of genus *Lactobacillus* into several new genera which was performed in year 2020 by Zheng et al. [30]. After this year several studies have been performed where species from some of these new genera were identified, such as *Lactiplantibacillus* and *Limosilactobacillus* in triticale sourdoughs from Belgium [31], *Lactiplantibacillus* and *Levilactobacillus* in different cereal sourdoughs from Bulgaria [32], *Fructilactobacillus*, *Lactiplantibacillus*, *Limosilactobacillus*, *Companilactobacillus*, *Lacticaseibacillus* in different cereals sourdoughs from Spain [33] and *Companilactobacillus*, *Levilactobacillus*, *Latilactobacillus* and *Lactiplantibacillus* in traditional sourdoughs in Turkey [34].

Apart of these mentioned studies, it lacks data about the identity and diversity of LAB present in sourdoughs of many European countries and therefore more studies are necessary because the isolation and characterization of LAB from different fermented flours and geographical locations would likely contribute to the discovery of a greater diversity of LAB [35] and will allow to design novel starters for different bread elaboration. Therefore, the aim of this study was the identification of LAB isolated from different cereal sourdoughs elaborated in North-Western Spain through MALDI-TOF MS and *pheS* gene analyses, which have been combined in previous works to identify LAB [36].

2. Materials and Methods

2.1. Strains Isolation

To isolate the LAB strains, we used on one hand artisan wheat sourdoughs elaborated in different bakeries in three provinces of North-Western Spain, Ávila, Salamanca and Zamora, which were also used to isolate yeasts in a previous study [37]. On the other hand, we isolated LAB strains from experimental firm and liquid sourdoughs elaborated by a baker (E. Mateos) with flours of wheat, spelt, rye and tritordeum (Tables S1 and S2). These experimental sourdoughs, obtained through spontaneous fermentation of cereal flours, were propagated for about one month by daily back-slopping, as described by Chiva et al. [37].

To perform the isolation, we weighed 10 g samples from each sourdough and added them to 90 mL of sterile peptone water (1 g/L peptone, 8.5 g/L NaCl) in 250 mL flasks. We then incubated the mixtures at 28 °C for 1 hour with agitation (200 rpm). Subsequently, 5 mL of each mixture was

centrifuged at low speed (3000 x g) to remove the starter culture residues. From the resulting supernatant, we prepared serial dilutions and inoculated them onto MRS agar plates (Man, Rogosa, and Sharpe medium, Sigma Co.). Finally, we incubated the plates at 28 °C for 4 days.

2.2. MALDI-TOF MS Performing and Data Analysis

The sample preparation and the MALDI-TOF MS analysis were carried out as was previously published [38] using a matrix of saturated solution of α -HCCA (Bruker Daltonics, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid. We used amounts of biomass between 5 to 100 mg to obtain the spectra as indicate the manufacturer. The calibration mass were the Bruker Bacterial Test Standards (BTS) which were as follows (masses as averages): RL36, 4365.3 Da; RS22, 5096.8 Da; RL34, 5381.4 Da; RL33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10300.1 Da; RNase A, 13683.2 Da and myoglobin, 16952.3 Da.

The score values proposed by the manufacturer are the following: a score value between 2.3 and 3.00 indicates highly probable species identification; a score value between 2.0 and 2.299 indicates secure genus identification and probable species identification, a score value between 1.7 and 1.999 indicates probable genus identification, and a score value <1.7 indicates no reliable identification.

Cluster analysis was performed based on comparison of strain-specific main spectra created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., USA) integrated in the MALDI Biotyper 3.0 software. The parameter settings were: 'Distance Measure=Correlation' and 'Linkage=Average'. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

2.3. Phylogenetic Analysis of *pheS* Gene

The amplification and sequencing of *pheS* genes were carried out as indicated by Doan et al. [39]. The sequences obtained were compared with those from the GenBank using the Blastn program [40]. The obtained sequences and those of related bacteria retrieved from GenBank were aligned using the Clustal W program [41]. The phylogenetic distances were calculated according to Kimura's two-parameter model [42]. The phylogenetic trees were inferred using the neighbour joining model [43] and MEGA 7.0 [44] was used for all the phylogenetic analyses.

3. Results

3.1. MALDI-TOF MS Analysis

The results of the analysis of LAB strains isolated from artisan wheat sourdoughs elaborated in bakeries from different provinces of North-Western Spain showed that these strains matched with scores higher than 2.0 with respect to *Lactocaseibacillus* (*Lc.*) *paracasei*, *Lactiplantibacillus* (*Lt.*) *plantarum*, *Lactiplantibacillus* (*Lt.*) *paraplantarum*, *Levilactobacillus* (*Lv.*) *brevis*, *Lactococcus lactis* and *Pediococcus pentosaceus* (Tables 1 and S3). The most abundant species in the wheat fermented sourdoughs from Ávila bakeries was *Lt. plantarum* with a 46% of isolated strains, followed by *Lc. paracasei* with 31%, *P. pentosaceus* with 13%, *Lt. paraplantarum* and *Lv. brevis* with less than 10% each one (Figure 1 and Table 2). In Salamanca bakeries also was *Lt. plantarum* the most frequently isolated species with 50% of strains followed by *L. lactis* with 29% of strains and *Lv. brevis*, *P. pentosaceus* and *Weissella paramesenteroides* with less of 10% in each case. In Zamora bakeries also was *Lt. plantarum* the most frequently isolated species with 43% of strains followed by *Lc. paracasei* with 35% and *Lv. brevis* with 22% (Figure 1 and Table 2).

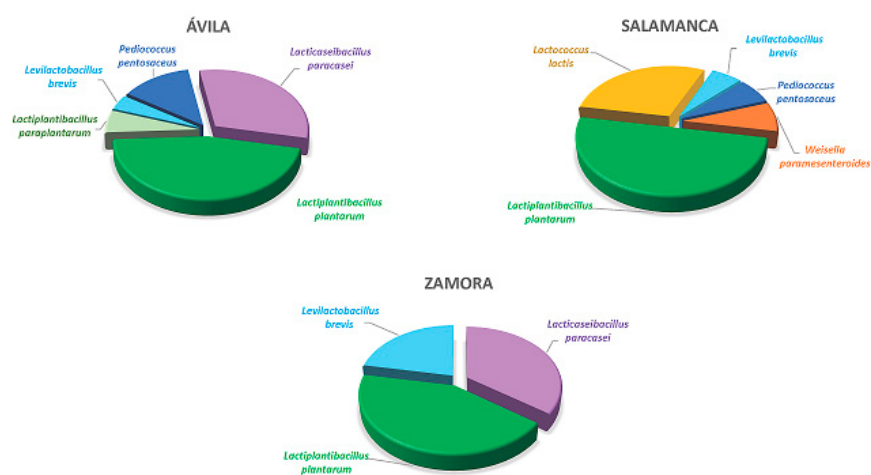


Figure 1. Pie charts showing the distribution of the different species of LAB strains isolated from artisan wheat sourdoughs elaborated in bakeries from different provinces of North-Western Spain.

Table 1. Results of the identification through MALDI-TOF and *pheS* gene sequence analyses of strains isolated from fermented sourdoughs elaborated with different cereals. *These values were calculated with respect to the type strains of each species which are indicated in Figure 3.

Sourdoughs codes (number of isolated strains)	Cereal/sourdough type	origin	Identified LAB species through MALDI-TOF	Representative strain/MALDI-TOF group	<i>pheS</i> gene sequence similarity values (%)*
mBAL5 (1), mBAL11 (5), mBAL12 (9)	wheat/firm	Avila bakeries	<i>Lactocaseibacillus paracasei</i>	mBAL11_1/III mBAL12_21/V	99.7% 99.7%
mBAL13 (7), mBAL17 (1), mBAL18 (20)	wheat/firm	Zamora bakeries	<i>Lactocaseibacillus paracasei</i>	mBAL18_15/I mBAL13_7/II mBAL18_20/IV	99.7% 99.7% 99.7%
mBAL4 (6), mBAL5 (4), mBAL11 (8), mBAL12 (3)	wheat/firm	Avila bakeries	<i>Lactiplantibacillus plantarum</i>	mBAL4_3/VI	100%
mBAL1 (17), mBAL10 (2), mBAL19 (23)	wheat/firm	Salamanca bakeries	<i>Lactiplantibacillus plantarum</i>	mBAL1_4/IX mBAL19_15/IV	100%
mBAL13 (1), mBAL14 (10), mBAL15 (14), mBAL17 (10)	wheat/firm	Zamora bakeries	<i>Lactiplantibacillus plantarum</i>	mBAL4_20/V	100%
BARI (15), BMOL (13)	wheat/firm	experimental	<i>Lactiplantibacillus plantarum</i>	BMOL_18/II	100%
BPARi (14), BPMOL (13)	wheat/liquid	experimental	<i>Lactiplantibacillus plantarum</i>	BPMOL_17/VIII	100%
BCEN (14)	rye/firm	experimental	<i>Lactiplantibacillus plantarum</i>	BCEN_10/VII	100%
BPCEN (13)	rye/liquid	experimental	<i>Lactiplantibacillus plantarum</i>	BPCEN_14/XI	100%
BESP (9)	spelt/firm	experimental	<i>Lactiplantibacillus plantarum</i>	BESP_5/III	100%
BPESP (12)	spelt/liquid	experimental	<i>Lactiplantibacillus plantarum</i>	BPESP_5/X	100%
BTRi (9)	tritordeum/firm	experimental	<i>Lactiplantibacillus plantarum</i>	BTRi_13/I	100%

BPTRi (12)	tritordeum/liq uid	experimental	<i>Lactiplantibacil lus plantarum</i>	BPTRi_13/XII	100%
mBAL4 (3)	wheat/firm	Avila bakery	<i>Lactiplantibacil lus paraplantarum</i>	mBAL4_6/II	100%
BESP (4)	spelt/firm	experimental	<i>Lactiplantibacil lus paraplantarum</i>	BESP_7/IV	97.9%
BPESP (4)	spelt/liquid	experimental	<i>Lactiplantibacil lus paraplantarum</i>	BPESP_2/III BPESP_13/V	97.9% 97.9%
BTRi (1)	tritordeum/fir m	experimental	<i>Lactiplantibacil lus paraplantarum</i>	BTRi_2/I	97.9%
mBAL1 (5)	wheat/firm	Salamanca bakery	<i>Levilactobacillu s brevis</i>	mBAL1_3/IV	98.7%
mBAL12 (2)	wheat/firm	Avila bakery	<i>Levilactobacillu s brevis</i>	mBAL12_11/IX	99.2%
mBAL13 (16), mBAL17 (2)	wheat/firm	Zamora bakeries	<i>Levilactobacillu s brevis</i>	mBAL13_8/VI	98.7%
BMOL (3)	wheat/firm	experimental	<i>Levilactobacillu s brevis</i>	BMOL_20/I	98.7%
BPARI (2), BPMOL (4)	wheat/liquid	experimental	<i>Levilactobacillu s brevis</i>	BPARI_7/XI	98.7%
BPCEN (2)	rye/liquid	experimental	<i>Levilactobacillu s brevis</i>	BPCEN_1/X	99.2%
BESP (5)	spelt/firm	experimental	<i>Levilactobacillu s brevis</i>	BESP_10/VII BPESP_11/III	99.2% 98.7%
BPESP (3)	spelt/liquid	experimental	<i>Levilactobacillu s brevis</i>	BPESP_14/V	99.2%
BTRi (10)	tritordeum/fir m	experimental	<i>Levilactobacillu s brevis</i>	BTRi_3/VIII	99.2%
BPTRi (4)	tritordeum/liq uid	experimental	<i>Levilactobacillu s brevis</i>	BPTRi_5/II	99.2%
mBAL4 (1), mBAL12 (5)	wheat/firm	Avila bakeries	<i>Pediococcus pentosaceus</i>	mBAL4_8/III mBAL12_24/II	99.7% 100%
mBAL9 (1), mBAL10 (5)	wheat/firm	Salamanca bakeries	<i>Pediococcus pentosaceus</i>	mBAL10_7/I	99.7%
mBAL9 (22), mBAL10 (2)	wheat/firm	Salamanca bakeries	<i>Lactococcus lactis</i>	mBAL9_4/I mBAL9_16/III mBAL10_23/II	100% 100% 100%
mBAL10 (7)	wheat/firm	Salamanca bakery	<i>Weissella paramesenteroi des</i>	mBAL10_8/II mBAL10_18/I	100% 100%

Table 2. Percentages of the LAB species isolated in this study. 1: *Lacticaseibacillus paracasei*, 2: *Lactiplantibacillus plantarum*, 3: *Lactiplantibacillus paraplantarum*, 4: *Lactococcus lactis*, 5: *Levilactobacillus brevis*, 6: *Pediococcus pentosaceus*, 7: *Weissella paramesenteroides*.

Sourdoughs	1	2	3	4	5	6	7
Fermented wheat sourdoughs elaborated in bakeries of different provinces							
Ávila province							
mBAL4, mBAL5, mBAL11, mBAL12	31%	46%	6%		4%	13%	
Salamanca province							
mBAL1, mBAL9, mBAL10		50%		29%	6%	7%	8%

Zamora province			
mBAL13, mBAL14, mBAL15, mBAL17, mBAL18	35%	43%	22%
Firm and liquid sourdoughs elaborated with different cereals			
Wheat	86%	14%	
Rye	93%	7%	
Spelt	56%	22%	22%
Tritordeum	58%	3%	39%

The results of the analysis of LAB strains isolated from liquid and firm sourdoughs elaborated with different cereals matched with scores higher than 2.0 with respect to *Lt. plantarum*, *Lt. paraplantarum* and *Lv. brevis* (Tables 1 and S3). The most abundant species (more than 50%) in all these sourdoughs was *Lt. plantarum*, particularly in those elaborated with wheat and rye where more than 85% of the isolated strains belonged to this species. In all sourdoughs, but in less amounts, we found the species *Lv. brevis* with less than 40% of the isolated strains. In wheat and rye sourdoughs the species *Lt. paraplantarum* was not found being its proportion low than 25% in the sourdoughs elaborated with spelt and tritordeum (Figure 2 and Table 2). Although some differences were found between firm and liquid sourdoughs from the same cereal, they were only found in the minority of species, such as *Lv. brevis* in the case of rye and *Lt. paraplantarum* in the case of tritordeum (Figure 2 and Table 2).

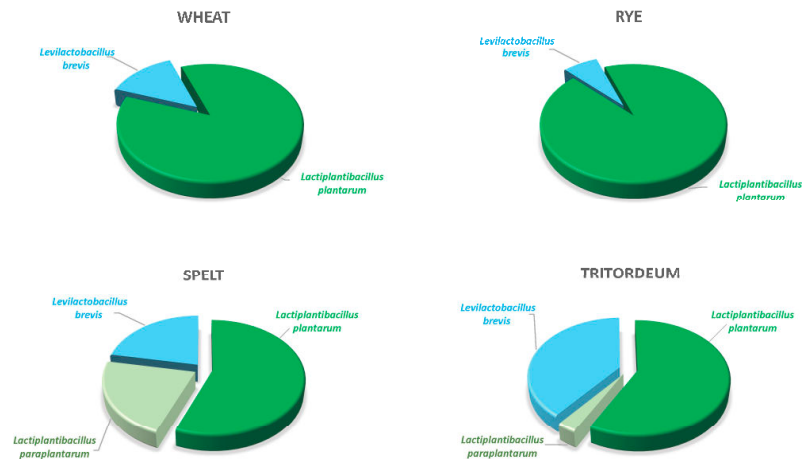


Figure 2. Pie charts showing the distribution of LAB strains isolated from experimental mature sourdoughs elaborated with different cereals.

3.2. *pheS* Gene Analysis

The analysis of partial sequences of *pheS* gene of strains isolated from different sourdoughs representative of different MALDI-TOF MS groups within each LAB species (Figs. S1 to S7, Table 1). The results of this analysis confirmed the identification obtained after MALDI-TOF MS analysis at genus and species levels for all strains isolated in this study with similarity values of *pheS* gene sequences ranging from 97.9% to 100% (Figure 3 and Table 2). All strains identified by MALDI-TOF MS as *Lt. plantarum*, *W. paramesenteroides* or *L. lactis* showed 100% similarity with respect to the type strain of this species. In the case of *Lv. brevis* all strains showed 98.9% with respect to the type strain of this species. In the remaining cases, the strains clustered into two separated groups, one of them showing 100% similarity with respect to the type strain of the species identified through MALDI-TOF MS and another one showing different similarity values with respect to those of the type strains. This was the case of *Lt. paraplantarum* with 97.8% similarity and *P. pentosaceus* and *Lc. paracasei* with 99.7% similarity in both cases (Table 2).

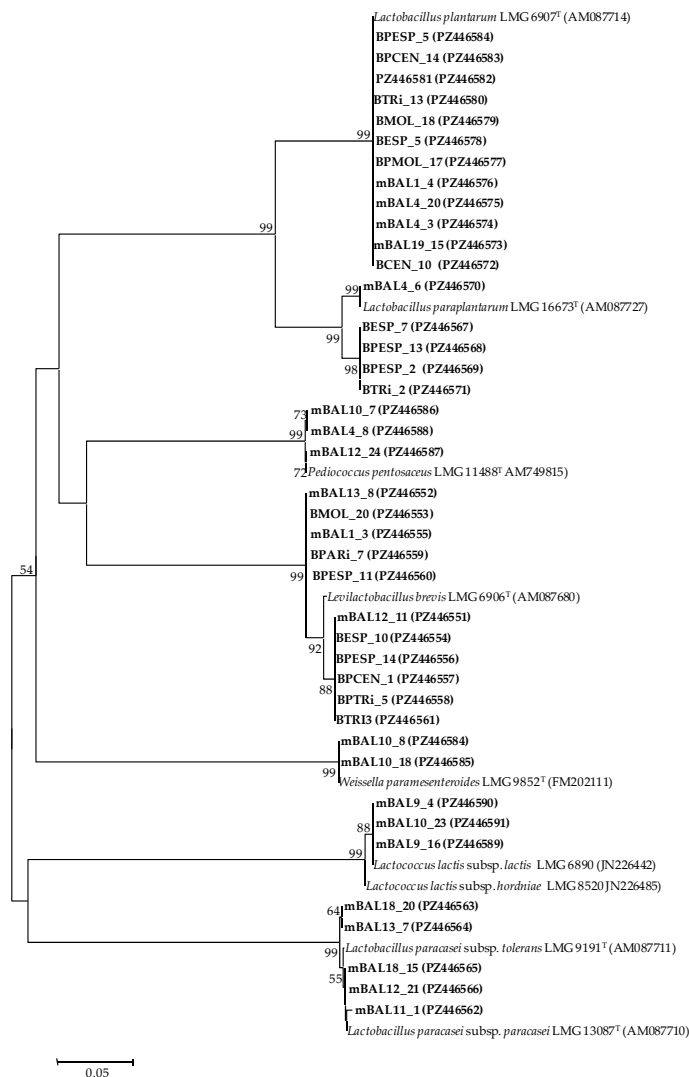


Figure 3. Neighbour-joining phylogenetic unrooted tree based on *pheS* gene partial sequences (400 nt) showing the taxonomic location of representative strains from different groups of MALDI-TOF MS found in this study. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 1000 nt. Accession numbers from Genbank are given in brackets.

4. Discussion

LAB isolated from sourdoughs are generally accepted as safe microorganisms that play important roles in food fermentation and preservation and could be used to formulate novel starters for the bakery industry [10]. To date more than 100 species from more than 25 genera of LAB have been described [13]. Nevertheless, it lacks studies about the LAB present in some countries and therefore, the identification of LAB isolated from different unstudied fermented matrices elaborated in different geographical locations would likely contribute to increase the knowledge of their diversity [28]. Along the time the identification of LAB has been carried out using different methodologies, but currently MALDI-TOF MS combined with *pheS* gene analysis has been used to identify LAB in fermented foods [36,38]. In the present study we used this combination to identify LAB present in sourdoughs from 11 bakeries (Table S1) and 10 sourdoughs one month old, experimentally elaborated with 4 different cereals in Spain (Table S2). We found differences between the bakery artisan sourdoughs and experimental wheat ones, since some species found in sourdough from artisan bakeries were not found in experimental ones, as occurs with *L. lactis*, *Lc. paracasei* (before *Lactobacillus paracasei*), *P. pentosaceus*, *W. paramesenteroides* and *Lt. paraplantarum* (before *Lactobacillus paraplantarum*) (Figures. 1 and 2, Table 2). This could be attributed to different practices of backslopping (the use of fermented sourdough from previous batches) in the different Spanish

bakeries and to the microorganisms naturally present in each bakery. It has been already observed in some works that the higher number of reinoculation cycles in bakeries facilitates a high level of biodiversity and microbial stability in their sourdoughs than in experimental ones which are started de novo or under restricted conditions that fail to replicate [45–47].

The obtained data agree with those of the remaining studies carried out in other European countries. In Italy, France and Austria the species *Lt. plantarum* (before *Lactobacillus plantarum*), *Lv. brevis* (before *Lactobacillus brevis*), *Lc. paracasei* and *P. pentosaceus* were found in wheat and rye sourdoughs [15–20,23,28]. These species were also found in wheat sourdoughs in Belgium [22,31]. In Bulgaria *Lt. plantarum* and *P. pentosaceus* were the most abundant species in wheat and rye sourdoughs [24] and *Lt. paraplantarum* and *Lv. brevis* in wheat and spelt flours [32]. In sourdoughs elaborated with *Triticum* alone or mixed with rye or spelt in Southwest Spain the species *Lt. plantarum* and *Lc. paracasei* were found [33]. The species *Lt. plantarum* and *Lv. brevis* were found in Estonia and Turkey [26,34]. All these results contrast with those of Ganchev et al. [27] who did not find these species in rye sourdoughs in Bulgaria.

Concerning the LAB present in tritordeum sourdoughs have been only analysed in Italy to date [48]. In agreement with our results the species *Lt. plantarum* was identified in Italian tritordeum sourdoughs. However, species of *Weissella* and *Leuconostoc*, which were identified in these sourdoughs, were not found in the Spanish tritordeum sourdoughs analysed in this study. In turn, the species *Lt. paraplantarum* and *Lv. brevis*, absent in Italian tritordeum sourdoughs, were identified in Spanish ones (Table 1)..

5. Conclusions

In this study we identified through MALDI-TOF MS and pheS gene sequence analyses six genera of LAB, *Lactiplantibacillus*, *Levilactobacillus*, and *Lacticaeibacillus* (before included into the genus *Lactobacillus*), *Lactococcus*, *Pediococcus* and *Weissella*, in sourdoughs experimentally elaborated with wheat, rye, spelt or tritordeum by the same baker in North-Western Spain (Table S2). The species *Lt. plantarum* and *Lv. brevis* were found in all analysed sourdoughs (considering together liquid and firm ones). In sourdoughs elaborated with tritordeum, a cereal developed in Spain and increasingly incorporated into bread production in some countries, such as Spain and Italy, in addition to *Lt. plantarum* and *Lv. brevis*, the species *Lt. paraplantarum* was found. These results agree with those found in other European countries, such as France, Italy and Austria, which are the geographically closest to Spain among those where LAB present in cereal sourdoughs have been analysed to date.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, M.T, E.V. M.A.S. and R.R.; methodology, M.T, E.V., M.A.S, R.R, R.C., L.C.L and J.A.U.; validation, M.T, E.V., M.A.S, R.R., R.C., L.C.L., and J.A.U.; investigation R.C., L.C.L., J.A.U., and A.J.L.; resources, A.J.L.; writing—original draft preparation, E.V.P. and M.T.; writing-review and editing, M.T., M.A.S, R.R., E.V., L.C.L and R.C.; visualization, M.T, E.V., M.A.S., R.R., R.C. and L.C.L.; supervision, M.T, E.V., and M.A.S.; project administration, M.T.; funding acquisition, M.T., M.A.S. and E.V. All authors have read and agreed to the final version of the manuscript.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

Informed Consent Statement: Not applicable.

Institutional Review Board Statement: Not applicable.

Abbreviations

The following abbreviations are used in this manuscript:
MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization Time-of-Flight), DNA (deoxyribonucleic acid), RNA (ribonucleic acid)

LAB	Lactic Acid Bacteria
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

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