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PROFILE OF LACTIC ACID BACTERIA IN RYE FLOUR AND SOURDOUGH

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Summary

In order to examine the composition of the lactic acid bacteria community of rye flour and sourdough made of it, 41 strains were isolated and characterized. The phenotypic characterization of the isolates, carried out using a set of 34 tests, allowed the identification of 11 clusters at the 80 % similarity level by hierarchical cluster analysis. All lactic acid bacteria isolates from rye flour were homofermentative cocci belonging to the genera Streptococcus (30 % of the lactic acid bacteria count) and Enterococcus (70 %). Homofermentative (Streptococcus sp. – 6 %, Enterococcus sp. – 26 %, and Pediococcus pentosaceus – 16 %) and heterofermentative (Leuconostoc mesenteroides – 13 %, and Weisella paramesenteroides – 7 %) cocci, as well as homofermentative bacilli (Lactobacillus sp. – 32 %) were isolated from sourdough.

The lactic acid bacteria were not the predominant microorganisms in the rye flour (comprising one-third of the entire bacterial population) but their acid resistance enabled them to continue growing during natural sourdough fermentation and to become the major bacterial population of sourdough.

Key words: *lactic acid bacteria, sourdough, phenotypic characterization.*

Introduction

Lactic acid bacteria (LAB) are an important group of industrial starter cultures, applied in the production of fermented foods. LAB contribute to the improvement of the taste properties of fermented foods, as well as to their preservation and microbial safety [3]. In nature, they are widely distributed and could be isolated from soils, waters, plants, silage, waste products, and the intestinal tract of animals and humans [20].

The baking of sourdough breads is one of the oldest biotechnological processes, and despite the traditionalism, it has a great potential because of its benefits. Sourdough is a mixture of wheat or rye flour and water that is fermented with LAB and yeast [7]. Sourdough fermentation contributes to the characteristic flavour of bread, improves bread texture, and delays staling and microbial spoilage of bread [8]. It can be pre-pared in bakeries through spontaneous fermentation with natural microbial flora or by use of special starter cultures. Using commercial starters is beneficial in the easier controlled process and the stable bread quality. The spontaneous fermentation makes production unsteady due to the variations of natural microbial flora that depends on raw material and process conditions.

The main aim of this study was to examine the biodiversity of LAB microbial flora and to purify and identify the most predominant LAB that had been isolated from rye flour and sourdough made by spontaneous fermentation of rye dough. The isolates were selected for latter examination of their potential as LAB starter cultures on the basis of their occurrence in the natural population.

Materials and Methods

Rye flour ("Žitopromet" Zaječar, Serbia) with 11.7 % and 0.993 % water and ash content, respectively, was used in all tests.

Enumeration and isolation of LAB. The LAB were isolated from rye flour (RF) and sourdough (SD) after spontaneous fermentation (24 h, 30 °C) of dough. The dough was made by mixing 100 g of rye flour and 60 cm^3 of sterile tap water in aseptic conditions (prior to work the mixer dish and blades were moistened with ethanol and flamed). For isolation and enumeration of bacteria, 10 g of rye flour or dough were homogenized with 90 cm^3 of sterile 0.85 % saline. Serial dilutions were spread plated on MRS agar (Torlak, Belgrade, Serbia) and incubated under anaerobic conditions (48 h, 30 °C, GasPak).

After enumeration, colonies were isolated randomly from plates, transferred in MRS broth and, after incubation (48 h, 30 °C), triple purified by streaking on MRS agar, checked for morphology, Gram-staining and catalase test. All Gram-positive and catalase-negative cultures were transferred on MRS agar and maintained under glycerin in a refrigerator until further examination.

Physiological characterization. A set of 34 tests (including morphology, Gram-staining characteristic and catalase test) was used to classify and identify the isolates. The determinations of catalase activity, gas production, arginine and esculine hydrolysis, ability to grow at different temperatures (15 and 45 °C) and at different concentrations of NaCl (4, 6.5 and 8 %) were performed by previously described methods [16, 19]. Other tests were also done: observing growth on Rocheux (entero) and citrate agar (HIMEDIA, Beograd, Serbia), and ability to form diacetyl [1] and exopolysaccharides - EPS (formation of slimy colonies on MRS agar with sucrose as the carbon source, 20 g/dm^3).

Acid production from carbohydrates (L-arabinose, D-xylose, galactose, mannitol, trehalose, mannose, raffinose, lactose, maltose, sucrose, glucose, fructose, rhamnose, sorbose, ribose, salicin, cellobiose, melibiose, sorbitol, Sigma) was evaluated by the following procedure: filter sterilized solution of sugar was added to basal MRS medium (with 0.16 g/dm^3 bromcresol-purple and without glucose) to a final concentration of 10 g/dm^3. One cm^3 of cell suspension (obtained by centrifugation of 5 cm^3 of 16-h old MRS broth culture and resuspending the sediment in 5 cm^3 of sterile saline) was used to inoculate 9 cm^3 of MRS basal medium with sugars. The occurrence of yellow colour in the medium after incubation (48 h at 30 °C) was considered as a positive result.

The strains were identified by comparing results with those previously published [2, 5, 9, 10, 12, 13, 21].

Determination of TTA and pH. The total titratable acidity (TTA) and pH of sourdough were determined in an aliquot of 10 g sourdough blended with 90 cm^3 distilled water. pH was determined in this suspension using HANNA HI 9025 meter. For TTA determination, the same aliquot was titrated against 0.1 M NaOH to final pH 8.5. TTA was expressed as the amount (cm^3) of NaOH used.

Statistical analysis. The relationships among the isolated strains were determined by hierarchical cluster analysis. The results of the phenotypic tests were coded as positive (+), negative (-), and weak positive or delayed (positive after 7 days of incubation) reaction (-,+). Two variables were used for the morphology: C/R (- cocci, + rods) and S/C (+ single cells, - chains, tetrads). Clustering was carried out in Statistica 5.0 for Windows (StatSoft Inc. USA) using the algorithm "Unweighed Pair-Group Average Linkage Analysis". The distances between clusters were assessed using "Percent of disagreement" and its translation in similarity level assuming that 0 % disagreement = 100 % similarity.

Results

The cell number of anaerobic or facultative anaerobic microorganisms increased during spontaneous sourdough fermentation (24 h, 30 °C) from the start number of 2.9×10^4 CFU/g to the final 1.0×10^8 CFU/g (Table 1). Since the dough was prepared with sterile tap water in aseptic conditions, it could be assumed that all microorganisms at the beginning of the spontaneous fermentation originated from the RF. That assumption was confirmed by the similar total number of microorganisms in the RF and in the dough made of it (Table 1).

Totally 29 of the 30 isolates from the RF and the 40 isolates from the SD were discarded as catalase-positive and Gram-negative bacteria. A final number of 41 Gram-positive, non-sporeforming, non-motile, catalase-negative, facultative anaerobic strains (10 from the flour and

31 from the dough) were available for identification by using a set of 31 phenotypic tests. Based on morphological, cultural, physiological and biochemical characteristics, the isolates from RF and SD were divided into 3 and 8 groups, respectively (Table 2). The isolates belonging to a given group were identified on the basis of species literature descriptions [2, 5, 9, 10, 12, 13, 21]. The profiles within the groups were not identical but very similar.

Table 1. Total number of microorganisms, number of isolated colonies and number of LAB (Gram-positive and catalase-negative bacteria), pH and TTA in rye sourdough at the beginning and after 24 h spontaneous fermentation at 30 °C.

Parameters	Rye flour	Sourdough	
		Start	After 24 h
Total number microorganisms, CFU/g	3.9×10^4	2.9×10^4	1.0×10^8
Number of isolated colonies	30		40
Number of Gram-negative or catalase-positive bacteria	20		9
LAB	10		31
Streptococcus sp.	3		2
Enterococus sp.	7		8
Leuconostoc sp.	-		4
Weisella sp.	-		2
Pediococcus sp.	-		5
Lactobacillus sp.	-		10
pH		6.5	5.5
TTA, cm^3 NaOH		3.3	6.7

Table 2. Differentiation of rye flour (RF) and sourdough (SD) LAB isolates based on morphological and physiological characteristics.

Characteristics	Groups										
	Rye flour			Sourdough							
	RF1	RF2	RF3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8
Number of LAB	3	3	4	2	2	2	5	8	2	8	2
Morphology	C	CC	C	C	CC	CC	TC	C	C	B	B
CO_2 from glucose	-	-	-	+	+	+	-	-	-	-	-
Growth at											
45 °C	-	+	+	-	-	-	+	+	-	-	+
15 °C	+	+	+	+	+	+	+	+	+	+	-
Arginine hydrolysis	-	-	+	-	-	-	+	+	-	-	-
Esculin hydrolysis	-,+	+	+	+	+	+	+	+	+*	-	-
Growth with											
4 % NaCl	+	+	+	+	+	+	+	+	+	+	+
6.5 % NaCl	-,+*	+	+	-	-	-	+	+	+	+	-
8 % NaCl	-	-	-	-	-	-	+	-	-	+*	-
Entero agar	-	+	+	-	-	-	+	+	-	-	-
Citrate agar	-	+	-	-	-	-	+	-*	-	-*	-
Diacetile	-*	+	+	-	-	-	+*	+*	-	w*	-
EPS production	-	-	-	+	+	-	-	-	-	-	-

Table 2. Continued.

Characteristics	Groups										
	Rye flour			Sourdough							
	RF1	RF2	RF3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8
Number of LAB	3	3	4	2	2	2	5	8	2	8	2
Acid from											
L-arabinose	-	-	-	-	+	-	+	w*	-	-*	+
D-xilose	-	-	-	w	-	-	w	-	-	-	-
galactose	-,+	+	-,+	+*	w	w	+	+	-	+	+
mannitol	+	+	+	+*	w	w	w*	+*	+	-	-
trehalose	+*	+	+	+	+	+	+	+	+	-*	+
mannose	+	+	+	+	+*	+	+	+	+	+	+*
rafinose	-	-	-	+	-,+	-	w	+*	-	-	+
lactose	-,+	+	+	-	w	+	+	+*	+	-	+
maltose	+	w	+	+	+	+	+	+	+	+	+
sucrose	-,+*	-	+	+	+	+	w*	+	-	+	+
glucose	+	+	+	+	+	+	+	+	+	+	w
fructose	+	+	+	+	+	+	+	+	+	+	+
rhamnose	-	w	-,+	-	-	-	-	-	-	-	-
sorbose	-	-	-	-	-	-	-	-	-	-	-
ribose	-	-	w	-,+	-*	w	w	-*	-	+*	-
salicine	-	+	+	-	+	w*	+	+	-	-	-*
celobiose	+	+	+	-	+	-	+	+	+	-	w
melibiose	w	-	-,+	+	+	+	w*	+	-	-	w
sorbitol	-,+	-	-	-	-	-	w*	-	-	-	-

Legend: cocci (C); coccoids (CC); cocci as tetrads (TC); rods (B); positive (+); negative (-); weakly positive (w); delayed reaction - positive reaction after 7 days (-,+);
* properties differ among strains of the same group.

All LAB isolates from the RF1 were homofermentative cocci incapable to grow at 45 °C (Table 2). They were related to the genus *Streptococcus*, species *S. alactolyticus,* because this bacterium was the only one among the streptococci that could grow in 6.5 % NaCl and showed a high similarity profile in the rest of the physiological properties (Table 2) with the species description [10].

The isolates from groups RF2 and RF3 belonged to the genus *Enterococcus* according to their ability to hydrolyze esculine and to grow at 15 and 45 °C, and in 6.5 % NaCl [5, 10, 12, 13]. The isolates of group RF3 could be identified as *E. faecium* by comparing with the species description [5, 10, 12, 13]. The identification of enterococci group RF2 was not possible because they could not be related to any known species description.

Homo- and heterofermentative cocci, as well as homofermentative bacilli, were isolated from sourdough (Tables 1 and 2). The heterofermentative cocci were identified as leuconostocs and weisellas according to their disability to grow at 45 °C and to hydrolyze arginin [2, 10]. The exopolysaccharide forming isolates of groups SD1 and SD2 belonged to *Leuconostoc mesenteroides*. Because of differences in the fermentation profile, the isolates SD1 were *L. mesenteroides* subsp. *dextranicum* (no acid production from L-arabinose, salicine and cellobiose), while the strains SD2 were *L. mesenteroides* subsp. *mesenteroides* (acid production from L-arabinose, salicine and cellobiose). The isolates of group SD3 that could not form exopolisaccharides (Table 2) were classified as *Weisella paramesenteroides* by comparing their properties with the species description [2].

A group of five homofermentative cocci (group SD4) were identified as *Pediococcus pentosaceus* with regard to their morphological (cocci in tetrads) and phenotypic (growth on 45 °C and 4 and 6.5 % NaCl, arginin hydrolysis, acid from maltose and trehalose) properties [10, 21].

The only LAB found in rye flour as well in sourdough were streptococci and enterococci. The strains of group SD6 were closely related with the isolates RF1 and were identified as *Streptococus* sp. (Table 2). The sourdough isolates SD5 showed similar results of the applied tests with the isolates of groups RF2 and RF3 and were presumed to be enterococci.

Two different groups of homofermentative lactobacilli (groups SD7 and SD8) were isolated from sourdough. The isolated lactobacilli showed incapability to form extracellular polysaccharides and they could not hydrolyze either arginin or esculin. Also, no growth on entero-agar was observed. The bacilli of group SD7 were mesophilic, while those in the SD8 group grew at 45 °C and were thermophilic (Table 2). The isolates of group SD7 were able to grow in 6.5 % NaCl, while two strains of group SD8 could grow only in 4 % NaCl. It was not possible to identify the lactobacilli isolates to a species level according to the results of the applied tests following the classical identification systems [9, 10].

To show the relationships among the LAB isolates, a dendrogram was produced applying hierarchical cluster analysis (Fig. 1). The cluster analysis confirmed the proposed grouping of isolates (Table 2) resulting in 11 different LAB groups at the 80 % similarity level.

The streptococci from RF and SD were not identical and were divided in two groups at similarity level of ca. 70 %. The differences resulted from the changed profiles of sugar fermentation (Table 2).

A similarity level of ca. 72 % was observed among leuconostocs and weisellas. They occurred in 3 clusters with 88 % (*L. mesenteroides* subsp. *dextranicum*), 82 % (*L. mesenteroides* subsp. *mesenteroides*) and 88 % (*W. paramesenteroides*) correlation. The leuconostocs and weisellas showed to be the most related to the streptococci (62 % similarity) and the lactobacilli (56 % similarity). The lactobacilli were divided in 2 clusters with intercluster correlation of ca. 62 %.

The clusters RF2, RF3, and SD5 were comprised of enterococci with intragenera correlation of ca. 72 %. The enterococci of group RF2, as well as those from group RF3 (*E. faecium*), showed 100 % intraspecies similarity as a consequence of the identical results of the applied tests. The analysis showed that the enterococci were best related with *P. pentosaceus* (65 % similarity level) included in the cluster SD4 at 84 % intraspecies similarity level (Fig. 1).

Discussion

The LAB were not the predominant microorganisms in rye flour making one-third of the entire bacterial population (Fig. 2), but their acid resistance enabled them to continue growing during the natural sourdough fermentation. Since LAB produce lactic acid and/or acetic acid, pH of sourdough decreased from 6.5 to 5.5 (Table 1). Rapid LAB growth and acidification (TTA increased from 3.3 to 6.7, see Table 1) were the basic aims of sourdough fermentation leading to the suppression of non-beneficial microbes. So, LAB from a minority in the rye flour (33 %) became the majority (78 %) in the 3.500 times greater bacterial population of the sourdough (Fig. 2). The number of bacteria found in the sourdough (10^8 CFU/g) was in accordance with previously published counts of LAB on MRS in sourdoughs, which ranged between 10^7 and10^8 [16, 17]

The anaerobe and/or facultative anaerobe isolates from rye flour and sourdough belonged to well-recognized LAB genera and some of them were related to particular species. The characterized RF strains were assigned to the genera *Streptococcus* and *Enterococcus,* comprising 30 % and 70 % in the counts of LAB, respectively (Fig. 2). The genera found in the RF were found also in the SD, but as a lower percent from the total LAB population. The participation of enterococci in the LAB count was significantly reduced from 70 % in RF to 26 % in the SD. Moreover, the SD fermentation reduced five times the contribution of streptococci to the LAB microflora (Fig. 2). The growth of other LAB that could not be detected in rye flour probably diminished the role of enterococci and lactococci in the SD. The natural habitat of the streptococci is vertebrates, but *Streptococcus* species have been previously isolated from sourdoughs [18]. Some enterococci were reported as common inhabitants of vegetables [13] and were isolated from rye flour (*E. faecium, E. avium, E. casseliflavus* and *E. durans* - [18]) and sourdoughs [16, 18]. However, the presence in the cited cases of *E. faecium* suggests that the enterococci have originated from soil or water.

A greater LAB diversity was observed in the SD because four more genera were detected: *Leuconostoc*, *Weisella*, *Pediococcus* and *Lactobacillus*. Leuconostocs and weisellas were the only heterofermentative LAB isolated

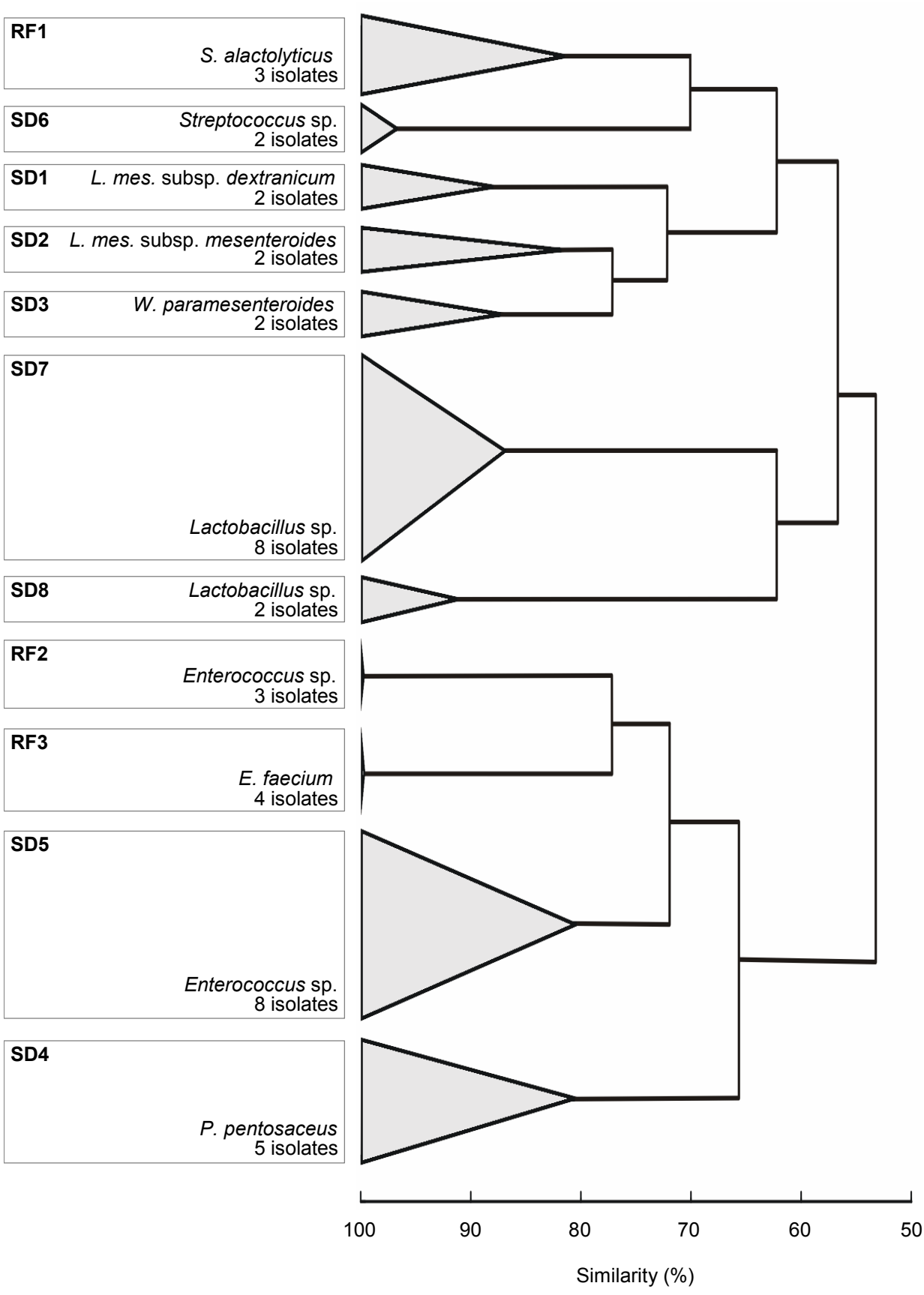

Fig. 1. Dendrogram showing the similarities based on phenotypic tests of 41 isolates of LAB from rye flour (RF) and sourdough (SD).

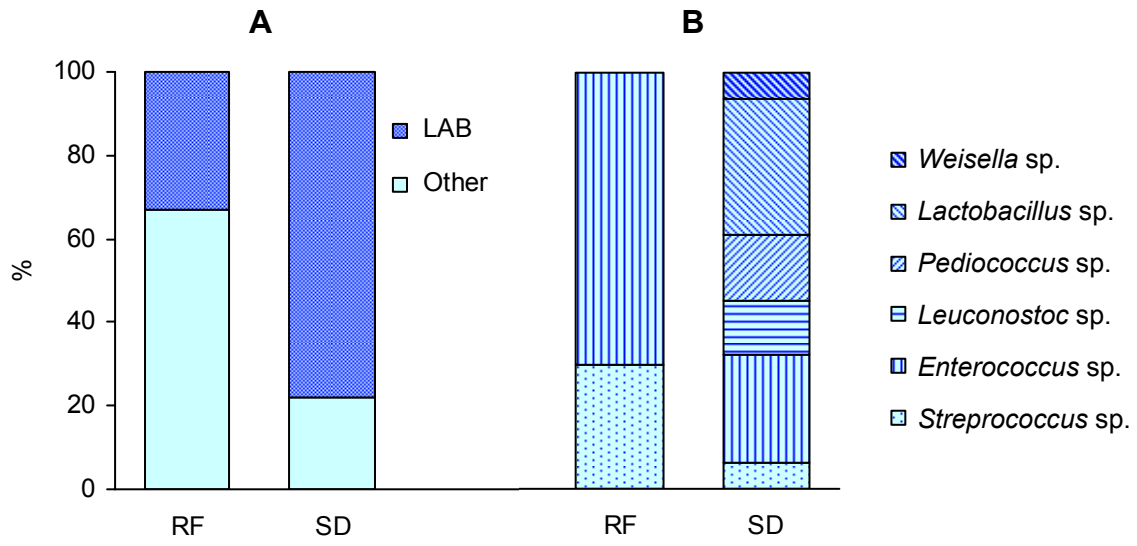

Fig. 2. Participation of LAB in the entire microflora (A) and particular genera in the lactic acid bacterial population (B) in rye flour (RF) and sourdough (SD).

from SD. Strains of those LAB genera were frequently isolated from sourdoughs [4, 7, 16, 18], and some leuconostocs [6, 11, 15] and *W. pseudomesenteroides* [15] were used for sourdough preparation as starters, because their role in sourdough processes was significant. Pediococci participated in the sourdough microflora with 16 % (Figure 2), and this percentage was significantly higher than the previously published 5 % [16].

Lactobacilli comprised the greatest group of LAB in the SD, totaling up one-third of the LAB community. This confirmed the already published records that rye and wheat sourdoughs microbial populations were dominated mainly by bacteria of the genus *Lactobacillus* [4, 16, 17]. The identification of lactobacilli strains was not possible because they did not confirm to any species description. A high percent of disagreement was accounted between our isolates and the homofermentative lactobacilli commonly isolated from sourdoughs: *L. amilovorus* [17], *L. plantarum* [4, 16, 17] and *L. acidophylus* [4, 17]. This could be explained by difficulties in standardizing the test protocols, or it could be due to the genetic heterogeneity of these species [16]. Contrary to previous studies [4, 16, 17], which have reported isolation of heterofermentative lactobacilli from SD, this group of LAB were not detected in this study. The fact that only MRS was used as an isolation medium may have introduced a bias. To confirm the proposed identification and to classify unidentified strains of LAB, molecular genetic techniques, such as 16S rRNA analysis, PCR, amplification of DNA sequences, should be applied.

Statistical procedures based on phenotypic properties have been commonly used for analysis of sourdough microbial communities [14, 16]. The statistical analyses in this study provided numerical indices to evaluate the distances among the population members and their diversity.

Conclusion

Sourdough fermentation begins with aerobic growth immediately upon mixing flour and water. Once oxygen is depleted, anaerobic fermentation begins with the growth of LAB. LAB produce acids which enhance their rapid growth when the pH value has dropped too low for other microorganisms to develop. So, the LAB become the most abundant microorganisms in the sourdough and they are therefore responsible for the final stages of the sourdough processing.

Most of the taxa to which the obtained 41 isolates belong had already been detected in rye flours and sourdoughs, although the profiles of the isolates were unique in this study. The results of this study also provide a basis to select LAB starter cultures for the production of sourdough breads.

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ПРОФИЛ НА МЛЕЧНОКИСЕЛИ БАКТЕРИИ В РЪЖЕНО БРАШНО И ТЕСТЕН КВАС

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Резюме

За да се проучи съставът на млечнокиселите бактерии в ръженото брашно и направеното от него тесто, са изолирани и изследвани 41 щама. Фенотипното характеризиране на изолатите, включващо 34 теста, позволява идентифицирането на 11 кластера при 80 % сходство на базата на йерархичен кластерен анализ. Всички изолати млечнокисели бактерии от ръженото брашно са хомоферментативни коки от родовете Streptococcus (30 %) и Enterococcus (70 %). От тестения квас са изолирани хомоферментативни (Streptococcus sp. – 6 %, Enterococcus sp. – 26 % и Pediococcus pentosaceus – 16 %) и хетероферментативни (Leuconostoc mesenteroides – 13 % и Weisella paramesenteroides – 7 %) коки, както и хомоферментативни бацилии (Lactobacillus sp. – 32 %).

Млечнокиселите бактерии не са преобладаващите микроорганизми в ръженото брашно (те са една трета от цялата бактериална популация), но тяхната киселиноустойчивост им позволява да продължат да се размножават по време на естествената ферментация на тестения квас и да станат основната бактериална популация в него.