

## Potential of *Lactobacillus reuteri* from Spontaneous Sourdough as a Starter Additive for Improving Quality Parameters of Bread

Dovilė Jonkuvienė\*, Lina Vaičiulytė-Funk, Joana Šalomskienė,  
Gitana Alenčikienė and Aldona Mieželiene

Kaunas University of Technology, Food Institute, Taikos av. 92, LT-51180 Kaunas, Lithuania

Received: February 23, 2015

Accepted: April 25, 2016

### Summary

Retardation of microbial spoilage of bread can be achieved by the use of spontaneous sourdough with an antimicrobial activity. This study was undertaken to identify lactic acid bacteria naturally occurring in spontaneous sourdough and use them for quality improvement and prolonging shelf life of rye, wheat and rye with wheat bread. Identification of isolates from spontaneous sourdough by pyrosequencing assay showed that *Lactobacillus reuteri* were dominant lactic acid bacteria. The isolates showed a wide range of antimicrobial activity and displayed a synergistic activity against other lactobacilli, some lactococci and foodborne yeasts. The best application of spontaneous sourdough was noticed in the rye bread with the lowest crumb firmness of the final product, although the sensory results of wheat and rye with wheat bread did not statistically differ from control bread. *L. reuteri* showed a high preserving capacity against fungi during storage. This may be due to bacteriocins and various fatty acids secreted into the growth medium that were identified by agar well diffusion assay and gas chromatography. *L. reuteri* showing high antimicrobial activity have the potential to be used as a starter additive that could improve safety and/or shelf life of bread.

*Key words:* spontaneous sourdough, *Lactobacillus reuteri*, antimicrobial activity, bread, protective properties, food safety

### Introduction

Fermented bakery products differ from region to region. The production of the majority of these products is based on traditional methods and their uniqueness depends on the raw materials used for sourdough fermentation (1). Sourdough used in bread making plays an important role in improving the texture and flavour of the product through metabolically active yeasts and lactic acid bacteria (LAB). The LAB developing in sourdough may originate from the raw materials (2).

LAB are the biological basis for the production of a multitude of fermented foods. During the fermentation

their metabolic activity determines the food quality. Furthermore, LAB strains may produce a wide range of antimicrobial metabolites (3) that have a potential to inhibit food pathogens (4). Thus, the strains producing antimicrobial substances can be used as biopreservatives, extending the shelf life and enhancing food safety (5–9).

In the bread industry, antimicrobial properties of LAB are particularly important in ensuring the stability of the sourdough. A microbially stable sourdough of good consistency has a significant impact on bread quality. Another important reason for the use of antimicrobially active sourdough is the potential to suppress bread defects such as ropiness or bread moulding (10). The sourdough

\*Corresponding author: Phone/Fax: 370 37 312 393; E-mail: dovile.jonkuviene@yahoo.com

fermentation applied for the production of rye and wheat bread prolongs the freshness of the bread and enhances its microbial safety. Physical, chemical and biotechnological means are applied for bread antistaling and microbial spoilage (11–13). Preservatives of chemical origin are not natural tools, but their usage in bread increases its microbiological safety and extends its shelf life. Recently, physical methods, *i.e.* various sterilisation methods, are used to protect bread from mould spoilage on its surface (14,15). Antimicrobial packaging of bread has also become popular. Additives from the packages diffuse slowly into the environment between the inner package layer and the bread during storage. This mechanism is effective in protecting the bread from mould spoilage or other defects of microbial origin (16). Antimicrobial agents that migrate from the packaging material to the food product prevent fungal outgrowth and extend the shelf life of the product, compared to conventional packaging (17). However, the physical and chemical tools for retarding bread staling are not natural and may have a negative impact on consumer health (18).

In contrast, *Lactobacillus* is a natural and effective tool for retarding bread staling and microbial spoilage. It is characterized by antimicrobial activity against microscopic fungi and spore-forming bacteria. This would appeal to health-conscious consumers looking for natural foods without chemical preservatives. This study was undertaken to identify LAB cultures from spontaneous sourdough and use them to improve the quality and prolong the shelf life of bread.

## Materials and Methods

### Composition and preparation of spontaneous sourdough

The spontaneous sourdough fermentation was done by mixing sifted rye flour type 1370 according to LST 1481:2004 (19) and tap water in a ratio of 1:1.5. The mixture was incubated at 27 °C under anaerobic conditions for 24 h by stirring periodically. After incubation, 10 % of ripe sourdough was used to inoculate a fresh flour and water mixture under the same conditions as described above. The re-inoculation procedure was carried out until moisture content reached 55 %. The obtained spontaneous sourdough was used for the isolation of naturally occurring lactic acid bacteria (LAB).

### Isolation and identification of LAB

To isolate LAB, 10 g of spontaneous sourdough was homogenised in 90 mL of sterile physiological solution and then suitable serial dilutions were plated on MRS agar (Biolife, Milano, Italy) and incubated at 30 and 37 °C (according to the optimal growth temperature of typical LAB species) under aerobic and anaerobic conditions. LAB colonies were selected according to their appearance, and the microscopic preparations of purified isolates were observed by optical microscope Laboval 4 (Carl Zeiss Jena GmbH, Jena, Germany) using 1000× magnification.

After culturing in MRS broth (Biolife) at 30 °C for 18 h, the isolates were centrifuged at 900×g for 15 min and

bacterial pellets were used for automated DNA extraction with QIAcube using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR reaction and pyrosequencing were performed following the manufacturer's instructions using a 3B Blacklight sepsis bacterial (3B Blackbio Biotech, Madrid, Spain) kit. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 20 s, at 54 °C for 20 s and at 72 °C for 30 s, with a final extension step at 72 °C for 5 min. PCR products were stored at –20 °C until use. Pyrosequencing was performed on the PyroMark Q24 automatic pyrosequencing instrument (Qiagen) using a Gold24 (Qiagen) kit with dATP $\alpha$ S, dCTP, dGTP, dTTP, enzyme mixture and substrate mixture. Nucleotide sequence analysis was performed using the PyroMark Q24 (Qiagen) software and the National Center for Biotechnology Information Basic Local Alignment Search Tool (20).

### Determination of antimicrobial activity

The pure LAB isolates inoculated into MRS broth were incubated for 72 h at 37 °C under anaerobic conditions and centrifuged at 6000×g for 15 min. The obtained cell-free supernatant was used for investigations.

The antimicrobial activity of LAB isolated from spontaneous sourdough was evaluated against the reference strains of *Bacillus subtilis* ssp. *spizizenii* ATCC 6633, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19111, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (Microbiologics Inc, St. Cloud, MN, USA). The antifungal activity was tested against the foodborne yeast *Candida parapsilosis* JS-S1, *Debaryomyces hansenii* JS-J1, *Kluyveromyces marxianus* JS-V1, *Pichia guilliermondii* JS-S2, *Yarrowia lipolytica* JS-S3 and micromycetes *Aspergillus brasiliensis* Mi-G-21, *Aspergillus versicolor* Mi-Pr-4, *Cladosporium herbarum* SR-11, *Penicillium chrysogenum* SR-12 and *Scopulariopsis brevicaulis* Mi-Gr-5 obtained from the collection of Institute of Botany, Nature Research Centre, Vilnius, Lithuania. We also evaluated antimicrobial activity of LAB isolates from spontaneous sourdough against *Lactococcus lactis* ssp. *lactis* 140/2 and 142/21, *Lactobacillus casei* 305 and 12, *Lactobacillus brevis* 43, *Lactobacillus acidophilus* L59-30 and L41-2B-2v, *Lactobacillus helveticus* 19 and 13, *Lactobacillus delbrueckii* ssp. *bulgaricus* R and 148/3 strains obtained from the collection of Food Institute.

The antimicrobial activity of LAB isolates from spontaneous sourdough was tested by agar well diffusion assay. The reference bacterial cultures were pre-cultivated on plate count agar (PCA; Liofilchem, Roseto degli Abruzzi, Italy) slants for 24 h at 30 or 37 °C and target LAB strains were pre-cultivated on MRS agar slants for 24 h at 30 or 37 °C. The cultures from the slants were transferred into 10 mL of 0.9 % NaCl solution to obtain the inoculum density equivalent to a McFarland standard no. 0.5. Adjusted suspension (1 mL) was transferred to 100 mL of PCA or MRS agar dissolved and cooled to 45 °C. Isolated micromycetes and yeast cultures were cultivated for 24–48 h at 25 °C, respectively on the malt extract agar (MEA; Liofilchem) and Sabouraud dextrose agar (SDA; Liofilchem) slants. An inoculum of 10<sup>6</sup> CFU/mL in 0.9 % NaCl solution was prepared. After that, 1 mL of the target culture was added to 100 mL of the melted MEA or SDA

cooled to 45 °C. The prepared mixture (10 mL) was poured into Petri dishes. A control solution (MRS broth), cell-free supernatant, neutralised cell-free supernatant and neutralised cell-free supernatant treated with enzymes (50 µL) were added to 7.5 mm-diameter wells made in the solidified agar. Antibacterial activity was assessed after 24 h of incubation at 30 or 37 °C, while antifungal activity was determined after 48 h of incubation at 25 °C. Antimicrobial activity was determined according to the diameter of inhibition zones surrounding the wells after incubation.

### Bread-making technology

The effectiveness of experimental sourdough containing *Lactobacillus reuteri* used for rye, wheat, and rye with wheat bread making was evaluated. *Lactococcus reuteri* for preparation of sourdough for the experimental bread and *Lactococcus lactis* ssp. *lactis* 140/2 for preparation of sourdough for the control bread were cultivated in 100 mL of MRS broth at 30 °C for 24 h. The MRS broth was then centrifuged at 5000×g for 5 min, washed twice in 0.9 % NaCl solution and the whole precipitate, approx. 10 g (10<sup>6</sup> CFU/g), was added into the first mixture of flour and water. The sourdough was prepared as described in the first paragraph of the Materials and Methods section. Bread was made without adding any bread quality-enhancing additives (Table 1). Both the experimental and control bread dough were prepared by mixing flour with water (95–98 °C) and leaving it to rest for 1.5–2.0 h. Then the experimental and control sourdough (containing respectively *L. reuteri* and *L. lactis* ssp. *lactis* 140/2) were added to the mixture and further fermentation was carried out for 12 h at 27 °C until total titratable acidity of the obtained mixture reached 11°. After fermentation, the remaining components were mixed and homogenised for 15 min in a spiral mixer SP-100A/NH-B (Metos, Kerava, Finland) and the dough was placed into the incubator for 1.5 h at 35 °C. Dough loaves (500 g) were baked for 20–25 min at 220 °C.

Table 1. Composition of experimental bread

Components	Type of bread		
	Rye	Wheat	Rye with wheat
	m(component)/g		
Sifted rye flour, type 700	175.0	–	125.0
Sifted rye flour, type 1370	75.0	75.0	75.0
Wheat flour from grains of <i>Triticum spelta</i> sp.	–	100.0	–
Wheat flour, type 812D t	100.0	175.0	150.0
Fermented rye malt	25.0	25.0	25.0
Sourdough	20.0	20.0	20.0
Plant oil	15.0	15.0	15.0
Sugar	40.0	40.0	40.0
Compressed yeast	15.0	15.0	15.0
Salt	5.0	5.0	5.0
Cumin	7.5	7.5	7.5
Water	200.0	200.0	200.0

After baking, the bread loaves were cooled at room temperature for 2 h and subsequently sealed in polyethylene bags.

### Bread analysis

The quality and sensory evaluation of control and experimental bread was performed 12 h after baking and during 7-day storage at room temperature. It was assumed that the bread was suitable for consumption for 7 days. The moisture content of bread crumbs was determined by drying the samples at 105 °C to the constant mass. Acidity was measured by the titration method, and porosity was determined according to method LST 1442:1996 (21).

Crumb firmness was measured using a universal testing machine Instron 3343 (Instron Engineering Group, High Wycombe, UK). Samples for analysis (50 mm×40 mm×26 mm) were prepared and slices of 13 mm thickness were compressed to 40 % of their original height at a cross-head speed of 1 mm/s (22).

Colour measurements of bread were carried out using a CR-400 Chroma Meter colourimeter (Konica Minolta Sensing Inc., Warrington, UK). The obtained results were expressed as colourspace according to the CIELAB scale.

Sensory properties of baked bread samples were measured using a quantitative descriptive analysis by creating sensory profiles for each bread sample. Assessors were seven panellists between 20 and 60 years old. All training and data collection sessions were held in the sensory analysis laboratory of Food Institute. Bread samples were kept in the constant climate chambers (Binder, Tuttingen, Germany) before testing. They were presented to the panel at 21 °C in closed plastic boxes coded with three digit numbers. For the development of sensory profiles, a fully balanced randomised sample presentation plan with two repetitions was applied. A set of six bread samples (three samples of control bread (rye, wheat and rye with wheat) prepared with *Lactococcus lactis* ssp. *lactis* 140/2 and three samples of experimental bread (rye, wheat and rye with wheat) prepared with *Lactobacillus reuteri*) was presented to all participants in duplicate. The participants' responses were recorded and collected using a computerised data system FIZZ (Biosystemes, Couternon, France). The 15 points of numbered scales with indented anchors (left anchor 'low intensity/absent', right anchor 'high intensity') were used to evaluate each sensory attribute. Evaluated attributes included odour of bread starter, richness of taste, and intensity of non-typical odour and taste.

### Determination of protective properties of spontaneous sourdough

The protective properties of spontaneous sourdough containing *Lactobacillus reuteri* were evaluated according to the microbiological parameters of a bread crumb after storage at room temperature for 7 days. The number of moulds was determined in Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Liofilchem) (23). The number of spores of mesophilic bacteria was determined by heat-

ing the dilutions for 10 min at 80 °C before plating on PCA (24). The total bacterial count was determined on PCA according to standard method (25).

*L. reuteri* isolated from spontaneous sourdough were tested for antimould activity by spraying the cell-free supernatant on the surface of bread obtained in the local supermarket. The two control variants were the bread loaves sprayed and not sprayed with MRS broth (in order to determine the effect of MRS broth ingredients on the antimould activity). The bread loaves were stored for 8 days at 24–25 °C and 15–16 °C in polyethylene bags and the rind of loaves was examined externally for the presence of moulds according to the scale: – no moulds, + small colonies, and ++ large colonies (26). The LAB count in MRS broth for neutralised cell-free supernatant preparation was determined using a UV-Vis spectrophotometer Spectronic Genesys 5 (Thermo Fisher Scientific, Madison, WI, USA) at a wavelength of  $\lambda=600$  nm and converted to a cell count (27).

#### *Analysis of components produced by Lactobacillus reuteri*

Neutralised cell-free supernatants were obtained by adjusting the pH to 6.5 using 1 mol/L of KOH. To confirm the production of a proteinaceous compound produced by *L. reuteri*, neutralised cell-free supernatants were treated separately with catalase (Sigma-Aldrich, St. Louis, MO, USA) and proteinase K (Sigma-Aldrich) at a concentration of 20 mg/mL and incubated respectively at 25 and 56 °C for 30 min. Antimicrobial activity of treated neutralised cell-free supernatants was determined by the agar diffusion bioassay as described above.

Fatty acids secreted in the growth medium by *L. reuteri* were extracted from 3 mL of cell-free supernatant by mixing it with *n*-hexane and methylating with KOH, yielding methyl esters according to the standard method (28). Fatty acid methyl esters were analysed with a Shimadzu GC-2010 (Shimadzu, Kyoto, Japan) gas chromatograph using a 120-metre column BPX-70 (29). Chromatographic peaks were identified by comparison with retention times of a mixture of a Supelco 37 Component FAME Mix reagent kit (Supelco Analytical, Thermo Scientific, Bellefonte, PA, USA). Analytical conditions were as follows: column temperature was 60 °C for 2 min, then increased to 230 °C at 13 °C/min and maintained the same for 55 min; injector temperature was 250 °C and detector temperature was 270 °C; nitrogen was used as the carrier gas. The content of each relevant fatty acid was calculated as a percentage of total fatty acids in each sample.

#### *Statistical analysis*

Baking experiments were repeated three times, while mean values of bread parameters were estimated from three replicates of a single batch of bread. The comparison of microbiological tests was performed by a one-way ANOVA (Tukey's HSD) test using SPSS v. 16.0 software (30). Sensory evaluation was performed in duplicate while instrument measurements were done in triplicate. All data were subjected to *t*-tests to assess the significance of treatment mean values at the 5 % significance level.

## Results and Discussion

### *Dominant lactic acid bacteria in spontaneous sourdough*

Twenty LAB cultures were isolated from spontaneous sourdough. The number of LAB reached  $8.6 \cdot 10^7$  CFU/g. All isolates were rod-shaped. The sequences generated for the tested LAB isolated from spontaneous sourdough were the following: V1: CACTGGTGAT CCATCGTCAA TCAGGTGCAA GCACCATCAA TGA, and V2: GTGACTTTCT GCTTGGATAC CGTCACTGGC TGAACA and V3: GTCATTGCGT CCCC GAAGGG AACGCTTAT CTC-TAAGGTTA. This enabled all tested LAB isolates to be identified as the same species among *Lactobacillus* spp. The obtained sequences clearly corresponded to *L. reuteri* when compared to GenBank sequences (KC700337.1 or KC561127.1).

*L. reuteri* was one of the dominant LAB species isolated from spontaneous sourdough. Molecular DNA-based methods like DNA sequencing and fingerprinting became the essential complement for identification of yeasts and LAB. Compared to phenotyping methods, molecular DNA-based identification methods offer a much higher taxonomic resolution at species up to strain level (31).

Several species belonging to the genera *Leuconostoc*, *Weissella*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Streptococcus* have been isolated from sourdough, while *Lactobacillus* strains were the most abundant (32). Yeasts such as *Saccharomyces exiguus*, *Torulopsis holmii*, *Candida krusei*, *Pichia norvegensis* and *Hansenula anomala*, are also present in sourdough, but *Saccharomyces cerevisiae* is frequently present or added (33). Only *Lactobacillus reuteri* isolated from spontaneous sourdough was considered to evaluate the antimicrobial properties, while yeasts were not taken into account in this study.

### *The antimicrobial activity of Lactobacillus reuteri*

When it was determined that all LAB isolates from spontaneous sourdough were identified as one species of *L. reuteri*, three mixtures of *L. reuteri* isolates (all of them were made from five randomly selected isolates) were used for analysis of antimicrobial activity. *L. reuteri* was characterised as active against the tested bacterial strains usually found in foods and foodborne micromycetes that are most commonly detected as spoilers of milk and bread products (Table 2).

MRS broth contains some substances that in combination with microbial acidifiers may exert antimicrobial activity. The control solution did not show antimicrobial activity against the tested microorganisms, therefore the observed effects of cell-free supernatant were attributed to *L. reuteri* metabolites.

Gram-negative *Escherichia coli* ATCC 25922 was the most resistant to the antimicrobial activity of *L. reuteri* and the resulting inhibition zones were statistically smallest ( $p \geq 0.05$ ) compared with the inhibition zones of the other tested bacterial strains. The inhibitory activity of neutralised cell-free supernatant after the addition of catalase indicates the possibility to produce bacteriocins that are antimicrobially active components of *L. reuteri*. However, the active components of *L. reuteri* in the neutralised supernatant affected and not affected by catalase inhibit-

Table 2. Antimicrobial and antifungal activity of *Lactobacillus reuteri*

Target microorganism	<i>d</i> (inhibition)/mm				
	Cells in MRS broth	Cell-free supernatant	Cell-free neutralised supernatant	Cell-free neutralised supernatant affected by catalase	Cell-free neutralised supernatant affected by proteinase K
Reference bacterial strain					
<i>Bacillus subtilis</i> ssp. <i>spizizenii</i> ATCC 6633	(18.3±0.4) <sup>a</sup>	(18.3±0.6) <sup>a</sup>	0.0±0.0	0.0±0.0	0.0±0.0
<i>Bacillus cereus</i> ATCC 11778	(19.3±0.7) <sup>b</sup>	(22.3±2.5) <sup>c</sup>	0.0±0.0	0.0±0.0	0.0±0.0
<i>Listeria monocytogenes</i> ATCC 19111	(22.8±0.9) <sup>c</sup>	23.3±0.9	(10.5±1.6) <sup>e</sup>	17.5±0.5	0.0±0.0
<i>Escherichia coli</i> ATCC 25922	(14.3±0.9) <sup>d</sup>	15.1±0.2	0.0±0.0	0.0±0.0	0.0±0.0
<i>Staphylococcus aureus</i> ATCC 25923	(19.7±0.5) <sup>b</sup>	(19.7±0.9) <sup>b</sup>	0.0±0.0	0.0±0.0	0.0±0.0
Foodborne micromycetes					
<i>Cladosporium herbarum</i> SR-11	(19.0±1.0) <sup>b</sup>	18.0±1.0 <sup>a</sup>	(10.0±0.5) <sup>e</sup>	12.0±0.2	0.0±0.0
<i>Penicillium chrysogenum</i> SR-12	(16.4±0.2) <sup>f</sup>	(16.0±0.0) <sup>f</sup>	(13.8±0.2) <sup>d</sup>	(14.0±0.1) <sup>d</sup>	0.0±0.0
<i>Scopulariopsis brevicaulis</i> Mi-Gr-5	13.0±0.6	(14.0±1.0) <sup>d</sup>	(10.0±0.4) <sup>e</sup>	9.0±0.0	0.0±0.0
<i>Aspergillus brasiliensis</i> Mi-G-21	(18.0±0.2) <sup>a</sup>	16.2±0.6	(10.0±0.2) <sup>e</sup>	(14.0±0.4) <sup>d</sup>	0.0±0.0
<i>Aspergillus versicolor</i> Mi-Pr-4	(22.2±0.6) <sup>c</sup>	20.2±0.6	(18.2±0.4) <sup>a</sup>	15.0±0.5	0.0±0.0

Mean values with the same letter are not significantly different ( $p < 0.05$ )

ed only the reference culture of *Listeria monocytogenes*. These data strongly support the observation that *Lactobacillus reuteri* isolated from spontaneous sourdough produce bacteriocins and the inhibitory activity of these compounds depends on the type of bacteria subjected for inhibition. The loss of the antimicrobial activity after treatment with proteinase K indicates that antimicrobial substances produced by the tested LAB have a proteinaeous nature. They might be bacteriocins because their protease sensitivity is a key criterion in the characterisation of bacteriocin-like inhibitory substances (34).

*L. reuteri* was found to have a synergistic effect with the other tested *Lactobacillus* and *Lactococcus* spp. The synergistic effect of a dominant LAB culture present in sourdough is important because LAB strains with antimicrobial properties could be used for the production of starters that prevent bread defects and achieve the good taste properties.

According to the results of antifungal activity of *Lactobacillus reuteri* isolates, the cell-free supernatant was more active against *Cladosporium herbarum*, *Penicillium chrysogenum* and *Scopulariopsis brevicaulis* when compared with the activity of cell cultures of these strains. The same data that the supernatants of the tested LAB were more active against *Penicillium nordicum* than the cell culture were found by other researchers (35). The results suggest that biologically active substances in supernatants have a greater effect on the spore formation than on the growth of mycelium. It is known that *Lactobacillus plantarum* supernatants had a wide spectrum of activity against the *Eurotium*, *Penicillium*, *Aspergillus* and fungi of other genera (36).

*Lactobacillus reuteri* isolates did not show an antifungal activity against *Candida parapsilosis*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Pichia guilliermondii* and *Yarrowia lipolytica*. Some LAB produce fungicidal substances that act against *Candida* spp. (37,38). *K. marxianus* was

most sensitive to the *L. plantarum* effect of the examined yeasts (39). On the other hand, the synergistic effect of the investigated *L. reuteri* with yeasts is useful, because the sourdough LAB are mainly responsible for its acidification, whereas yeasts are very important for the production of flavour compounds and for a balanced flavour in combination with acids.

Although *L. reuteri* showed only antilisterial activity after neutralising the acidity of culture supernatant, they nevertheless could be attributed to LAB demonstrating superior antimicrobial activity against micromycetes and high inhibition by cell culture against bacterial strains. In order to determine *L. reuteri* as a starter culture that improves bread quality, its application was investigated in rye, wheat and rye with wheat bread making.

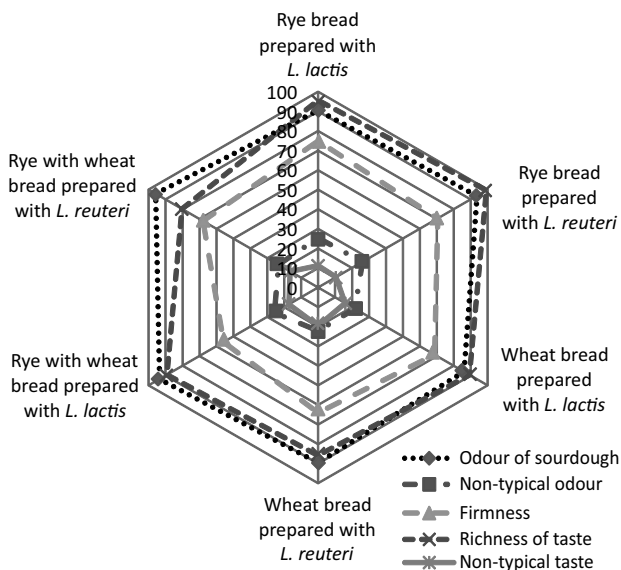
#### *The effect of Lactobacillus reuteri on bread quality*

The experimental bread technological parameters complied with the requirements of standard bread quality (40) and matched the quality of all types of bread. *Lactococcus lactis* ssp. *lactis* 140/2 was chosen for control bread making because this *Lactococcus* is suitable for all types of bread and it retains its specific composition after many generations during sourdough preparation.

According to the obtained colour measurement data (data not shown), it was found that the surfaces of all types of bread loaves were darker than bread slices, the crusts of fresh bread were less red-toned, while the crumbs were more yellow-toned. The colour characteristics of experimental and control bread samples did not change during storage for 7 days at 18 °C. The yeasts and LAB present in the dough reduced a certain amount of sugar, while during baking, the remaining sugar is involved in the Maillard reaction, in which aromatic substances are formed and give a desirable flavour to the final bread and a yellowish brown tone to the bread crust. This

shows that *L. reuteri* present in spontaneous sourdough are closely involved in the fermentation process and do not affect the colour characteristics of experimental bread (41).

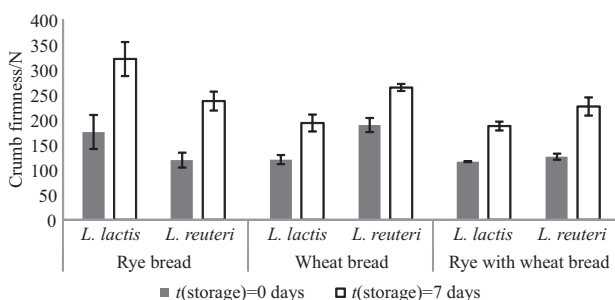
The results of the sensory analysis of the bread samples revealed that the overall quality of bread prepared using *L. reuteri* was not significantly different compared with control bread. Experimental bread samples were acceptable for all assessors (Fig. 1) and sensory attributes did not change significantly during the 7-day storage.



**Fig. 1.** Effect of bacterial starter (*Lactobacillus reuteri* vs. *Lactococcus lactis* ssp. *lactis* 140/2) and flour (rye, wheat, and rye with wheat) on fresh bread sensory characteristics

Although the results revealed the differences among the different types of bread, these findings are in agreement with the results of other researchers (42) who determined the effectiveness of using baking yeasts, kefir or *Lactobacillus casei* immobilized on brewer's spent grains in wheat bread production without any significant negative effect on bread taste or aroma.

The bread crumb textural profile of samples (Fig. 2) showed a significant effect of sourdough on bread crumb firmness compared with the control samples.



**Fig. 2.** Effect of bacterial starter (*Lactobacillus reuteri* vs. *Lactococcus lactis* ssp. *lactis* 140/2) and type of flour (rye, wheat, and rye with wheat) on experimental bread crumb firmness

Freshly baked experimental rye bread was softer than the control bread. An analogous trend was found after rye bread storage for 7 days. This suggested that the effect of sourdough on the bread firmness was statistically significant ( $p < 0.05$ ) and a lower crumb firmness of rye bread could be expected when using spontaneous sourdough for bread preparation. Experimental wheat bread and rye with wheat bread had a higher crumb firmness. Bread crumb firmness underwent changes during storage. The chemical structure of wheat and rye is quite similar, so only small differences could be observed in starch retrogradation of rye and wheat bread (43). The structure of A-type starch switches into hydrated crystalline form after bread baking and during storage, but fermented rye bread was less crystalline. The less intense increase of wheat bread crumb firmness than of rye bread demonstrated the dependence of bread firmness on the used flour, but not on the used sourdough composition.

There is some literature data about the use of *Lactobacillus reuteri* for bread making. It was noticed that the firmness of bread made with *L. reuteri* LTH5448 was higher when compared with bread made with *Weissella cibaria* 10M (44). Regardless of the possibility that bread consistency is affected by *L. reuteri*, certain LAB strains may be added as specific LAB cultures into sourdough to improve some parameters of the final bread. The use of a mixture of *Kluyveromyces marxianus* and *Lactobacillus plantarum* as a sourdough starter cultures provides the possibility to improve the specific volume of bread without decreasing its sensory quality or acceptability (45).

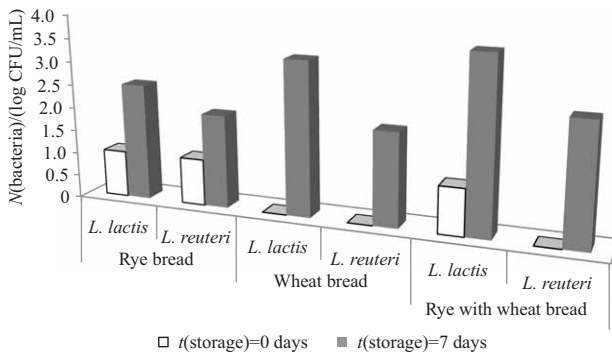
Results of the textural and sensory analysis of experimental rye, wheat and rye with wheat bread prepared with *L. reuteri* show a significant reduction of bread firmness compared to the control rye bread prepared with *L. lactis* ssp. *lactis* 140/2 as a starter culture suggesting its wide application for the rye bread making.

#### Protective properties of *Lactobacillus reuteri*

Following the determination of antimicrobial activity of *L. reuteri* isolated from spontaneous sourdough, it is important to estimate the effectiveness of this activity under natural fermentation conditions and heat treatment during baking. The protective properties of spontaneous sourdough fermented by *L. reuteri* were assessed by determining the number of extraneous microorganisms in baked bread samples. It was found that the fresh samples were not contaminated by microscopic fungi and aerobic mesophilic bacteria; only some bacterial colonies were detected (Fig. 3).

The positive protective effect of spontaneous sourdough was revealed during bread storage, because the total bacterial count was 1–20 times lower in the experimental bread than in the control bread prepared with *Lactococcus lactis* ssp. *lactis* 140/2 after 7 days of storage at room temperature. The fact that spontaneous sourdough possesses antimicrobial activity is also demonstrated by the absence of microscopic fungi and aerobic spore-forming bacteria in bread samples during storage over the course of the experiment.

LAB strains producing antimicrobial active substances secrete them into the growth environment, so anti-



**Fig. 3.** The effect of the spontaneous sourdough on the microbiological parameters of experimental bread crumb

mould activity of *L. reuteri* was determined by spraying the surfaces of bread loaves with cell-free supernatant containing active substances produced by these LAB. Spraying a culture supernatant on bread slices is not a suitable approach for bread preservation since it was prepared in the MRS broth, which is composed of non-food grade chemicals. However, this method allows the assessment of the more detailed effect of metabolites produced by *L. reuteri* on fungal spoilage. Results of spoilage determination of bread loaves sprayed with MRS broth were coincident with those of unsprayed loaves and it suggested that only metabolites of *L. reuteri* were effective against moulding, not the components of MRS. Cell-free supernatant protected the commercial bread against mould during storage for 15 days, while the commercial bread that was not sprayed with the tested solution became mouldy after 8 days of storage at 25 °C and after 12 (wheat bread) and 15 days (rye bread) during storage at 15 °C (Table 3).

Under certain conditions, some lactobacilli and lactococci possessing lipolytic activities may produce significant amounts of fatty acids (46). The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on the chain length, concentration and pH of the medium (47). Fatty acid analysis (Table 4) showed that *L. reuteri* produced 42 % of saturated, 31 % of monounsaturated, 26 % of polyunsaturated, 0.3 % of *trans*-fatty acids, 5 % of omega-3

**Table 3.** Antimould activity of active substances produced by *Lactobacillus reuteri* on the surfaces of bread obtained in local supermarket

Type of bread	Bread sample	t(storage) day at 15 °C			t(storage) day at 25 °C		
		8	12	15	8	12	15
Wheat	C1	-	+	+	-	+	++
	C2	-	+	+	-	+	++
	S	-	-	-	-	-	-
Rye	C1	-	-	+	+	+	++
	C2	-	-	+	+	+	++
	S	-	-	-	-	-	+

Control samples: C1=bread not sprayed and C2=sprayed with MRS broth, S=bread sprayed with supernatant of *L. reuteri*; - no moulds, + small colonies of moulds, ++ large colonies of moulds

**Table 4.** Fatty acid profiles of *Lactobacillus reuteri* isolated from spontaneous sourdough

Fatty acid	w(fatty acid)/%
butyric	1.33±0.50
valeric	0.97±0.21
capronic	1.89±0.30
caprylic	0.70±0.05
undecanoic	0.36±0.10
lauric	0.37±0.04
tridecanoic	0.50±0.01
pentadecanoic	1.07±0.06
<i>cis</i> -10-pentadecenoic	0.70±0.08
palmitic	15.20±1.05
palmitoleic	2.27±0.64
hexadecadienoic	1.49±0.50
heptadecanoic	3.16±0.80
<i>cis</i> -10-heptadecanoic	0.79±0.10
6,9-heptadecadiene	4.85±1.00
stearic	6.67±1.25
oleic	27.33±2.30
linoleic	9.77±1.50
$\alpha$ -linoleic	5.36±1.48
$\gamma$ -linoleic	0.32±0.05
heneicosanoic	4.96±1.20
behenic	3.83±0.60
docosadienoic	2.40±0.90
adrenic	1.44±0.50

and 15 % of omega-6 fatty acids. According to the inhibition zones (Table 2), *L. reuteri* had a greater impact on the growth inhibition of Gram-positive bacteria (*Bacillus subtilis* ssp. *spizizenii*, *B. cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*) than Gram-negative (*Escherichia coli*). This could happen because of high total amount of unsaturated fatty acids (57.3 %) produced by *L. reuteri*.

LAB suspension on the bread surface can be used for inhibition of the undesirable microorganism growth and for prevention of bread moulding (22). The same researchers applied the spraying of *Pediococcus acidilactici* and *P. pentosaceus* cell suspensions on the bread surface and a positive result of prolonging the shelf life of bread up to 8 days was determined. They proposed the LAB count of  $10^4$ – $10^5$  CFU/cm<sup>2</sup> for spraying the surface of bread. In our research, cell-free supernatant obtained by decanting approx.  $1.8 \cdot 10^9$  cells (according to  $A_{600\text{ nm}}=2.3$ ) was used for spraying bread loaves. The appearance of mould colonies was observed from 4 to 8 days later on the bread surfaces than on the control bread samples (not sprayed). Meanwhile, baked bread prepared with spontaneous sourdough containing *L. reuteri* was characterised by good antifungal properties and the antimould activity lasted during the storage for 15 days, when the small colonies of moulds appeared on the loaves of control rye

with wheat bread in the end of experiment. The mould contamination may be weaker in experimental bread prepared under laboratory conditions than in bread prepared under manufacturing conditions. Spores of microscopic fungi that get into the sourdough from the raw materials or from the air are killed during baking, while the spores may re-enter the surface of the bread during cooling, packaging, transportation or storage of the product and cause crumb damage through crust cracking.

## Conclusions

*Lactobacillus reuteri* was isolated and identified as lactic acid bacteria present in spontaneous sourdough. It expressed a good inhibiting effect against reference bacterial strains *Bacillus subtilis* ssp. *spizizenii* ATCC 6633, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19111, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and foodborne micromycetes *Cladosporium herbarum* SR-11, *Penicillium chrysogenum* SR-12, *Scopulariopsis brevicaulis* Mi-Gr-5, *Aspergillus brasiliensis* Mi-G-21 and *Aspergillus versicolor* Mi-Pr-4. The use of spontaneous sourdough for rye bread making results in significantly lower firmness of bread in comparison with wheat and rye with wheat bread. The less intense increase of wheat bread crumb firmness than the firmness of rye bread during storage demonstrated the dependence of bread firmness on the used flour, but not on the used sourdough composition. Antimicrobial substances produced by *L. reuteri* were observed to have a significant antimould activity on the bread surfaces during 12–15 days of storage. The use of antimicrobial active sourdough could prevent bread defects such as ropiness or moulding and prolong the shelf life of bread.

## Acknowledgements

We express our gratitude to dr. Jūratė Repečkienė (Institute of Botany, Nature Research Centre, Vilnius, Lithuania) for cultures of yeasts and moulds.

## References

- Brandt MJ. Starter cultures for cereal based foods. *Food Microbiol.* 2014;37:41–3. <http://dx.doi.org/10.1016/j.fm.2013.06.007>
- De Vuyst L, Neysens P. The sourdough microflora: biodiversity and metabolic interactions. *Trends Food Sci Technol.* 2005;16:43–56. <http://dx.doi.org/10.1016/j.tifs.2004.02.012>
- Gerez CL, Torino MI, Rollán G, Font de Valdez G. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control.* 2009;20:144–8. <http://dx.doi.org/10.1016/j.foodcont.2008.03.005>
- Hammes WP, Hertel C. New developments in meat starter cultures. *Meat Sci.* 1998;49:S125–38. [http://dx.doi.org/10.1016/S0309-1740\(98\)90043-2](http://dx.doi.org/10.1016/S0309-1740(98)90043-2)
- Paul Ross R, Morgan S, Hill C. Preservation and fermentation: past, present and future. *Int J Food Microbiol.* 2002;79:3–16. [http://dx.doi.org/10.1016/S0168-1605\(02\)00174-5](http://dx.doi.org/10.1016/S0168-1605(02)00174-5)
- Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol.* 2001;71:1–20. [http://dx.doi.org/10.1016/S0168-1605\(01\)00560-8](http://dx.doi.org/10.1016/S0168-1605(01)00560-8)
- Gálvez A, Abriouel H, López RL, Ben Omar N. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol.* 2007;120:51–70. <http://dx.doi.org/10.1016/j.ijfoodmicro.2007.06.001>
- Nes IF. History, current knowledge, and future directions on bacteriocin research in lactic acid bacteria. In: Drider D, Rebuffat S, editors. *Prokaryotic antimicrobial peptides: from genes to applications.* New York, NY, USA: Springer Science + Business Media, LLC; 2001. pp. 3–12. [http://dx.doi.org/10.1007/978-1-4419-7692-5\\_1](http://dx.doi.org/10.1007/978-1-4419-7692-5_1)
- Nilsson L, Hansen TB, Garrido P, Buchrieser C, Glaser P, Knöchel S, et al. Growth inhibition of *Listeria monocytogenes* by a nonbacteriocinogenic *Carnobacterium piscicola*. *J Appl Microbiol.* 2005;98:172–83. <http://dx.doi.org/10.1111/j.1365-2672.2004.02438.x>
- Saranraj P, Geetha M. Microbial spoilage of bakery products and its control by preservatives. *Int J Pharm Biol Arch.* 2012; 3:38–48.
- Farkas J. Physical methods for food preservation. In: Doyle MP, Beuchat LR, editors. *Food microbiology: fundamentals and frontiers.* Washington, DC, USA: ASM press; 2001. pp. 567–92. <http://dx.doi.org/10.1128/9781555815912.ch32>
- Dantigny P, Guilmar A, Radoi F, Bensoussan M, Zwietering M. Modelling the effect of ethanol on growth rate of food spoilage moulds. *Int J Food Microbiol.* 2005;98:261–9. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.07.008>
- Katina K, Salmenkallio-Marttila M, Partanen R, Forssell P, Autio K. Effects of sourdough and enzymes on stalling of high-fiber wheat bread. *LWT – Food Sci Technol.* 2006;39: 479–91. <http://dx.doi.org/10.1016/j.lwt.2005.03.013>
- Suhem K, Matan N, Nisoa M. In vitro and in vivo antifungal activities of various gas species under plasma jet treatment against brown rice cereal spoilage molds. *Int Food Res J.* 2013;20:947–51.
- Hasan SM, Abdolgader RA. Study of weak acid preservatives and modified atmosphere packaging (MAP) on mold growth in modal agar system. *Food Nutr Sci.* 2012;3:802–9. <http://dx.doi.org/10.4236/fns.2012.36108>
- Nielsen PV. Packaging, quality control, and sanitation of bakery products. In: Hui YH, Meunier-Goddik L, Solvejg Hansen Å, Josephsen J, Nip WK, Stanfield PS, Toldrá F, editors. *Handbook of fermented food and beverage.* New York, NY, USA: Marcel Dekker Inc.; 2004. pp. 901–18.
- Han JH. Antimicrobial food packaging. In: Ahvenainen R, editor. *Novel food packaging techniques.* Boca Raton, FL, USA: CRC Press; 2000. pp. 50–70. <http://dx.doi.org/10.1533/9781855737020.1.50>
- Robertson A, Tirado C, Lobstein T, Jermini M, Knai C, Jensen JH, et al. *Food and health in Europe: a new basis for action.* Copenhagen, Denmark: WHO Regional Publications, European Series, No. 96; 2004.
- LST 1481:2004. Rye flour. Vilnius, Lithuania; The Lithuanian Standards Board; 2004 (in Lithuanian).
- GenBank®. Bethesda, MD, USA: National Center for Biotechnology Information (NCBI), US National Library of Medicine; 2016. Available from: <http://www.ncbi.nlm.nih.gov/>.
- LST 1442:1996. Bread, rolls and buns. Determination of porosity. Vilnius, Lithuania: The Lithuanian Standards Board; 1996 (in Lithuanian).
- Bourne MC. Texture profile analysis. *Food Technol.* 1978;32: 62–72.
- ISO 21527-1:2008. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds – Part 1: Colony count technique in products with water activity greater than 0.95. Geneva, Switzerland:



- International Organization for Standardization (ISO); 2008.
24. LST 1963:2004. Enumeration of spores of mesophilic bacteria (ICC 144:1992). Vilnius, Lithuania: The Lithuanian Standards Board; 2004 (in Lithuanian).
  25. ISO 4833-1:2013. Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 degrees C by the pour plate technique. Geneva, Switzerland: International Organization for Standardization (ISO); 2013.
  26. Cizeikiene D, Juodeikiene G, Paskevicius A, Bartkiene E. Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control*. 2013;31:539–45. <http://dx.doi.org/10.1016/j.foodcont.2012.12.004>
  27. OD600 calculator for *E. coli*. LabTools; 2016. Available from: <http://www.labtools.us/bacterial-cell-number-od600/>.
  28. LST EN ISO 12966-2:2011. Animal and vegetable fats and oils – gas chromatography of fatty acid methyl esters – Part 2: Preparation of methyl esters of fatty acids. Vilnius, Lithuania: The Lithuanian Standards Board; 2011 (in Lithuanian).
  29. LST EN ISO 15304:2003. Animal and vegetable fats and oils – determination of the content of trans fatty acid isomers of vegetable fats and oils – gas chromatographic method (ISO15304:2002/Cor.1:2003). Vilnius, Lithuania: The Lithuanian Standards Board; 2011 (in Lithuanian).
  30. SPSS for Windows, v. 16.0; Chicago, IL, USA: SPSS Inc; 2007.
  31. De Vuyst L, Van Kerrebroeck S, Harth H, Huys G, Daniel HM, Weckx S. Microbial ecology of sourdough fermentations: diverse or uniform? *Food Microbiol.* 2014;37:11–29. <http://dx.doi.org/10.1016/j.fm.2013.06.002>
  32. Hammes WP, Vogel RF. The genus of *Lactobacillus*. In: Wood BJB, Holzapfel WH, editors. The genera of lactic acid bacteria. London, UK: Chapman&Hall; 1995. [http://dx.doi.org/10.1007/978-1-4615-5817-0\\_3](http://dx.doi.org/10.1007/978-1-4615-5817-0_3)
  33. Gobbetti M, Corsetti A, Rossi J. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. *Microbiol Biotechnol.* 1994;10:275–9. <http://dx.doi.org/10.1007/BF00414862>
  34. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Lett.* 1993;12:39–85. <http://dx.doi.org/10.1111/j.1574-6976.1993.tb00012.x>
  35. Schillinger U, Villarreal JV. Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. *Food Control*. 2010;21:107–11. <http://dx.doi.org/10.1016/j.foodcont.2008.11.010>
  36. Lavermicocca P, Valerio F, Evidente A, Lazzaroni S, Corsetti A, Gobbetti M. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Appl Environ Microbiol.* 2000;66:4048–90. <http://dx.doi.org/10.1128/AEM.66.9.4084-4090.2000>
  37. Melvydas V, Šakalytė J, Paškevičius A, Gedminienė G. Search for biological control agents against *Candida* yeasts and other dermatomycetes. *Biologija*. 2007;18:45–9.
  38. Smaoui S, Elleuch L, Bejar W, Karray-Rebai AI, Jaouadi B, Mathieu F, et al. Inhibition of fungi and Gram-negative bacteria by bacteriocin BacTN635 produced by *Lactobacillus plantarum* sp. TN635. *Appl Biochem Biotechnol.* 2010;162:1132–46. <http://dx.doi.org/10.1007/s12010-009-8821-7>
  39. Ström K, Sjögren J, Broberg A, Schnürer J. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl Environ Microbiol.* 2002;68:4322–7. <http://dx.doi.org/10.1128/AEM.68.9.4322-4327.2002>
  40. LST 1129:2003. Bread. General requirements. Vilnius, Lithuania: The Lithuanian Standards Board; 2003 (in Lithuanian).
  41. Koceva Komlenić DK, Slačanac V, Jukić M. Influence of acidification on dough rheological properties. In: De Vicente, editor. *Rheology*. Rijeka, Croatia: Intech; 2008. pp. 265–92. <http://dx.doi.org/10.5772/35829>
  42. Plessas S, Trantallidi M, Bekatorou A, Kanellaki M, Nigam P, Koutinas AA. Immobilization of kefir and *Lactobacillus casei* on brewery spent grains for use in sourdough wheat bread making. *Food Chem.* 2007;105:187–94. <http://dx.doi.org/10.1016/j.foodchem.2007.03.065>
  43. Mihhalevski A, Heinmaa I, Traksmaa R, Pehk T, Mere A, Paalme T. Structural changes of starch during baking and staling of rye bread. *J Agric Food Chem.* 2012;60:8492–500. <http://dx.doi.org/10.1021/jf3021877>
  44. Schwab C, Mastrangelo M, Corsetti A, Gänzle M. Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. *Cereal Chem.* 2008;85:679–84. <http://dx.doi.org/10.1094/CCHEM-85-5-0679>
  45. Martinbianco F, Martins AR, Rech R, Hickmann Flôres S, Ayub MAZ. Sensory evaluation of natural fermentation breads with innovative starter cultures. *Ciênc Rural.* 2013; 43:1701-6 (in Portuguese). <http://dx.doi.org/10.1590/SO103-847201000900026>
  46. Sanz B, Selgas D, Parejo I, Ordóñez JA. Characteristics of lactobacilli isolated from dry fermented sausages. *Int J Food Microbiol.* 1988;6:199–205. [http://dx.doi.org/10.1016/0168-1605\(88\)90012-8](http://dx.doi.org/10.1016/0168-1605(88)90012-8)
  47. Gould GW. Antimicrobial compound. In: Goldberg I, Williams R, editors. *Biotechnology and food ingredients*. New York, NY, USA: Springer, Science & Business Media; 1998. pp. 461–82.