Cereals and pseudocereals as substrates for growth and metabolism of a probiotic strain *Lactobacillus rhamnosus* GG

MONIKA KOCKOVÁ – JURAJ MENDEL – ALŽBETA MEDVEĎOVÁ – ERNEST ŠTURDÍK – ĽUBOMÍR VALÍK

Summary
The aim of this work was to evaluate suitability of cereals and pseudocereals as carriers for probiotic bacteria, leading to the development of new probiotic foods for consumers allergic to milk protein or gluten. Before fermentation experiments, content of water, reducing saccharides, proteins, lipids and crude fibre in selected cereals (rye, barley, oat, millet) and pseudocereals (amaranth, buckwheat) were evaluated. The growth and metabolic activity of the probiotic strain *Lactobacillus rhamnosus* GG during fermentation of mixtures of selected cereals and pseudocereals with water at two initial inoculation levels were monitored. Viable cell count, pH value, titratable acidity and contents of organic acids were analysed during static fermentation of cooked substrates at 37 °C for 48 h. *Lb. rhamnosus* GG was able to grow up to the counts higher than 6 log CFU·g^{-1} and metabolized each kind of substrates. The inoculation level had no significant influence on the growth of *Lb. rhamnosus* GG, but metabolic activity was affected by density of cells inoculated at the beginning of the process, in particular in substrates prepared from amaranth flour, milled amaranth grain, buckwheat flour and whole barley flour.

Keywords
cereal; pseudocereal; *Lactobacillus rhamnosus* GG; fermentation; probiotic; functional food

Cereals and pseudocereals belong to the most important and easily produced food for the majority of mankind as they are a source of saccharides, proteins, vitamins, minerals, fibre and biologically active compounds [1–3]. On the other hand, natural presence of antinutritive compounds should be taken into the account at evaluation of their nutrition quality [4, 5]. However, nutrition and health benefits of cereals and pseudocereals can be improved by fermentation, providing higher sensorial value and extended shelf-life at the same time [6, 7]. In addition to these facts, application of a probiotic strain in the cereal and pseudocereal fermentation process may contribute to the development of a new functional food. Cereal- and pseudocereal-based fermented foods could be potential vehicles for many functional compounds, such as antioxidants, dietary fibre, minerals, probiotics and vitamins [8].

There are several possibilities how the nutritional quality of cereals and pseudocereals could be improved by their fermentation: production of bioactive peptides that may stimulate immune system [9]; elimination of cereal gluten [10, 11]; production of γ-aminobutyric acid, the major inhibitory neurotransmitter of the central nervous system [12]; increasing total phenolic content and antioxidant capacity [13]; decreasing of antinutritional factors, such as phytic acid, tannins and enzyme inhibitors [5].

Lactic acid bacteria contribute to improving of the organoleptic quality of fermented products by

Monika Kocková, Alžbeta Medveďová, Ernest Šturdík, Ľubomír Valík, Department of Nutrition and Food Assessment, Institution of Biochemistry, Nutrition and Health Protection, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovakia.
Juraj Mendel, Department of Food Safety and Nutrition, Food and Trade Division, Ministry of Agriculture and Rural Development of the Slovak Republic, Dobrovičová 12, 812 66 Bratislava, Slovakia.

Correspondence author:
Monika Kocková, e-mail: monika.kockova@stuba.sk

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producing substances like alcohols, organic acids, carbonyl substances and another, which improve taste and flavour of end products, and by producing of exopolysaccharides, which improve texture of fermented foods and have prebiotic properties [14, 15]. Lactic acid bacteria produce many antimicrobial substrates such as organic acids (mainly lactic, acetic, formic, propionic, phenyllactic acid), CO_2, ethanol, hydrogen peroxide, diacetyl, fatty acids, bacteriocins and fungicins, which contribute to the extension of shelf-life [6, 16, 17].

Lactobacillus rhamnosus GG may be used in food industry not only as a probiotic but also as a protective culture in fermented and non-fermented dairy products, beverages, ready-to-eat foods, dry sausages and salads, because of its probiotic and antimicrobial activities [18]. The growth of this strain was quantitatively described [19. 20]. The recent scientific opinion of the EFSA Panel on Dietetic Products, Nutrition and Allergies considered the maintenance of defence against pathogenic gastrointestinal microorganisms as a beneficial physiological effect, though not including the treatment of gastrointestinal infections [21].

Traditional cereal and pseudocereal fermented products are made from various kinds of substrates and they are widespread around the world, mainly in developing countries of Asia and Africa. They have various applications, some of these are used as colouring or spice, others are consumed as beverages, breakfast cereals or staple foods [22]. The fact that the majority foods containing probiotic bacteria are based on milk is commonly accepted by consumers. Contradictory to this, our work was inspired by the need of probiotics for consumers who suffer from allergy to milk proteins or gluten. That is why the selection of cereal and pseudocereal wet heat-treated substrates was performed regarding the growth and metabolic activity of the probiotic strain *Lactobacillus rhamnosus* GG. Based on the results provided by the study, we intend to prepare probiotic snacks or side-dishes using the most suitable pseudocereal or cereal matrices.

MATERIALS AND METHODS

Materials

Nine samples of cereal and pseudocereal flour and/or grain were used in this work. Rye flour (RF), barley flour (BF), amaranth flour (AF), whole barley flour (WBF), buckwheat flour (BWF), whole oat flour (WOF) and rye grain (RG) were obtained from mill house (Mlyn Zrno, Šišov, Slovakia); amaranth grain (AG; Primeal, Peaugres, France) and millet grain (MG; Marianna wholesale, Ivanka pri Dunaji, Slovakia) were obtained from the market in Bratislava.

Preparation of media and fermentation process

Whole grains (with hull) were milled and sieved (pore size 0.5 mm) before their use in the fermentation experiments. Five grams of samples were mixed with 95 ml of deionized water, autoclaved for 15 min at 121 °C, cooled down and inoculated with an overnight culture of *Lb. rhamnosus* GG. Static fermentation was performed for 48 h at 37 °C. Samples for analyses were taken every 24 h.

Microorganisms, inoculation and cultivation conditions

The strain *Lb. rhamnosus* GG was kept in de Man – Rogosa – Sharpe broth (MRS; Merck, Darmstadt, Germany) at 5 ± 1 °C. The standard suspension of the microorganism was prepared from an 18-h culture grown in MRS broth at 37 °C. This culture was inoculated to the cereal and pseudocereal substrates (to give approximately 5 log CFU·g^{-1} or 6 log CFU·g^{-1} of the substrate after inoculation).

Bacteriological analyses

Bacterial counts were determined after dilution and cultivation on MRS agar (Merck) according to STN ISO 15214 [23].

Chemical analyses

Dry matter of cereals and pseudocereals was determined according to the STN ISO 712 [24]. Chemical composition (reducing saccharides, proteins, lipids and crude fibre) of flours and milled grains was evaluated according to STN ISO 56 0512 [25]. Water activity was measured by a_w Sprint, TH-500 (Novasina, Lachen, Switzerland).

The pH of samples was measured by pH-meter CG 843 (Schott, Mainz, Germany). Titratable acidity (TTA) was established according to STN ISO 56 0512 [25]. The result was expressed as content of lactic acid.

The quality and quantity of the produced organic acids were measured by isotachophoretic analysis by using the Isotachophoretic Analyser ZKI 01 (Villa Labeco, Spišská Nová Ves, Slovakia). Electrolytic system according to Zalán et al. [26] with some modifications was used: 0.01 mol·l^{-1} HCl as leading electrolyte, ε-aminocaproic acid as a counter ion, ethylhydroxymethylcellulose as additive (0.1%), pH 4.3; terminating electrolyte: 5×10^{-3} mol·l^{-1} caproic acid, 5×10^{-3} mol·l^{-1} Tris (3-hydroxymethyl-aminomethane), pH 4.5–5.0;

driving current of 250 μA in the pre-separation column. For quantitative analysis, calibration was performed using a standard solution of lactic, acetic, citric, formic and succinic acids (Lachema, Brno, Czech Republic) [27].

Statistical analyses

Each experiment was performed in three separate trials. Results represented means with standard deviations. Statistical analyses were carried out using Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA). Data were treated by independent two-samples Student t-test (unequal variances) and confirmed with ANOVA test with a least significant difference of 95%.

RESULTS AND DISCUSSION

Chemical composition of used cereals and pseudocereals

Chemical composition of selected cereal and pseudocereal flours and grains is shown in Tab. 1. Only small differences in water content, which is expressed as dry matter, were observed – dry matter ranged from 87.1% to 87.9% for most substrates, only in whole oat flour, content of water was lower (only 10.3%). Similar situation was at values of water activity – water available for microorganisms, which ranged from 0.514 for whole oat flour to 0.606 for amaranth flour. Greater differences in content of reducing saccharides, proteins, lipids and crude fibre were observed in different samples. The lowest content of reducing saccharides was measured in millet grain (6 g·kg^{-1}), the highest in amaranth flour (46 g·kg^{-1}). Proteins content ranged from 8 g·kg^{-1} (rye flour) to 76 g·kg^{-1} (amaranth grain). Similar range of values was observed in crude fibre content – from 4.6 g·kg^{-1} for rye flour to 77.3 g·kg^{-1} for millet grain. Higher variability in the contents of chemical components was observed at lipids – millet grain contained the lowest amount of lipids (27.4 g·kg^{-1}) and amaranth grain had the highest content (129.0 g·kg^{-1}). Cereals and pseudocereals contain also other components, such as polysaccharides, vitamins, mineral and others. For growth and metabolic activity of cultures of lactic acid bacteria, contents of water and utilizable saccharides are the most important.

Growth of *Lactobacillus rhamnosus* GG and changes in pH value of substrates

According to the results shown in Fig. 1, all substrates were suitable for growth of *Lb. rhamnosus* GG. At the end of fermentation process, maximum population density reached counts of 6.15–8.34 log CFU·g^{-1}, higher cell counts were measured in whole barley flour and amaranth grain inoculated at a higher inoculation level (8.11 CFU·g^{-1} and 8.34 CFU·g^{-1}, respectively). Similar cell counts of a potentially probiotic strain *Lb. reuteri* 11951 in 5% malt suspension, probiotic strains *Lb. plantarum* (NCIMB 8826) and *Lb. acidophilus* (NCIMB 8821) in malt, barley and malt-barley flour were reported to be reached [28, 29]. HELLAND et al. [30] evaluated growth and metabolism of probiotic strains in milk- and water-based cereal (maize and rice) puddings. It was found that *Lb. rhamnosus* GG was the only strain with an acceptable survival in water-based puddings. The same research team studied growth and metabolism of probiotic strains in maize porridge, in which all strains reached maximum cell counts

Tab. 1. Chemical composition of cereals and pseudocereals.

Sample	Dry matter [%]	a_w	Reducing saccharides [%]	Proteins [%]	Lipids [%]	Crude fibre [%]
RF	87.7 ± 0.0	0.541 ± 0.005	2.5 ± 0.3	0.8 ± 0.1	2.9 ± 0.5	0.5 ± 0.0
RG	87.6 ± 0.1	0.567 ± 0.012	1.0 ± 0.0	1.5 ± 0.1	6.5 ± 0.8	1.0 ± 0.0
BF	87.8 ± 0.2	0.533 ± 0.009	1.7 ± 0.1	1.7 ± 0.0	5.4 ± 0.5	0.8 ± 0.0
WBF	87.9 ± 0.1	0.559 ± 0.010	2.7 ± 0.1	2.3 ± 0.3	7.9 ± 0.5	2.7 ± 0.1
AF	87.3 ± 0.1	0.606 ± 0.015	4.6 ± 0.2	7.4 ± 0.0	11.2 ± 0.5	5.1 ± 0.0
AG	87.5 ± 0.2	0.561 ± 0.007	2.0 ± 0.1	7.6 ± 0.2	12.9 ± 0.5	3.2 ± 0.1
BWF	87.7 ± 0.2	0.585 ± 0.007	1.9 ± 0.1	4.8 ± 0.1	7.9 ± 0.5	6.4 ± 0.0
WOF	89.7 ± 0.0	0.514 ± 0.009	1.5 ± 0.1	3.5 ± 0.2	8.7 ± 0.5	7.1 ± 0.0
MG	87.1 ± 0.2	0.559 ± 0.012	0.6 ± 0.1	4.7 ± 0.2	2.7 ± 0.5	7.7 ± 0.2

The results are means ± standard deviation (n = 3).
RF – rye flour, RG – rye grain, BF – barley flour, WBF – whole barley flour, AF – amaranth flour, AG – amaranth grain, BWF – buckwheat flour, WOF – whole oat flour, MG – millet grain; a_w – water activity.

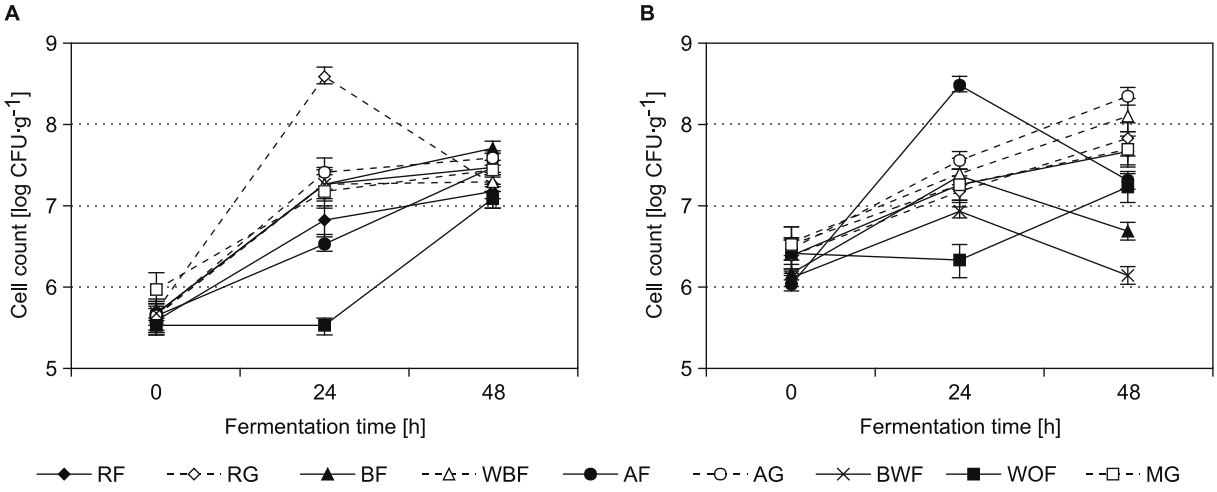

Fig. 1. Cell counts during fermentation of cereal and pseudocereal substrates.

A – initial density 5 log CFU·g^{-1}, B - initial density 6 log CFU·g^{-1}.
Results are means (n = 3), vertical lines represent standard deviations.

of 7.2–8.2 log CFU·g^{-1} [31]. Influence of milk addition to pseudocereal suspensions on the growth of probiotic and potentially probiotic strains was studied by PELIKANOVÁ et al. [32]. Among lactobacilli, *Lb. rhamnosus* GG was found to be the fastest growing. For example, its growth rates were 0.912 log CFU·ml^{-1}h^{-1} and 0.126 log CFU·ml^{-1}h^{-1} in milk- and water-based buckwheat porridge, respectively.

Generally, in substrates inoculated at lower inoculation density, the pH value at the end of the fermentation process did not drop below 5.0, except for rye flour (Fig. 2). Samples with higher inoculation concentration had pH at 48 h lower, in case of barley flour, amaranth flour and whole buckwheat flour below pH 4.0 (Fig. 2). During the first 24 h, the reduction of pH was significantly greater for most samples. The fastest and greatest reduction was seen for amaranth flour at the higher inoculation level. Acidification caused by the metabolic activity of *Lb. rhamnosus* GG was not significant compared to other lactic acid bacteria in similar substrates. For instance, metabolic activity of *Lb. plantarum* and *Lb. acidophilus*

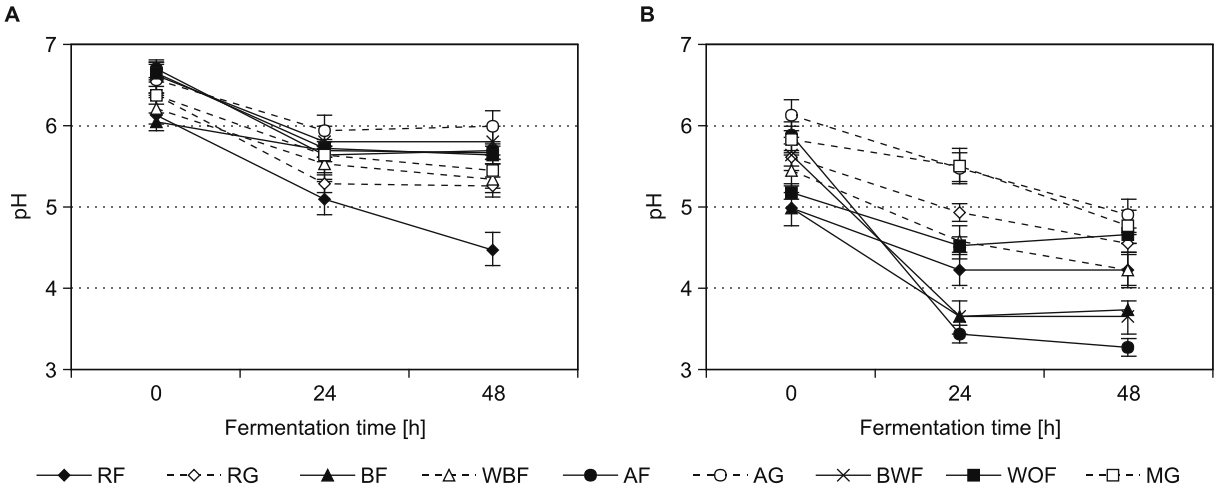

Fig. 2. pH values during fermentation of cereal and pseudocereal suspensions.

A – initial density 5 log CFU·g^{-1}, B - initial density 6 log CFU·g^{-1}.
Results are means (n = 3), vertical lines represent standard deviations.

caused a decrease in pH to below 3.5 after a 24-h fermentation of malt, barley and malt-barley flour [29].

Production of organic acids

Titratable acidity (TTA, expressed as a percentage of organic acids, in our case of lactic acid as the predominant acid) was depended on type of cereal or pseudocereal flours and inoculation levels also (Tab. 2). Higher influence of inoculation level was shown in following samples: amaranth flour, whole barley flour, whole buckwheat flour and amaranth grain, in which titratable acidity increased about 2–4 multiply. The fastest and highest titratable acidity increase was observed in amaranth flour at higher inoculation rate.

The difference in initial inoculation of *Lb. rhamnosus* GG and used substrate may be the reason for differences in production or reduction of most analysed organic acids (Tab. 3–7). The levels of organic acids were evaluated by isotachophoretic method.

Lower nutrition content in this kind of substrates might cause slower lactic acid production

Tab. 2. Changes in titratable acidity in fermented cereal and pseudocereal suspensions.

Substrate	Titratable acidity [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	336.9 ± 26.52^{a,y}	275.6 ± 0.00^{a,x}	321.6 ± 0.00^{a,y}	398.2 ± 53.05^{a,x}	505.3 ± 45.94^{a,x}	490.0 ± 26.52^{a,x}
RG	367.5 ± 0.00^{a,x}	551.3 ± 0.00^{b,y}	551.3 ± 0.00^{b,y}	551.3 ± 45.94^{c,x}	796.3 ± 70.18^{b,y}	903.5 ± 26.52^{b,y}
BF	666.1 ± 32.49^{d,x}	918.8 ± 0.00^{d,y}	918.8 ± 0.00^{d,y}	719.7 ± 26.52^{d,x}	1 500.7 ± 26.52^{d,z}	1 255.7 ± 70.18^{d,y}
WBF	627.9 ± 26.52^{d,x}	689.1 ± 0.00^{c,y}	589.1 ± 45.94^{c,y}	842.2 ± 26.52^{e,x}	1 362.9 ± 70.18^{c,y}	1 730.4 ± 26.52^{e,z}
AF	1 562.0 ± 45.94^{f,x}	3 292.4 ± 26.52^{f,x}	3 323.1 ± 26.52^{f,y}	1 638.6 ± 26.52^{h,x}	6 462.3 ± 53.05^{g,y}	7 855.9 ± 137.82^{h,z}
AG	566.6 ± 26.52^{c,x}	719.7 ± 26.52^{c,y}	765.7 ± 26.52^{c,y}	750.4 ± 26.52^{d,x}	1 332.3 ± 0.00^{c,y}	2 924.9 ± 26.52^{f,z}
BWF	1 271.0 ± 26.52^{e,x}	1 546.7 ± 26.52^{e,y}	1 653.9 ± 0.00^{e,z}	1 087.3 ± 26.52^{g,x}	3 154.6 ± 53.05^{f,y}	3 124.0 ± 91.94^{g,y}
WOF	673.8 ± 26.52^{d,x}	735.1 ± 0.00^{c,y}	781.0 ± 0.00^{c,z}	934.1 ± 26.52^{f,x}	1 699.8 ± 45.94^{e,z}	1 271.0 ± 26.52^{d,y}
MG	459.4 ± 0.00^{b,x}	551.3 ± 0.00^{b,y}	673.8 ± 26.52^{c,z}	459.4 ± 0.00^{b,x}	888.2 ± 26.52^{b,y}	1 087.3 ± 26.52^{c,z}

Refer to Tab. 1 for substrates. The results are means ± standard deviation (n = 3).
5% suspension of cereal/pseudocereal and water has been used for fermentation by *Lb. rhamnosus* GG at initial density of 5 log CFU·g^{-1} and 6 log CFU·g^{-1}. Process has been led stationary at 37 °C for 48 hours.
a–h – means within a column with different superscript letters are significantly different (P < 0,05); x, y, z – means within a row with different superscript letters are significantly different (P < 0,05).

Tab. 3. Changes in lactic acid content in fermented cereal and pseudocereal suspensions.

Substrate	Lactic acid [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	ND	131.9 ± 1.27^{b,y}	93.8 ± 0.00^{a,x}	89.4 ± 3.80^{b,x}	167.8 ± 4.57^{a,y}	191.9 ± 7.06^{b,z}
RG	ND	224.2 ± 2.54^{d,y}	164.1 ± 2.20^{b,x}	95.3 ± 12.10^{b,x}	213.2 ± 19.69^{b,y}	208.8 ± 1.27^{c,y}
BF	ND	170.7 ± 15.84^{c,y}	101.1 ± 4.57^{a,x}	58.7 ± 0.00^{a,x}	609.3 ± 7.06^{d,z}	483.3 ± 6.71^{f,y}
WBF	ND	233.7 ± 1.27^{e,y}	197.8 ± 1.27^{d,x}	84.3 ± 1.27^{b,x}	421.1 ± 2.20^{e,y}	414.5 ± 9.57^{e,y}
AF	68.2 ± 15.58^{a,x}	1 061.8 ± 12.49^{g,y}	1 043.5 ± 1.27^{g,y}	104.8 ± 5.81^{b,x}	3 993.4 ± 29.0^{g,x}	5 572.7 ± 40.82^{h,z}
AG	ND	237.3 ± 3.36^{e,y}	183.2 ± 2.54^{c,x}	121.7 ± 7.06^{c,x}	391.8 ± 5.07^{d,y}	473.1 ± 7.06^{f,z}
BWF	58.7 ± 4.39^{a,x}	364.0 ± 5.81^{f,z}	266.6 ± 7.06^{f,y}	74.1 ± 2.20^{b,x}	1 682.7 ± 46.97^{f,y}	1 873.8 ± 48.94^{g,z}
WOF	ND	123.8 ± 1.27^{a,y}	105.5 ± 1.27^{a,x}	80.6 ± 3.80^{b,x}	368.4 ± 10.07^{c,z}	299.6 ± 7.06^{d,y}
MG	ND	171.4 ± 1.27^{c,x}	208.8 ± 3.36^{e,y}	86.5 ± 20.41^{b,x}	167.0 ± 6.71^{a,y}	104.1 ± 8.88^{a,x}

Refer to Tab. 1 for substrates. The results are means ± standard deviation (n = 3).
ND – not detected (detection limit for lactic acid was 40 mg·kg^{-1}).
a–h – means within a column with different superscript letters are significantly different (P < 0,05); x, y, z – means within a row with different superscript letters are significantly different (P < 0,05).

Tab. 4. Changes in acetic acid content in fermented cereal and pseudocereal suspensions.

Substrate	Acetic acid [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	ND	$52.1 \pm 2.08^{a,x}$	$109.7 \pm 2.75^{c,y}$	$83.9 \pm 7.27^{b,x}$	$70.1 \pm 4.53^{a,x}$	$73.1 \pm 5.19^{a,x}$
RG	$90.5 \pm 3.12^{d,x}$	$110.9 \pm 1.04^{c,y}$	$129.5 \pm 2.08^{d,z}$	$76.7 \pm 10.39^{b,x}$	$105.5 \pm 4.16^{b,y}$	$158.9 \pm 1.80^{d,z}$
BF	ND	$57.5 \pm 8.50^{a,x}$	$81.5 \pm 6.49^{a,y}$	$78.5 \pm 2.75^{b,x}$	$112.1 \pm 3.12^{b,y}$	$131.9 \pm 1.80^{c,z}$
WBF	$65.9 \pm 1.04^{b,x}$	$105.5 \pm 5.50^{c,y}$	$112.7 \pm 2.75^{c,y}$	$69.5 \pm 4.53^{b,x}$	$126.5 \pm 1.80^{b,y}$	$170.9 \pm 9.06^{d,z}$
AF	$61.7 \pm 1.80^{a,x}$	$111.5 \pm 1.04^{c,y}$	$167.3 \pm 1.04^{g,z}$	$43.7 \pm 6.49^{a,x}$	$130.1 \pm 1.80^{b,y}$	$275.8 \pm 9.35^{e,z}$
AG	$57.5 \pm 4.53^{a,x}$	$104.3 \pm 4.53^{c,z}$	$80.9 \pm 1.04^{a,y}$	$115.1 \pm 2.08^{c,x}$	$137.9 \pm 2.08^{c,y}$	$316.6 \pm 3.75^{f,z}$
BWF	ND	$122.9 \pm 14.05^{c,x}$	$157.7 \pm 1.04^{e,y}$	ND	119.3 ± 4.76^{b}	ND
WOF	$68.3 \pm 1.04^{c,x}$	$71.9 \pm 1.04^{b,z}$	$88.1 \pm 1.04^{b,z}$	$141.5 \pm 2.75^{d,z}$	$142.7 \pm 3.12^{c,y}$	$127.1 \pm 1.64^{b,x}$
MG	ND	$57.5 \pm 7.27^{c,y}$	$104.3 \pm 2.08^{c,y}$	$64.7 \pm 10.54^{b,x}$	$72.5 \pm 1.80^{a,x}$	$67.1 \pm 4.76^{a,x}$

Refer to Tab. 1 for substrates. The results are means ± standard deviation (n = 3).
ND – not detected (detection limit for acetic acid was 35 mg·kg^{-1}).
a–h – means within a column with different superscript letters are significantly different ($P < 0,05$); x, y, z – means within a row with different superscript letters are significantly different ($P < 0,05$).

(Tab. 3). The content of lactic acid at the end of fermentation process ranged from 93.82 mg·kg^{-1} to 1043.46 mg·kg^{-1} in samples inoculated at lower density of cells and from 104.07 mg·kg^{-1} to 5572.72 mg·kg^{-1} in samples inoculated at higher density of cells. The greatest production of lactic acid was seen in case of amaranth, in all samples and both inoculation levels. During fermentation period, significantly less lactic acid was produced in substrates inoculated with approximately 5 log CFU·g^{-1}, compared to samples inoculated at approximately 6 log CFU·g^{-1} (with exception of millet grain).

Helland et al. [30] demonstrated that addition of milk (as source of proteins, peptides and vitamins) to cereal substrates caused increase of metabolic activity of lactic acid bacteria. Substitute of water by milk in cereal puddings caused increasing in lactic acid production from 560–2600 mg·kg^{-1} to 4300–9800 mg·kg^{-1} [30]. On the other hand, production of organic acids by *Lb. rhamnosus* GG during cultivation in milk at temperatures from 6 °C to 46 °C was not significant, as was described by Valík et al. [33]. Production of lactic acids by probiotic strains *Lb. reuteri* SD 2112, *Lb. acidophilus* LA5, *Lb. acidophilus* NCDO 1748 and *Lb. rhamnosus* GG in maize-barley porridge after 24 hours fermentation was ranged from 1360 mg·kg^{-1} to 4000 mg·kg^{-1}, largest production was observed in case of *Lb. rhamnosus* GG [31]. Content of lactic acids after 24 hours fermentation of barley substrates by *Lb. plantarum* and *Lb. acidophilus* (inoculated alone and together in ratio 1:1) did not reach 100 mg·l^{-1} of beverages [29]. Lactic acid content in malt suspension after fermentation by *Lb. reuteri* was approximately 1200 mg·kg^{-1}. Production of lactic acid in togwa (Tanzanian cereal fermented food or beverage) ranged from cca 2500 mg·kg^{-1} to cca 5000 mg·kg^{-1}, according to used starter culture [34]. In naturally fermented togwa product, content of lactic acid was ranging between 4000 mg·kg^{-1} and 7000 mg·kg^{-1} after 24h fermentation, according to the used cereal substrates (maize, millet, sorghum or combination of maize and sorghum) [35].

Influence of cereal or pseudocereal type and inoculation level was not so significant in acetic acid production (Tab. 4). The content of acetic acid at the end of process ranged from 81.51 mg·kg^{-1} to 167.28 mg·kg^{-1} for samples with low inoculation level and from 67.12 mg·kg^{-1} to 316.61 mg·kg^{-1} for higher inoculated suspensions. In whole buckwheat flour at a high inoculation level, the content of acetic acid was below the detection limit of the isotachophoretic measurements. The highest influence of inoculation was observed in amaranth flour and amaranth grain, in which the differences were about 3–4 multiply. In samples prepared from millet grain, content of acetic acid was about 30% lower in higher inoculated samples in comparison to the samples with a lower inoculation level.

Production of acetic acid and other acids in fermented substrates is conditioned by used strains of lactic acid bacteria and their metabolisms. Homofermentative strains metabolize glucose mainly to lactic acid. Heterofermentative lactic acid bacteria produce only 50% of lactic acid from glucose

[36]. *Lb. rhamnosus* GG belongs to the facultative heterofermentative lactobacilli, so during the fermentation not only lactic acid is produced. According to the measurements conditions, formic acid, succinic acid and citric acid were detected. Also other acids and metabolites could be produced during process, but they were not detected by our methods at used conditions.

Lb. rhamnosus GG is citrate negative and does not utilize citrate in milk [37]. However, when it grew in cereal and pseudocereal substrates, except for amaranth grain, it was able to metabolize citric acid. Decreasing the content of citric acid level (Tab. 5) was approximately 80–210 mg·kg^{-1} (except amaranth grain) for samples with a lower inoculation level, and approximately 40–870 mg·kg^{-1} for samples inoculated at 6 log CFU·g^{-1}. No citric acid was detected after 48 h of fermentation in the following samples: rye flour at both inoculation levels, and whole barley flour, whole oat flour, rye grain and millet grain at low inoculation density (Tab. 5). Partial reduction of citric acid was observed during fermentation of maize porridge by *Lb. rhamnosus* GG [27]. Reduction of citric acid by lactic acid bacteria was observed in cereal fermented food togwa, mainly in case of natural fer-

Tab. 5. Changes in citric acid content in fermented cereal and pseudocereal suspensions.

Substrate	Citric acid [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	100.9 ± 1.94^{b}	ND	ND	$215.5 \pm 5.15^{a,y}$	$173.9 \pm 3.37^{b,x}$	ND
RG	115.5 ± 1.94^{c}	ND	ND	$355.8 \pm 3.37^{b,y}$	$226.7 \pm 5.15^{c,x}$	$217.7 \pm 12.15^{c,x}$
BF	$316.5 \pm 3.89^{f,z}$	$160.4 \pm 6.74^{c,y}$	$103.2 \pm 0.00^{a,x}$	$433.3 \pm 3.37^{d,z}$	$276.1 \pm 7.01^{d,y}$	$258.1 \pm 3.37^{d,x}$
WBF	177.3 ± 0.00^{d}	ND	ND	$509.7 \pm 5.15^{e,z}$	$406.4 \pm 3.37^{g,y}$	$361.4 \pm 9.72^{f,x}$
AF	$477.1 \pm 5.83^{h,z}$	$391.8 \pm 7.01^{f,x}$	$379.4 \pm 3.37^{d,x}$	$975.7 \pm 3.37^{g,z}$	$540.0 \pm 18.55^{i,y}$	$107.7 \pm 10.29^{a,x}$
AG	$204.2 \pm 5.83^{e,x}$	$226.7 \pm 1.94^{d,z}$	$221.1 \pm 0.00^{b,z}$	$435.6 \pm 7.78^{d,z}$	$322.1 \pm 0.00^{e,y}$	$313.2 \pm 1.94^{e,x}$
BWF	$346.9 \pm 7.78^{g,z}$	$264.9 \pm 3.37^{e,z}$	$233.4 \pm 3.89^{c,x}$	$520.9 \pm 3.37^{f,y}$	$443.4 \pm 20.49^{h,x}$	$483.8 \pm 17.50^{g,x}$
WOF	$86.3 \pm 0.00^{a,y}$	$75.1 \pm 1.94^{a,x}$	ND	$403.0 \pm 3.37^{c,z}$	$334.5 \pm 1.94^{f,y}$	$313.2 \pm 7.01^{e,x}$
MG	ND	102.1 ± 1.94^{b}	ND	$198.6 \pm 10.29^{a,x}$	$151.5 \pm 12.75^{a,x}$	$175.0 \pm 19.45^{b,x}$

Refer to Tab. 1 for substrates. The results are means ± standard deviation ($n = 3$).
ND – not detected (detection limit for citric acid was 60 mg·kg^{-1}).
a–h – means within a column with different superscript letters are significantly different ($P < 0,05$); x, y, z – means within a row with different superscript letters are significantly different ($P < 0,05$).

Tab. 6. Changes in formic acid content in fermented cereal and pseudocereal suspensions.

Substrate	Formic acid [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	ND	$32.1 \pm 0.77^{a,x}$	$31.2 \pm 0.00^{b,x}$	$77.0 \pm 2.04^{f,y}$	$35.6 \pm 3.08^{a,x}$	$40.1 \pm 0.77^{b,x}$
RG	$48.1 \pm 0.77^{b,y}$	$62.3 \pm 0.77^{d,z}$	$31.6 \pm 0.77^{b,x}$	$73.9 \pm 1.33^{f,y}$	$36.5 \pm 0.00^{a,x}$	$122.3 \pm 2.04^{h,z}$
BF	$52.5 \pm 2.67^{b,x}$	$49.4 \pm 2.04^{c,x}$	$53.8 \pm 2.31^{c,x}$	$56.5 \pm 0.00^{e,x}$	$95.2 \pm 1.33^{e,z}$	$65.6 \pm 0.77^{f,y}$
WBF	ND	$36.9 \pm 0.77^{b,y}$	$27.2 \pm 0.00^{a,x}$	$52.1 \pm 2.04^{c,y}$	$43.2 \pm 0.00^{c,x}$	$56.5 \pm 0.00^{e,z}$
AF	ND	ND	32.9 ± 2.04^{b}	$40.5 \pm 1.33^{a,x}$	$51.2 \pm 1.33^{d,y}$	$51.2 \pm 2.31^{d,y}$
AG	ND	ND	ND	ND	ND	153.5 ± 0.77^{i}
BWF	ND	ND	50.7 ± 2.78^{c}	$42.7 \pm 0.77^{a,x}$	$40.5 \pm 1.33^{b,x}$	$45.0 \pm 0.77^{c,y}$
WOF	$40.5 \pm 0.00^{a,y}$	ND	$35.2 \pm 0.00^{b,x}$	$45.4 \pm 0.77^{b,z}$	$33.8 \pm 1.33^{a,y}$	$30.3 \pm 0.77^{a,x}$
MG	ND	ND	ND	ND	ND	67.2 ± 1.33^{g}

Refer to Tab. 1 for substrates. The results are means ± standard deviation ($n = 3$).
ND – not detected (detection limit for formic acid was 25 mg·kg^{-1}).
a–h – means within a column with different superscript letters are significantly different ($P < 0,05$); x, y, z – means within a row with different superscript letters are significantly different ($P < 0,05$).

Tab. 7. Changes in succinic acid content in fermented cereal and pseudocereal suspensions.

Substrate	Succinic acid [mg·kg^{-1}]					
	Initial density 5 log CFU·g^{-1}			Initial density 6 log CFU·g^{-1}		
	0 h	24 h	48 h	0 h	24 h	48 h
RF	ND	ND	ND	ND	ND	ND
RG	ND	ND	ND	ND	ND	ND
BF	ND	ND	ND	ND	ND	ND
WBF	ND	ND	ND	ND	ND	ND
AF	$124.8 \pm 11.46^{b,z}$	50.3 ± 1.52^{y}	37.2 ± 1.52^{x}	$77.5 \pm 7.89^{b,y}$	$34.5 \pm 1.52^{a,x}$	$34.5 \pm 1.52^{a,x}$
AG	ND	ND	ND	$57.3 \pm 3.04^{a,y}$	$39.8 \pm 5.47^{a,x}$	ND
BWF	73.0 ± 9.95^{a}	ND	ND	ND	$39.8 \pm 8.03^{a,x}$	$40.7 \pm 6.95^{a,x}$
WOF	ND	ND	ND	ND	ND	ND
MG	ND	ND	ND	ND	ND	80.1 ± 7.43^{b}

Refer to Tab. 1 for substrates. The results are means ± standard deviation (n = 3).
ND – not detected (detection limit for succinic acid was 30 mg·kg^{-1}).
a–b – means within a column with different superscript letters are significantly different ($P < 0,05$); x, y, z – means within a row with different superscript letters are significantly different ($P < 0,05$).

mentation, fermentation by *Lb. plantarum* and *Lb. fermentum* during 4 h [34, 35].

Formic acid was detected in all fermented substrates, except for amaranth and millet grains at low inoculation level (Tab. 6). Content of formic acid at the end of the fermentation period ranged from 27.15 mg·kg^{-1} to 53.84 mg·kg^{-1} for samples inoculated at 5 log CFU·g^{-1}, and from 30.27 mg·kg^{-1} to 153.47 mg·kg^{-1} for substrates at higher inoculation level. Production of formic acid was also observed in case of fermented millet, sorghum, maize and maize-sorghum products. Content of formic acid after 24 h natural fermentation process ranged from 30 mg·kg^{-1} to 70 mg·kg^{-1}, fermentation by various lactic acid bacteria being similar [34, 35].

The content of succinic acid was also followed during the fermentation process (Tab. 7). It was detected only in few substrates: amaranth flour, whole buckwheat flour and amaranth grain at both inoculation levels, and millet grain at higher inoculation level. During fermentation of millet, sorghum, maize and maize-sorghum substrates (naturally fermented or with selected lactic acid bacteria), a reduction of succinic acid was observed [34, 35].

Relations between chemical composition of substrates and fermentation parameters

According to Helland et al. [30], fermentation of cereals and pseudocereals is influenced mainly by contents of fermentable substrate, nutrients, growth factors and minerals. The main characteristic that influences the growth of microbial cultures is availability of water, measured as water activity (a_w). a_w-values of dry flour or grain were low, as required to ensure the stability of flour and grain during storage. Adding water to create optimal a_w was the starting point for flour fermentation. For growing of cultures lactic acid bacteria, content of reducing saccharides is a limiting factor, because saccharides are important for metabolic activity of lactic acid bacteria. In comparison to milk, which is the most used substrate for lactic acid fermentation, cereals and pseudocereals have lower contents of saccharides . On the other hand, cereals and pseudocereals contain more vitamins and minerals, which are also necessary for growth and metabolism of cultures of lactic acid bacteria [30, 38].

Considering the influence of chemical composition of the substrate on fermentation, there was no evidence of impact of saccharides, proteins or lipids content on density of *Lb. rhamnosus* GG at the end of fermentation process in substrates at both inoculation levels (Fig. 3 and Fig. 4).

Contents of proteins and lipids in substrates did not influence pH value of fermented products at both inoculation levels at the end of the process (Fig. 3B, 3C and Fig. 4B, 4C). Higher contents of reducing saccharides in amaranth flour (in comparison to other cereals and pseudocereals) had a positive impact on the decrease of pH at the end of the process in case of higher inoculation density of *Lb. rhamnosus* GG (Fig. 3A and Fig. 4A).

Influence of the contents of reducing saccharides on titratable acidity at the end of the process was significant only in case of amaranth flour, in

which content of reducing saccharides was evidently higher than in other substrates (Fig. 3A and Fig. 4A). Higher content of proteins influenced the final titratable acidity only in case of amaranth flour, but not in case of amaranth grain, in which amount of proteins was similar (Fig. 3B and Fig. 4B). Similar situation was at the effect of the content of lipids on titratable acidity (Fig. 3C and Fig. 4C).

Influence of the content of saccharides on production of organic acids was significant mainly in case of lactic acid production. It was mainly clear

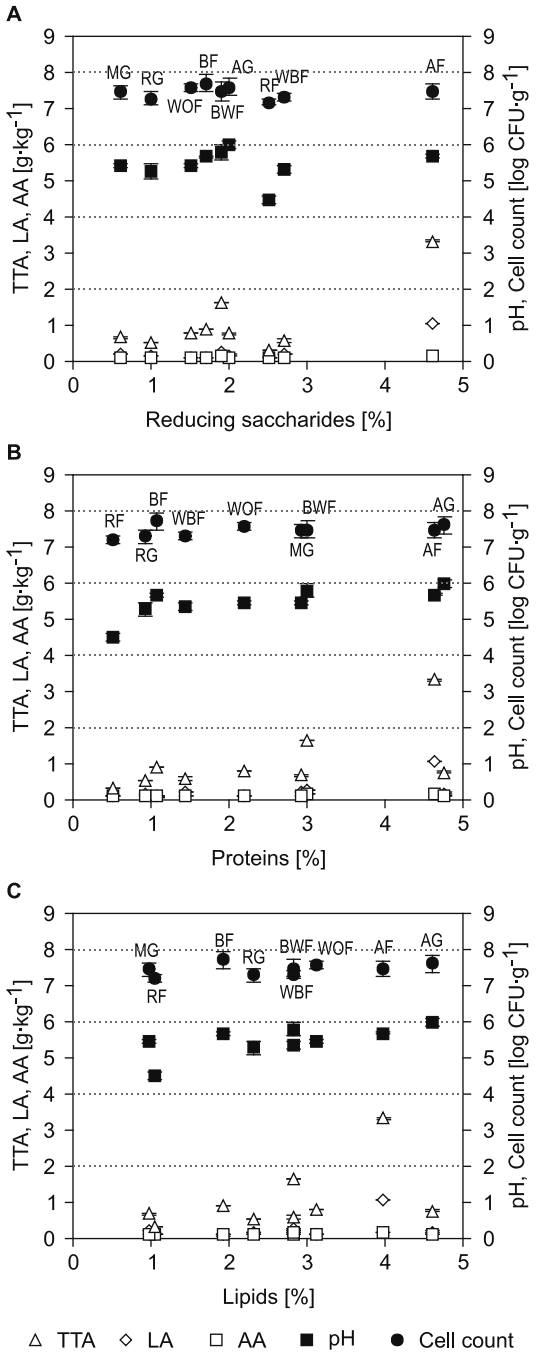

Fig. 3. Influence of chemical composition on fermentation at inoculation level 5 log CFU·g^{-1}.

Results are means (n = 3), vertical lines represent standard deviations.
TTA – titratable acidity, LA – lactic acid, AA – acetic acid.

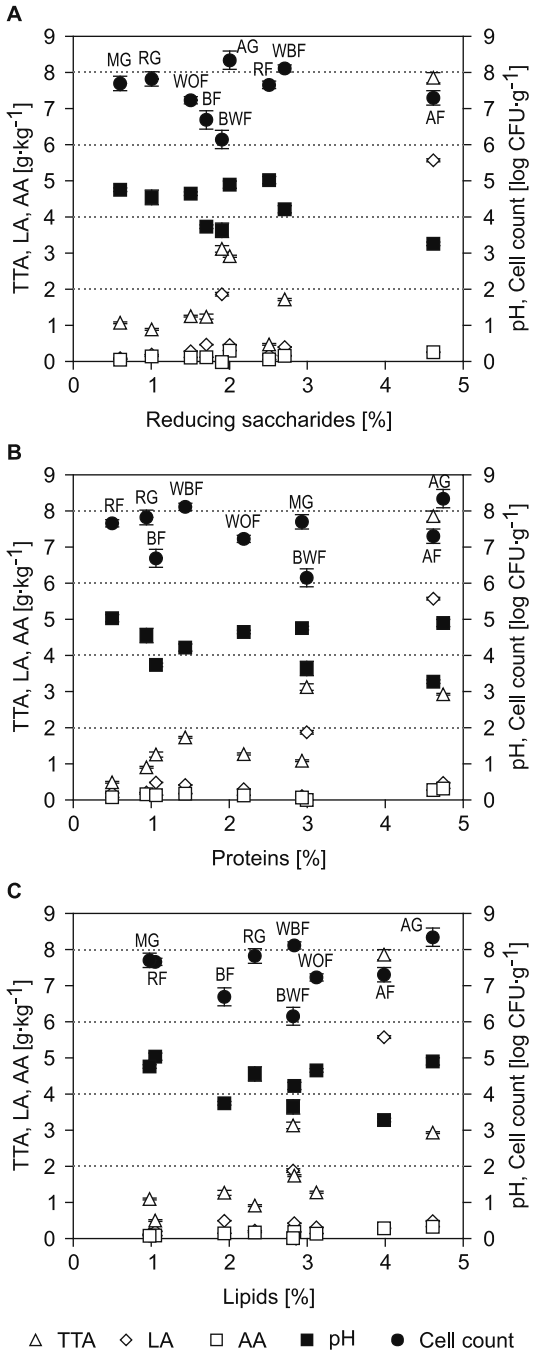

Fig. 4. Influence of chemical composition on fermentation at inoculation level 6 log CFU·g^{-1}.

Results are means (n = 3), vertical lines represent standard deviations.
TTA – titratable acidity, LA – lactic acid, AA – acetic acid.

at both inoculation levels in case of amaranth flour, which is richer in reducing saccharides in comparison to other substrates (Fig. 3A and Fig. 4A). Content of proteins also influenced the production of lactic acid in case of amaranth flour, but no influence was observed in case of amaranth grain, similar to the situation at total titratable acidity (Fig. 3B and Fig. 4B). The same situation was observed at the influence of the contents of lipids on the production of lactic acid. Impact of chemical composition on the production of other acids was not observed (Fig. 3C and Fig 4C).

CONCLUSIONS

The probiotic strain *Lactobacillus rhamnosus* GG was able to grow and metabolize in cereal and pseudocereal substrates. No significant differences were observed in cell counts during the fermentation process among different samples at both inoculation levels. The highest culture density was reached in substrates prepared from whole barley flour and amaranth grain at higher inoculation rate. Culture density in the beginning of fermentation had a significant influence on metabolic activity of *Lb. rhamnosus* GG. In substrates inoculated at higher density, pH value dropped rapidly, in substrates as barley, amaranth and whole buckwheat flour even to below 4.0. Influence of inoculation density was significant in production of organic acids, in particular lactic acid. In substrates prepared from amaranth flour and grain, whole buckwheat flour and whole barley flour, at higher inoculation level the titratable acidity was several times higher in comparison of the value reached at the lower inoculation level. Production of lactic acid was faster in substrates at the higher inoculation level. Production of acetic acid was significantly higher only in substrates from amaranth flour and amaranth grain. No influence of substrates and inoculation level on the production of formic, citric and succinic acids was observed. Impact of chemical composition of cereals and pseudocereals on the metabolic activity of *Lb. rhamnosus* GG was observed only in case of amaranth flour, in which the comparatively higher content of reducing saccharides, proteins and lipids had a positive effect on the metabolic activity. According to these results, none of the studied substrates will be excluded from further development and we plan to use them for preparation of heat-treated fermented products.

Acknowledgements

The strain of *Lb. rhamnosus* GG was provided by Dr. Salminen (University of Turku, Turku, Finland) through mediation of Dr. Lauková (State Veterinary and Food Institute, Košice, Slovakia).

The work was supported by The Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic for the Structural Funds of EU, Operational Programme Research and Development of European Regional Development Fund in frames of the project "Evaluation of natural substances and their selection for prevention and treatment of lifestyle diseases" (ITMS 26240220040) and The Scientific Grant Agency in project "The mutual relations among undesirable and health promoting microorganisms in cereal and milk matrices fermented by lactic acid bacteria: the quantitative analysis leading to the development of fermented products for people with nutritional handicaps" (VEGA 1/0495/13).

This work was also supported by Slovak Research and Development Agency in projects: "Chemical, biological and engineering aspects of research and preparation of cereal based health beneficial food products with extended shelf life" (APVV VMSP-II-024-09).

Financial support for the project was from the Slovak University of Technology Programme for the young researchers support: "Potential of cereals, pseudocereals and leguminous substrates for growth and metabolic activity of probiotic strains" (ZAK 6425).

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Received 28 January 2013; revised 14 February 2013; accepted 15 February 2013.