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EVALUATION OF LEGUMES AS A SUBSTRATE FOR PROBIOTIC STRAIN *LACTOBACILLUS RHAMNOSUS* GG

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The aim of this study was to evaluate suitability of legumes as carriers for probiotic strain *Lactobacillus rhamnosus* GG, leading to the development of new probiotic foods for consumers who have to restrict or dislike dairy products. The growth and metabolic activity of the probiotic strain *Lactobacillus rhamnosus* GG during fermentation of water-based leguminous porridges, prepared from soy bean, soy flour, green lentil, husked lentil, white bean, speckled bean, red bean, yellow pea, chickpea, and chickpea flour, were monitored. Viable cell counts, pH values, and contents of organic acids were analysed during static fermentation of autoclaved substrates at 37 °C for 48 h. *Lactobacillus rhamnosus* GG was able to grow up to the counts higher than 6 log CFU g^{-1} (measured values in the range of 7.8–8.9 log CFU g^{-1}), which is legislative limit for labelling food as probiotic. pH values of fermented substrates varied between 4.0–6.0, concentration of lactic acid ranged from 99.9 to 687.7 mg kg^{-1}, and level of acetic acid varied from 266.1 to 1182.0 mg kg^{-1}.

Keywords: legumes, fermentation, *Lactobacillus rhamnosus* GG, probiotic

Legumes are plants (legume family; *Leguminosae*) that produce seeds for human consumption. They are good sources of proteins, carbohydrates, vitamins, and minerals and play important role in human nutrition. They are subdivided into: pulses, with high level of carbohydrates and low amount of lipids, and leguminous oilseeds, with higher lipid content and lower carbohydrate level (Michaels, 2004; Boschin & Arnoldi, 2011; Sreerama et al., 2012). Legumes are consumed mainly in developing countries, in which they substitute animal proteins in diet. Legumes in daily diet have physiological effects in controlling or preventing some metabolic diseases due to high content of dietary fibre (Tharanathan & Mahadevamma, 2003).

Legumes are consumed after some processing, e.g. dehulling, soaking, germination, heat treatment, fermentation, or combination of these (Tharanathan & Mahadevamma, 2003). Fermentation is probably one of the oldest methods of processing legumes. Fermented leguminous products are widespread mainly in Asian and African countries. The fermentation is spontaneous, caused by natural microflora, often under unhygienic conditions. Controlled fermentation could lead to production of safe foods, with higher nutritional and sensory quality and extended shelf-life of end products, which fulfil consumers' requirements for natural, minimally processed, and additive-free products (Frias et al., 2005). During fermentation process, reduction of anti-nutritional factors (oligosaccharides, allergenic proteins, proteinase inhibitors, lectins, and cyanogenic glycosides) and immunoreactivity can occur (Yadav & Khetarpaul, 1994; Egounlety & Aworh, 2003; Song et al., 2008). In

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additional, fermented legume foods could be carrier of probiotic bacteria and variegate market with probiotic foods, which are usually milk based and thus not suitable for people who have to restrict dairy product consumption (due to lactose intolerance, cow's milk allergy, low-protein diet, phenylketonuria) or who dislike dairy products (Němečková et al., 2011).

Lactobacillus rhamnosus belongs to the group of heterofermentative lactic acid bacteria. Evidence of the ability of *Lb. rhamnosus* to tolerate presence of bile salts and ability to survive during gastric and duodenal digestion exist. *Lactobacillus rhamnosus* GG effectively colonizes the gastrointestinal tract after three days of use (Görner & Valík, 2004; Succi et al., 2005; Lam et al., 2007; Pitino et al., 2010). According to Valík and co-workers (2008) and Kocková and co-workers (2013a; 2013b), *Lb. rhamnosus* GG has good growth properties in milk, cereal, and pseudocereal substrates. Based on these results and the increasing interest in plant probiotic foods, we decided to evaluate growth and metabolic activity of *Lb. rhamnosus* GG in leguminous porridges. Moreover, this kind of products could extend offers of probiotic foods on market, which could be suitable for people who have to restrict consumption or dislike the dairy products.

1. Materials and methods

1.1. Materials

Soybean flour, whole soybean, and chickpea flour obtained from mill house (Mill house Zrno, Šíšov, Slovakia), and green lentil, white bean, speckled bean (Svitko, Banská Bystrica, Slovakia), red bean, chickpea (Lagris, Dolní Lhota u Luhačovic, Czech Republic), husked lentil (Marianna, Ivánka pri Dunaji, Slovakia), and yellow pea (Kroner, Bratislava, Slovakia) obtained from market were used in this work.

1.2. Microorganisms, inoculation, and cultivation conditions

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

1.3. Preparation of media and fermentation process

Twenty grams of milled and sieved (0.5 mm sieve size) leguminous sample were mixed with 180 ml of distiller water and autoclaved (121 °C, 15 min). After cooling, inoculation with overnight culture of *Lb. rhamnosus* GG at initial density of cells at approximately 5 log colony forming units per gram (CFU g^{-1}) was done. Fermentation was done for 48 h at 37 °C. Samples for determination of viable counts, pH value, and organic acid content were collected every 24 h.

1.4. Microbial analysis

Bacterial counts were determined on MRS-agar (Merck, Darmstadt, Germany), according to the Slovak Technical Standard STN ISO (2002) (spread-plate method).

1.5. Chemical analysis

The pH of gruels was measured by pH-meter CG 843 (Schott, Mainz, Germany). The quality and quantity of the organic acids (lactic, acetic, and citric) were measured by isotachophoretic analysis by Isotachophoretic Analyzer ZKI 01 (Villa Labeco, Spišská Nová Ves, Slovak Republic) with electrolytic system according to KOCKOVÁ and co-workers (2013a).

1.6. Statistical analysis

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

2. Results and discussion

For evaluation of leguminous suitability for fermentation technology, the fermentation experiments were realized. Changes in the number of viable cells counts, pH value, titratable acidity, and organic acids concentration during a 48-h fermentation process were studied.

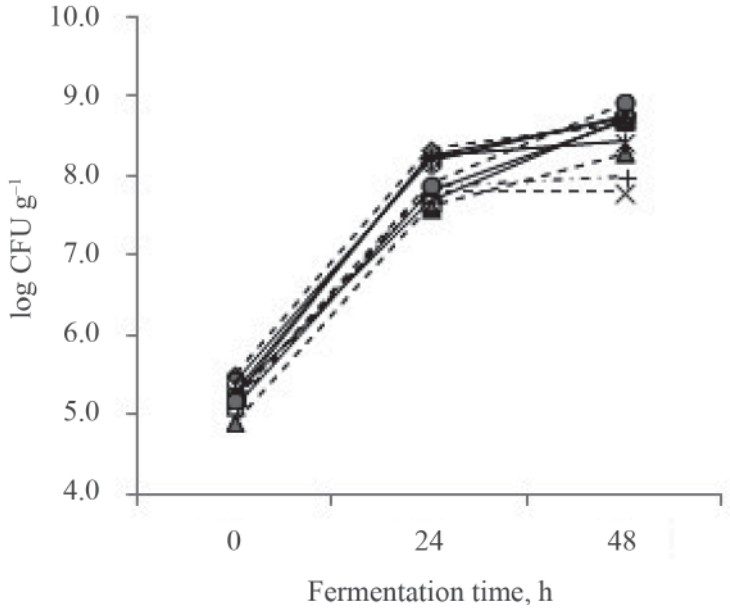

Fig. 1. Cell counts of *Lb. rhamnosus* GG during fermentation of leguminous substrates
The results are means (n=3). SF: soya flour; SW: soya whole; GL: green lentil; HL: husked lentil; WB: white bean; SB: speckled bean; RB: red bean; YP: yellow pea; C: chickpea whole; CF: chickpea flour

2.1. Growth of Lb. rhamnosus *GG*

According to the results shown in Fig. 1, all substrates were suitable for growing of *Lb. rhamnosus* GG. At the beginning of the fermentation process, the density of cells ranged from 4.9 to 5.5 log CFU g^{-1}. At the end of the fermentation process, the population density ranged from 7.8 to 8.9 log CFU g^{-1}, which is similar to the growth properties of *Lb. rhamnosus* GG in cereal substrates (Kocková et al., 2013a; 2013b), cereal water-based puddings (Helland et al., 2004a), maize porridges with malt addition (Helland et al., 2004b), and milk (Valík et al., 2008). In vegetable substrates fermented by mesophilic culture CCDM17, *Lb. delbrueckii* CCDM707 and *Lb. fermentum* CCDM154, microbial density reached about 7–8 log CFU g^{-1} (Němečková et al., 2011). According to Pelikánová and co-workers (2011) *Lb. rhamnosus* GG showed the best growing properties in amaranth and buckwheat water- and milk-based puddings in comparison to other probiotic strains. In addition, *Lb. rhamnosus* GG is able to survive in cereal substrates during cold storage (Helland et al., 2004a; Pelikánová et al., 2011; Kocková et al., 2013b).

2.2. Metabolic activity of Lb. rhamnosus *GG*

The metabolic activity of *Lb. rhamnosus* GG in leguminous substrates led to the decrease of pH values (Fig. 2). The pH values dropped from the initial 5.7–6.4 to a final 4.0–6.0. In cereal and pseudocereal substrates, fermented by the same probiotic strain, the decrease in pH values was more significant (Kocková et al., 2013a; 2013b). Even though, acidification caused by metabolic activity of *Lb. rhamnosus* GG was not significant compared to the other lactic acid bacteria in similar substrates. For example, pH value of malt, barley, and malt-barley porridges fermented by *Lb. acidophilus* and *Lb. plantarum* decrease below 3.5 (Rathore et al., 2012). Final pH in fermented vegetable substrates varied between 3.7–4.5 (Němečková et al., 2011).

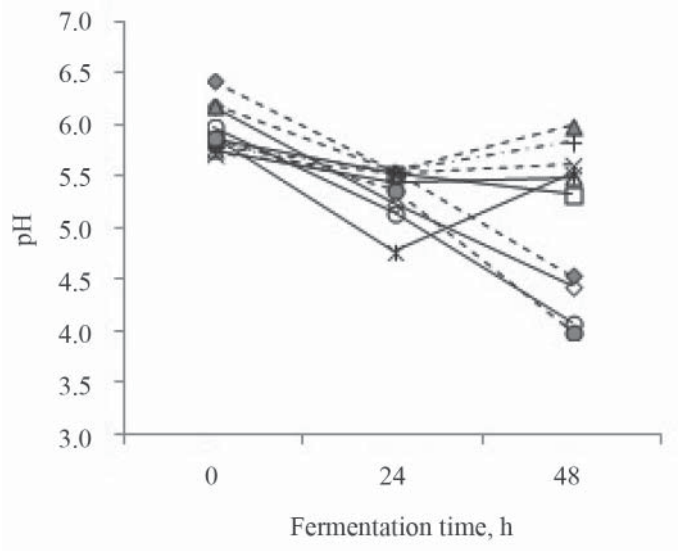

Fig. 2. pH values during fermentation of leguminous substrates
The results are means (n=3). SF: soya flour ; SW: soya whole ; GL: green lentil ; HL: husked lentil ; WB: white bean ; SB: speckled bean ; RB: red bean ; YP: yellow pea ; C: chickpea whole ; CF: chickpea flour

Organic acids produced during the fermentation process were qualified and quantified by isotachophoretic method (Tables 1–3).

The initial lactic acid level ranged from 99.9 to 687.7 mg kg^{-1}. The concentration of lactic acid at the end of the fermentation process ranged from 76.3 to 3444.3 mg kg^{-1}. Production of lactic acid in cereal substrates (Kocková et al., 2013a; 2013b) and milk (Valík et al., 2008) fermented by the same probiotic strain was lower compared to leguminous substrates. According to Helland and co-workers (2004a), lactic acid content in water-based cereal puddings fermented by *Lb. acidophilus* La5, *Lb. acidophilus* NCIMB 701748, *Bifidobacterium animalis* Bb12 and *Lb. rhamnosus* GG was from 560 to 2600 mg kg^{-1}. Addition of milk to these puddings increased lactic acid levels to 4300 to 9800 mg kg^{-1}. Concentration of lactic acid in malt-barley porridges fermented by *Lb. reuteri* SD 2112, *Lb. acidophilus* LA5, *Lb. acidophilus* NCDO 1748, and *Lb. rhamnosus* GG ranged from 1300 to 4000 mg kg^{-1}. According to the used starter cultures, the highest value was measured in case of *Lb. rhamnosus* GG (Helland et al., 2004b).

Production of acetic acid was higher compared to lactic acid production, except substrates prepared from soya flour, whole soya, chickpea flour, and chickpea. The acetic acid content increased from the initial 158.9–332.8 mg kg^{-1} to a final 266.1–1182.0 mg kg^{-1}, which was higher than in case of cereal and pseudocereal substrates (Kocková et al., 2013a; 2013b).

Lb. rhamnosus GG is citrate negative during growing in milk (Østlie et al., 2003). However, reduction of citric acid in cereal and pseudocereal substrates (Kocková et al., 2013a; 2013b), water- and milk-based cereal puddings (Helland et al., 2004a), and malt-maize porridges (Helland et al., 2004b) fermented by *Lb. rhamnosus* GG was observed. In leguminous substrates, reduction in citric acid content was observed, except those prepared from white, speckled, and red beans, probably due to higher content of glucose, which may regulate the citrate permease (Jyoti et al., 2004). Reduction of citric acid occurs from the initial 632.1–1620.2 mg kg^{-1} to a final 231.2–469.2 mg kg^{-1}, the citric acid content in chickpea flour was under detection limit.

Table 1. Production of lactic acid during fermentation of leguminous substrates by *Lb. rhamnosus* GG. The results are means (n=3)

Substrate	0 h	24 h	48 h
SF	$130.4\pm6.7^{c,x}$	$1159.9\pm7.1^{h,y}$	$1899.4\pm4.4^{f,z}$
WS	$216.8\pm5.8^{e,x}$	$1124.0\pm7.6^{g,y}$	$2573.0\pm11.1^{g,z}$
GL	$99.9\pm3.5^{b,x}$	$213.5\pm2.3^{a,z}$	$134.3\pm5.8^{b,y}$
HL	$102.6\pm9.6^{b,y}$	$534.6\pm15.4^{e,z}$	$76.3\pm5.8^{a,x}$
WB	$209.7\pm3.5^{e,x}$	$199.1\pm14.0^{a,x}$	$225.0\pm4.0^{c,x}$
SB	–	$347.7\pm8.0^{c,x}$	$300.4\pm23.9^{d,x}$
RB	$158.7\pm6.9^{d,x}$	$300.4\pm15.8^{b,y}$	$285.2\pm6.6^{d,y}$
YP	$687.7\pm3.5^{a,x}$	$302.7\pm10.5^{b,y}$	$455.9\pm2.3^{e,z}$
C	$103.3\pm18.9^{b,x}$	$671.5\pm4.4^{f,y}$	$3444.3\pm22.0^{h,z}$
CF	$102.3\pm1.3^{b,x}$	$493.3\pm3.5^{d,y}$	$2024.0\pm114.4^{f,z}$

SF: soya flour; WS: whole soya; GL: green lentil; HL: husked lentil; WB: white bean; SB: speckled bean; RB: red bean; YP: yellow pea; C: chickpea; CF: chickpea flour. $^{a–h}$: 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 ($P<0.05$)

Table 2. Production of acetic acid during fermentation of leguminous substrates by *Lb. rhamnosus* GG. The results are means (n=3)

Substrate	0 h	24 h	48 h
SF	$158.9\pm7.2^{a,x}$	$600.3\pm3.8^{d,y}$	$786.2\pm2.8^{c,z}$
WS	$269.8\pm5.2^{d,x}$	$934.3\pm37.7^{f,y}$	$888.2\pm4.5^{d,y}$
GL	$254.0\pm35.5^{d,x}$	$426.3\pm8.4^{a,y}$	$266.1\pm16.1^{a,x}$
HL	$172.1\pm7.3^{a,x}$	$806.0\pm34.9^{e,y}$	$930.1\pm21.3^{d,z}$
WB	$332.8\pm8.8^{d,x}$	$932.2\pm23.7^{f,z}$	$638.8\pm19.8^{b,y}$
SB	$321.7\pm14.1^{d,x}$	$1008.9\pm27.2^{g,z}$	$791.6\pm30.4^{c,y}$
RB	$262.5\pm32.6^{d,x}$	$835.5\pm45.5^{e,z}$	$642.5\pm37.5^{b,y}$
YP	$177.9\pm5.1^{a,x}$	$530.5\pm18.4^{c,y}$	$637.2\pm26.0^{b,z}$
C	$220.7\pm5.5^{c,x}$	$753.8\pm35.2^{e,y}$	$1182.0\pm29.1^{e,z}$
CF	$193.2\pm2.8^{b,x}$	$467.0\pm9.0^{b,y}$	$805.9\pm22.5^{c,z}$

SF: soya flour; WS: whole soya; GL: green lentil; HL: husked lentil; WB: white bean; SB: speckled bean; RB: red bean; YP: yellow pea; C: chickpea; CF: chickpea flour. $^{a–h}$: 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 ($P<0.05$)

Table 3. Changes of citric acid during fermentation of leguminous substrates by *Lb. rhamnosus* GG. The results are means (n=3)

Substrate	0 h	24 h	48 h
SF	$1158.7\pm20.3^{g,z}$	$358.1\pm7.0^{a,y}$	$266.0\pm19.7^{a,x}$
WS	$1620.2\pm5.2^{i,z}$	$515.3\pm15.2^{e,y}$	$231.2\pm12.2^{a,x}$
GL	$904.0\pm46.3^{f,z}$	$454.7\pm16.6^{d,y}$	$400.8\pm16.6^{b,x}$
HL	$955.5\pm44.1^{f,y}$	$437.8\pm19.7^{d,x}$	$398.5\pm5.2^{b,x}$
WB	$266.0\pm10.8^{b,x}$	$403.0\pm0.0^{c,y}$	$426.6\pm3.4^{c,z}$
SB	$553.5\pm1.9^{c,x}$	$652.3\pm8.9^{f,z}$	$623.1\pm1.9^{e,y}$
RB	$211.0\pm25.4^{a,x}$	$711.8\pm53.5^{f,y}$	$738.7\pm25.3^{f,y}$
YP	$809.5\pm14.0^{e,z}$	$408.6\pm17.0^{c,x}$	$469.2\pm5.2^{d,y}$
C	$1257.5\pm75.1^{h,z}$	$379.4\pm3.4^{b,y}$	$273.9\pm19.7^{a,x}$
CF	632.1 ± 0.0^{d}	-	-

SF: soya flour; WS: whole soya; GL: green lentil; HL: husked lentil; WB: white bean; SB: speckled bean; RB: red bean; YP: yellow pea; C: chickpea; CF: chickpea flour. $^{a–h}$: 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 ($P<0.05$)

3. Conclusions

Probiotic strain *Lactobacillus rhamnosus* GG was able to grow in selected leguminous substrates, the best growing properties were observed in substrate prepared from chickpea.

Metabolic activity of the probiotic strain was observed in all substrate, mainly in case of porridges prepared from soya flour, whole soya, chickpea flour, and chickpea. Future study will examine whether *Lb. rhamnosus* GG is able to survive in these kinds of substrates during refrigerated storage and whether these porridges are sensorically acceptable.

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