Evaluation of The Potential of Amaranth Flour for Lactic Acid Fermentation

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Abstract: Although cereals and pseudocereals are deficient in some basic components, fermentation process is the most economical and simple way, how to improve nutritional value, functional qualities and sensory properties of the final products. In our study, we focused on the evaluation of amaranth flour for preparation of new probiotic functional foods suitable for celiac patients. That is why the growth dynamics of several *Lactobacillus* sp. in amaranth mashes were evaluated. All the monitored strains showed sufficient growth in mashes (growth rates of lactobacilli ranged from 0.73 to 1.52 h^{-1}). Based on the rates, only *Lb. rhamnosus* VT1 was able to grow with the values higher than 1.38 h^{-1} in both milk and water based mashes.

In the second part of our study, we described behaviour of *Lb. rhamnosus* GG in amaranth water- or milk- based mashes after 8 h of co-cultivation with Fresco DVS 1010 culture (37 ± 1 °C, 5 % CO_2). Final counts after the fermentation reached values 10^8 CFU.ml^{-1} and no decrease was recorded during 2-week storage period at 6 ± 1 °C. Thus we may conclude that densities of lactobacilli were able to maintain above the limit of >10^6 CFU.ml^{-1} essential from the legislation point of view.

Keywords: Amaranth, pseudocereal substrates, probiotics, lactic acid bacteria, celiac disease.

INTRODUCTION

Lactic acid bacteria (LAB) are defined as a large group of heterogeneous, gram-positive, catalase-negative, non-sporeforming cocci or rods, producing lactic acid as a major catabolic product of fermentable carbohydrates. The representative species of LAB are predominantly *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* [1]. LAB are used not only as starter cultures for fermentation of different types of substrates, but also in beverage production and in manufacture of medicaments [2]. Probiotics are single or mixed cultures of live lactic acid bacteria that are associated with beneficial health effects on human being, play important role in managing of intestinal disorders, such as lactose intolerance, constipation, or inflammatory bowel diseases, and may have a significant role in immunological functions [3]. There are many requirements in the selection of suitable probiotic strain. A key factor is its ability to survive acidic environment of the final fermented products and the adverse conditions of the gastrointestinal tract. Survival of probiotic strain during gastrointestinal transit is also important when probiotics have to overcome low pH values, enzymes, bile acids and low surface tension [4, 5]. Most probiotic foods on market are dairy based, however recent studies have been focused on non-dairy variant of probiotic fermented cereal and pseudocereal products [6]. Natural fermentation of cereals by lactic acid bacteria brings a wide range of benefits, including extension of shelf life, decreasing in the level of non-digestible poly- and oligosaccharides or carbohydrates and provides optimum pH conditions for enzymatic degradation of phytate that may increase the amount of soluble iron, calcium and zinc [7]. Lactic acid bacteria also improve organoleptic quality – taste and flavour of final products by producing alcohols, organic acids or carbonyl substances [8]. Products such as sake, cereal beer, and spirits are known all over the world and new types of fermented cereal foods are continuously being developed [6]. As described above, there is a considerable potential in manufacturing fermented functional products for specific groups of consumers that are cereals based. The main aim of this work was to evaluate important growth characteristics of selected lactic acid bacteria in amaranth substrates. Remaining of viable probiotic bacteria in final products is important since products may be consumed refrigerated after several weeks of storage. Thus, final amaranth products were therefore stored and analysed for viable cell counts of probiotic strain *Lb. rhamnosus* GG.

MATERIALS AND METHODS

Microorganisms, Inoculation and Cultivation Conditions

Fresco DVS 1010 culture (consists of *Lactococcus lactis* spp. *lactis*, *L. lactis* spp. *cremoris, Streptococcus*

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thermophilus) and *Lactobacillus acidophilus* 145 are commercial cultures from Christian and Hansen (Hørsholm, Denmark).

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

Potentially probiotic strain *Lb. rhamnosus* VT1, isolated from tartar sauce, from the microbial collection of Institute of Chemical Technology (Prague, Czech Republic), was provided for this study by doc. Ing. M. Plocková, PhD.

Strain *Lb. paracasei* subsp. *paracasei* was obtained from the collection of strains of Masaryk University (Brno, Czech Republic).

Fresco DVS 1010 culture was kept in a deep-freezer. Pure cultures of lactobacilli were maintained in MRS broth (Biokar Diagnostics, Beauvais, France) at 5 ± 1 °C.

The starter culture was prepared from a 24 h culture of selected strain of lactobacilli grown in MRS broth at 37 ± 1 °C (5 % CO_2). The standard suspension of Fresco culture was prepared from a 24 h culture grown in M17 broth (Biokar Diagnostics, Beauvais, France) and was incubated at 30 °C. 24 h starter cultures were centrifuged (6000 rpm) for 5 min, washed in 10 ml of sterile distilled water and centrifuged again under the same conditions. After centrifugation, supernatant was decanted and pellets were re-suspended in distilled water to its original volume [9].

Amaranth Substrate Preparation, Fermentation, and Storage

Pseudocereal mashes were prepared from amaranth flour (Mlyn Zrno, Šišov, Slovakia). To maintain the same consistency, milk based mashes were prepared from 14 % of amaranth flour, while the content of flour in water mashes was higher (20 %) with addition of 2 % sucrose in both.

After boiling for 20 min and autoclaving at 121 °C (20 min) the mashes were cooled down and inoculated with the starter culture of selected strains of lactobacilli to achieve approximately initial number of 10^3 CFU.ml^{-1}.

In case of co-cultivation, amaranth mashes were inoculated with starter Fresco DVS 1010 culture and *Lactobacillus rhamnosus* GG to achieve approximately 10^5 to 10^6 CFU.ml^{-1}. Static fermentations were performed for 14 hours at 37 ± 1 °C (5 % CO_2) and were carried out in duplicate trials. The samples for analyses of counts and pH values were measured every 2 hours. After 8 h of co-cultivation pseudocereal amaranth mashes were carried out and stored at 6 ± 1 °C for 14 days.

Viable Cell Enumeration

Viable counts of lactobacilli were determined using ten-fold dilutions on Vegiton MRS agar plates (Sigma-Aldrich Chemie GmbH, Switzerland) and counts of cocci from Fresco DVS 1010 culture on M17 agar plates (Biokar Diagnostics, Beauvais, France) in accordance with ISO 15214 [10]. Inoculated Petri dishes with lactobacilli were cultivated at 37 ± 1 °C (5 % CO_2) for 48 hours and mesophilic Fresco culture at aerobic conditions for 24 hours (30 ± 1 °C).

Evaluation of Growth and pH Values

Growth parameters of lactic acid bacteria in amaranth mashes were fitted and calculated using the mechanistic model DMFit by Baranyi and Roberts (1994) [11]. Growth and metabolic parameters were calculated from each growth curve. Specific growth rates (μ) were recalculated from the growth rates (g) according the equation $\mu = \ln 10 \times g$.

The pH levels were measured during fermentation and storage using a pH meter with a penetration electrode (Knick Portamess, Berlin, Germany).

Statistical Analysis

Specific growth rates of 4 tested lactobacilli strains were statistically evaluated using the Analyse-it Method Validation package ver. 3.50 (Analyse-it Software, Leeds, United Kingdom). Obtained growth rates of 4 tested lactic acid bacteria were analysed using Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA). The data were treated by Student t-test with the least significant difference of 95 %.

RESULTS AND DISCUSSION

The aim of this study was to determine and compare the growth characteristics of probiotic and potentially probiotic strains in amaranth milk or water based mashes with sucrose. The growth and acidity curves of tested strain *Lactobacillus rhamnosus* GG are shown in Figure **1** and all calculated growth parameters of observed strains are summarized in Table **1**. All strains investigated, attained high cell

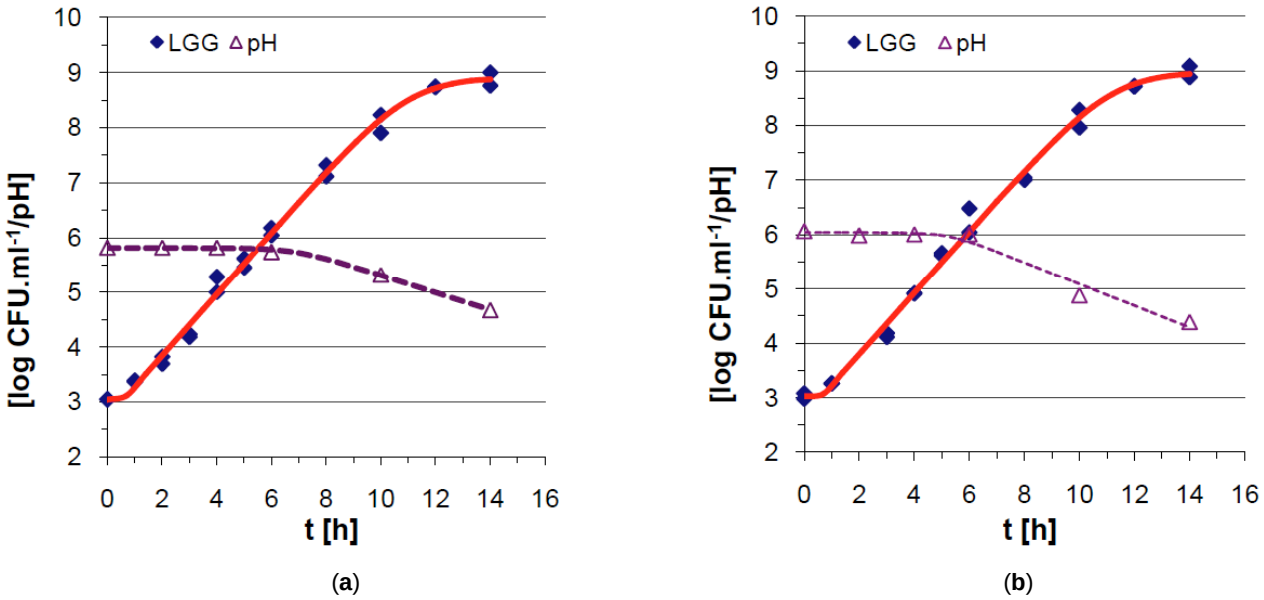

Figure 1: Growth dynamics and acidity changes of *Lb. rhamnosus* GG in milk based-amaranth (**a**) and water based-amaranth (**b**) mash during fermentation at 37 ± 1 °C.

populations when growing in amaranth mashes at optimal temperature 37 ± 1 °C, reaching maximum densities of 10^7 – 10^8 CFU.ml^{-1} from initial 10^3 CFU.ml^{-1} in 14 hours. Helland *et al*. (2004) [6] evaluated growth dynamics of *Lactobacillus rhamnosus* GG in mashes prepared by combining rice and maize flour that after 12 h of fermentation at 37 °C observed densities varied from 10^8 to 10^9 CFU.g^{-1}.

Salmerón *et al*. (2014) [12] evaluated growth of *Lb. plantarum* in non-dairy cereal beverages during fermentation at 37 °C for 10 h. The highest density of observed strain was evaluated in the oat and barley media (N_0 = 8.2 log CFU·ml^{-1} and N_B = 7.9 log CFU·ml^{-1}), respectively.

Both amaranth mashes were suitable for the growth of lactobacilli when growth rates in milk mashes ranged from 0.76 to 1.43 h^{-1} and in water based mashes from 0.73 to 1.52 h^{-1}. Despite the claim that lactobacilli require for their growth complex nutrients such as carbohydrates, amino acids, peptides, vitamins, especially those of B group and minerals present more in milk, the growth rates in milk amaranth mashes were similar to those in water. This fact was confirmed also in the study by Pelikánová *et al*. (2011a) [5].

In both mashes, *Lb. rhamnosus* VT1 entered immediately into the exponential phase of growth and showed the highest specific growth rates of all tested strains. In milk mash, growth rate of strain VT1 was characterized about 6 % slower than in water product (μ = 1.52 h^{-1}). No important difference between the growth rates of probiotic strain *Lb. rhamnosus* GG in amaranth milk and water mash (1.29 h^{-1} and 1.30 h^{-1}) was determined, respectively.

Farnworth *et al*. (2007) [13] also reported *Lb. rhamnosus* GG reaching density higher than 10^8 CFU.ml^{-1} in soy beverage at 41 ± 1 °C/12 h what was finally evaluated as better in comparison with milk (2% fat).

For the strain *Lb. acidophilus* 145, the greatest difference in growth between milk and water amaranth mash (18 %) was recorded. The lowest growth rate was calculated in case of *Lb. paracasei* subsp. *paracasei* 1753 in water product (0.73 h^{-1}). Kask *et al*. (2003) [14] evaluated maximal specific growth rate of *Lb. paracasei* in MRS broth, that ranged from 0,40 to 0,57 h^{-1}. For this strain, growth rate in milk amaranth mash compared to UHT milk (1.5% fat content) was about 39 % higher [15]. Amaranth is a good source of proteins, amino acids, minerals and vitamins [16]. This fact indicates that the composition of flour promotes the growth of *Lb. paracasei* subsp. *paracasei* 1753.

Presented results show changes in pH during a fermentation period of 14 h, when final pH ranged between 5.52 – 4.39 in water mashes that was comparable to milk mashes (5.73 – 4.67). Pelikánová *et al*. (2011a) [5] recorded changes of pH levels in amaranth substrates at 1.0 – 1.5 units depended on the strain used. Martensson *et al*. (2002) [17] reported pH 3.9 – 4.5 after fermentation (16 h) of an oat base product with commercial mixed dairy cultures.

Table 1: Growth Parameters of Tested Lactobacilli in Amaranth Mashes, 14 h Fermentation at 37 ± 1 °C

Substrate	Microorganism	μ [h^{-1}]	λ [h]	k_{pH} [h^{-1}]
Amaranth milk mash	*Lb. acidophilus* 145	1.03[a]	0.58	-0.20
	Lb. rhamnosus GG	1.29[c]	0.57	-0.16
	Lb. rhamnosus VT1	1.43[d]	-	-0.20
	Lb. paracasei subsp. *paracasei* 1753	0.76[e]	-	-0.10
Amaranth water mash	*Lb. acidophilus* 145	0.84[b]	2.09	-0.04
	Lb. rhamnosus GG	1.30[c]	0.63	-0.20
	Lb. rhamnosus VT1	1.52[d]	-	-0.21
	Lb. paracasei subsp. *paracasei* 1753	0.73[e]	2.99	-0.17

μ – specific growth rate, λ - lag-phase duration, k_{pH} – rate constant for the decrease of pH; the same letters in the superscript indicate that three *Lactobacillus* spp. ([c, d, e]) did not showed any differences at the 0.5 % significance level between the specific growth rates in the water- and milk-based mashes

The pH levels of final products depended not only on the strain used but also on the composition of mash (milk/water). Due to lower buffering capacity of the water-based mashes, reduction in pH was faster in comparison with milk-based mashes except probiotic strain *Lb. acidophilus* 145. Amaral Santos *et al*. (2014) [18] observed a rapid drop of pH value in peanut-soy milk substrate fermented only by *Lb. acidophilus* (LACA 4), reaching pH value 4.6 at 12 h of fermentation. Determined limited pH value for survival of probiotic strain *Lb. acidophilus* ranged from 3.4 to 3.6 depending on type of substrate used [19].

The highest rate of pH decrease was found in case of *Lb. rhamnosus* VT1 (-0.21 h^{-1}) while the lowest final pH value of 4.39 was recorded in water amaranth mash fermented by *Lb. rhamnosus* GG. On the other hand, [20] determined the final pH of 4.9 in milk (1.5 % fat) fermented by *Lb. rhamnosus* GG after 10 h of fermentation process.

Kocková and Valík (2014) and Pelikánová *et al*., (2015) [21, 22] evaluated growth dynamic of *Lb. rhamnosus* GG in cereal and pseudocereal flours. Population density reached counts 10^7 to 10^8 CFU.g^{-1} at the end of 10 h fermentation process (37 ± 1 °C). Sterr *et al*. (2009) [23] have noticed the change of *Lb. plantarum* about 1 to 3 log CFU.ml^{-1} in pseudocereal product prepared form buckwheat flour to final densities (10^8 – 10^{10} KTJ.g^{-1}). In our case during 8 h of

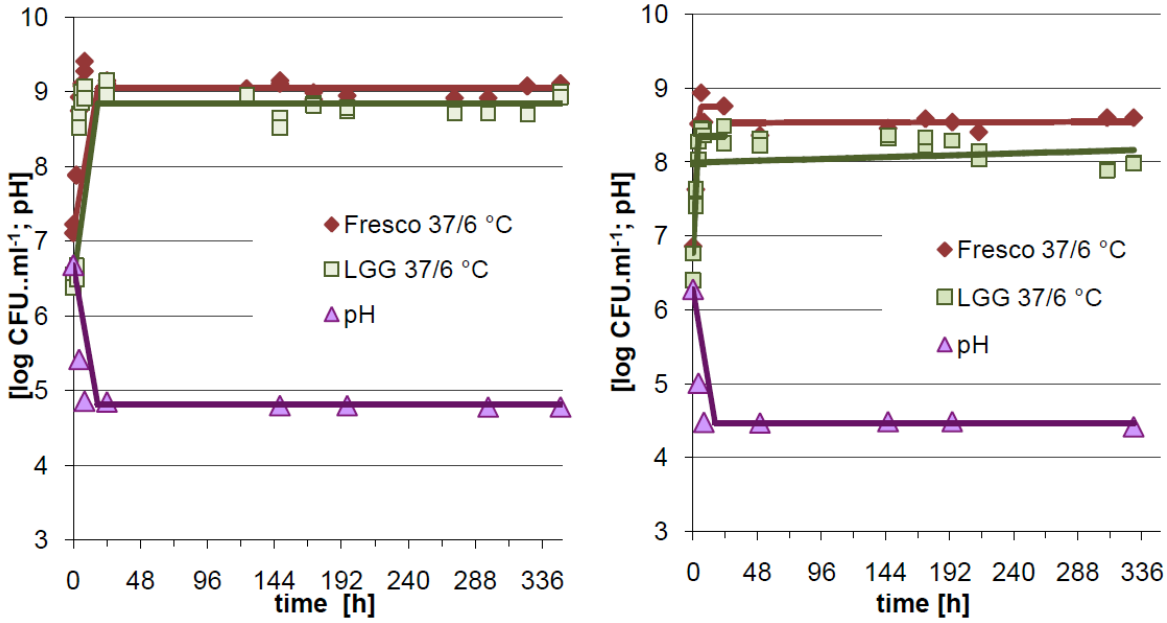

Figure 2: Evaluation of cell counts of Fresco DVS 1010 culture and *Lb. rhamnosus* GG in milk based-amaranth (**a**) and water based-amaranth (**b**) mashes during fermentation at 37 ± 1 °C and cold storage at 6 ± 1 °C.

Table 2: Growth Parameters of *Lb. rhamnosus* GG and Cocci from Fresco DVS 1010 Culture During 8 h of Co-Cultivation at 37 ± 1 °C in Amaranth Mashes

Substrate	Microorganism	μ_f [h^{-1}]	λ [h]	k_{pH} [h^{-1}]
Amaranth milk mash	Fresco DVS 1010	0.98	-	-0.32
	Lb. rhamnosus GG	2.91	2.21	
Amaranth water mash	Fresco DVS 1010	0.99	-	-0.32
	Lb. rhamnosus GG	0.99	-	

(μ_f - specific growth rate in co-culture with Fresco culture, λ – lag phase, k_{pH} – rate constant for the decrease of pH).

co-cultivation, *Lb. rhamnosus* GG was able to grow from initial counts $N_0 = 10^6$ to 10^7 CFU.ml^{-1} to final $N_{end} = 10^8$ to 10^9 CFU.ml^{-1} that was similar to density of Fresco DVS 1010 reached in both mashes (Figure **2**).

An overview of growth parameters of studied strains in water and milk based mashes is summarized in Table **2**.

Rathore *et al*. (2012) [24] observed rapid growth of *Lb. plantarum* in single malt, barley and barley-malt (mixed) substrates at 30 °C for 28 h at the beginning of fermentation (6 h). On the other hand, Leponen *et al*. (2007) [25] confirmed also in oat brans that lactic acid bacteria with dominance of *Lactobacillus rhamnosus* reached 10 log CFU.g^{-1} at 37 °C after 12-h.

The addition of milk had no positive impact on the growth rates of Fresco DVS 1010 culture, that grew at about the same in the amaranth mash based on milk (μ = 0.98 h^{-1}) and water (μ = 0.99 h^{-1}), despite of higher content of specific nutrients in milk. Fresco culture entered directly after the beginning of fermentation into the exponential phase of growth. Only in case of milk based mash, lag phase of *Lb. rhamnosus* GG was observed (2.21 h).

The highest growth rate of lactobacilli was calculated in milk mash (μ = 2.91 h^{-1}) which was characterized about 66 % higher than in water product. Viable counts of cocci from Fresco DVS 1010 and probiotic strain *Lb. rhamnosus* GG after 14 days at 6 ± 1 °C were not reduced and were similar to those reached after the fermentation process (10^8 to 10^9 CFU.ml^{-1}). Thus, density of *Lb. rhamnosus* GG after 14 days of storage was over the limit required for probiotic food > 10^6 CFU.ml^{-1} (CFU.g^{-1}). Shah *et al*. (1995) [26] observed only a slight decrease of counts of probiotic strain *Lb. acidophilus* 145 in curdled milk at 6 °C during 21 days of storage. Pelikánová *et al*. (2011a) [5] confirmed the ability of probiotic strain *Lb. rhamnosus* GG to survive in amaranth mashes for 21 days during cold storage at 6 °C.

Calculated rates of reducing pH were the same (-0.32 h^{-1}) in milk and water mash. At the end of 14 d cold storage in water mash, final pH level (4.42) dropped from initial 6.28 in milk fermented mash to final 4.86.

CONCLUSION

Our results pointed out that all four tested strains of lactobacilli showed good growth in milk and water amaranth products. In fermentation of amaranth substrates by 4 LAB strains, no statistically significant differences in impact of water or milk environment were observed, except probiotic strain *Lactobacillus acidophilus* 145.

The milk base of mashes proved to be a better substrate for growth of microbial strains *Lb. paracasei* subsp. *paracasei* 1753 and *Lb. acidophilus* 145. Potentially probiotic strain *Lb. rhamnosus* VT1 and probiotic strain *Lb. rhamnosus* GG showed the highest specific growth rates in water-based mashes. Overall, strain *Lb. rhamnosus* VT1 showed the highest growth rates in all types of substrates and it showed the fastest decrease in pH values. We also evaluated densities of probiotic strain *Lb. rhamnosus* GG during 14 days storage period. Based on the microbiological results obtained, probiotic mashes may be beneficial for consumers, if they are sensory accepted.

Such development of new non-dairy foods containing probiotics may lead to enrichment of product range suitable for handicapped consumers – people suffering from lactose intolerance, allergy to milk proteins or people on low protein diet because of the restriction in consumption of dairy products.

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