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Survival of *Lactobacillus rhamnosus* GG during Simulated Gastrointestinal Conditions Depending on Food Matrix

Riin Karu[1] & Ingrid Sumeri[1]

[1]Competence Center of Food and Fermentation Technologies, Akadeemia tee 15A, 12618 Tallinn, Estonia

Correspondence: Ingrid Sumeri, Competence Center of Food and Fermentation Technologies, Akadeemia tee 15A, 12618 Tallinn, Estonia. E-mail: ingrid@tftak.eu

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Abstract

When developing new probiotic foods, their protective properties in maintaining viability of probiotics under gastrointestinal conditions should be evaluated. In the current study, human upper gastrointestinal tract simulator (GITS) was used to compare the effect of different food matrixes on the survival of *Lactobacillus rhamnosus* GG (LGG). pH-auxostat was chosen for the cultivation of LGG cells to obtain culture samples in the same physiological state at maximum growth rate for the GITS experiments. The LGG culture was centrifuged and fast frozen in liquid nitrogen in various liquid food matrixes (commercial UHT milk, soymilk, apple juice, titrated apple juice, whey protein powder drink and M.R.S. Broth as reference) and stored at -40^{0}C. During 3-month storage, reduction of viability was significant only for apple juice. In the GITS experiments, bile had a greater negative impact on LGG than acid conditions, also the effect of food matrix was noted - in the case of milk, soymilk and whey protein powder drink only the highest concentration of bile (0.4%) caused a significant drop in the viability of bacteria when compared to apple juice. To maximize the health benefits of foodstuffs, it should be taken into account that the survival of probiotics during fast freezing, storage and gastrointestinal passage is dependent on the food matrix.

Keywords: bile, food matrix, *in vitro* gastrointestinal simulator, *Lactobacillus rhamnosus* GG, probiotic

1. Introduction

1.1 Food in Human Health

The basic function of a food is to provide vitamins, minerals and energy derived from the proteins, carbohydrates and lipids required for the well-being of the human body (Bigliardi & Galati, 2013). The emphasis on the benefits beyond the basic nutritional functions in the role of foodstuffs has given rise to the term `functional foods ´(Chen, Ma, Liang, Peng, & Zuo, 2011; Eussen *et al*., 2011).

The most important and the most frequently used functional food compounds can be listed as probiotics, prebiotics, plant antioxidants, vitamins and calcium (Grajek, Olejnik, & Sip, 2005). Probiotics are usually defined as microbial food supplements that have beneficial effects on consumers (Pang, Xie, Chen, & Hu, 2012).

1.2 Probiotics as Food Components

A probiotic must remain viable through the processing and storage of the food product prior to consumption as well as to be resistant to the gastrointestinal conditions during passage (Puupponen-Pimiä *et al.*, 2002). There are variable recommendations for the minimum viable count threshold to provide health benefits (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011; Karimi, Mortazavian, & Amiri-Rigi, 2012). The usual recommendation is that probiotic bacteria be present in products in a minimal viable concentration of 10^6 -10^7 CFU/ml (Cruz, Faria, & Van Dender, 2007; Vasiljevic & Shah, 2008), with the daily intake approximately of 10^8 CFU·g^{-1}, taking into account reductions in numbers during the gastrointestinal passage (Martins *et al.*, 2013).

Food matrix properties have a significant impact on the viability of probiotics, the most influential factors being the pH and buffering capacity, but also the chemical composition of food. The latter includes fat and carbohydrate content, concentration and type of proteins, presence of polyunsaturated fatty acids, plant extracts *etc* (Ranadheera, Baines, & Adams, 2010).

1.3 Different Food Matrixes Used for Incorporation of Probiotics

Traditionally probiotics were mainly incorporated into dairy products but lately there has been increasing interest in manufacturing non-dairy probiotic products, such as fruit juices and soy products. Effect of lactose intolerance and the cholesterol content are listed as the main disadvantages related to consumption of fermented dairy products. Also, there is a rising demand for vegetarian probiotic products (Prado, Parada, Pandey, & Soccol, 2008; Granato, Branco, Nazzaro, Cruz, and Faria, 2010). Soy products are considered to have good nutritional value for being a source for good quality proteins in high content, but also isoflavones, folic acid and some vitamins (Ng, Lye, Easa, and Liong, 2008; Kaur & Das 2011).

The protective effect of milk against low pH and bile in relation to lactic acid bacteria has been well documented (Saarela, 2011). In addition, soy milk has been reported to have protective buffering capacities as well - use of fermented soymilk as a delivery vehicle for probiotic *Lactobacillus casei* Zhang increased the tolerance to simulated gastric and intestinal juice (Wang *et al.*, 2009).

However, in case of fruit juices, the growth and the strain-specific viability of cells depends on pH, oxygen content, final acidity and the concentration of lactic acid and acetic acid of the product (Rivera-Espinoza & Gallardo-Navarro, 2010). In general, *Lb. rhamnosus* has been reported to maintain desirable viable counts when tested on fruity matrix (do Espirito Santo, Perego, Converti, & Oliveira, 2011).

Due to technological advancements high-protein and low-fat functional whey ingredients, such as whey protein concentrates have been developed (Smithers, 2008) and because of the nutritional and functional properties, whey proteins have been successfully formulated into energy and sports drinks (Wright, 2007).

1.4 Hypothesis and Research Design

The aim of the current study was to investigate the effects of various food matrixes on the survival of the probiotic bacterium *Lactobacillus rhamnosus* GG in simulated gastrointestinal conditions, at different bile concentrations.

2. Materials and Methods

2.1 Microorganisms and Media

The probiotic lactobacillus strain *Lactobacillus rhamnosus* GG isolated from Gefilus daily dose drink (Valio Ltd.) was selected for the study.

2.2 pH-auxostat Cultivation

Bacterial cultures were obtained using pH-auxostat cultivation, as described by Sumeri *et al.* (2010). In short, anaerobic cultivation was performed at 37 ℃ and pH = 6.0 with culture volume of 500 ml, using an Applikon 1 L fermenter; controlled by an ADI 1030 biocontroller (‘‘Applikon’’, The Netherlands) and cultivation control program ‘‘BioXpert’’ (‘‘Applikon’’). MRS medium (LAB M, UK) was used as feeding. Upon achieving a stable specific growth rate D = 0.8 h^{-1} sample collection from the fermenter was started.

2.3 Freezing in Liquid Food Matrix

After reaching steady state at the pH-auxostat cultivation, samples of 200 ml (collected in 50ml tubes) were taken from the fermenter, followed by a centrifugation step (10 min 8000 rpm 10^0C) and resuspending in different food matrixes in equal volume. Commercial food products were chosen – UHT milk (Tere AS, Tallinn, Estonia), soymilk (Alpro, Gent, Belgium), apple juice (AS Põltsamaa Felix, Tallinn, Estonia) and whey protein powder drink (Func Food Finland OY, Tampere, Finland) with M.R.S. Broth (Lab M Limited, Lancashire, UK) employed as the reference matrix.

Composition of the products is listed in Table 1, with the pH values determined with Mettler Toledo MP 125 pH Meter (Mettler Toledo Inc., Schwarzenbach, Switzerland).

Prior to resuspending, the pH of 200 ml apple juice (originally pH 3.30) was adjusted to pH 6.70 with 2M $NAHCO_3$ titration, to lessen the impact of acidic environment on long-term storage (in comparison, untitrated apple juice was also used). The whey protein powder drink was prepared by mixing 20 g of whey protein powder with 200 ml of destilled water (according to manufacturer´s instructions).

The bacterial suspension was fast frozen by dripping into liquid nitrogen. The resulting `probiotic beads` (1-2mm in size) were stored at -40^0C until subjected to GITS experiments.

Table 1. Composition of the food products used in the study, according to manufacturers' labels and pH measurements.

Component	Milk	Soymilk	Whey protein drink	M.R.S	Apple juice
Fat	3.2%	1.8%	8.8%	0.1%	0%
Protein	3%	3%	75%	1.4%	0.5%
Lactose	4.7%	0%	3.3%	-	0%
Sugar	-	2.5%	0.5%	2%	10.7%
Fiber	0%	0.5%	0%	-	0%
pH	6.75	6.89	6.46	6.86	3.30

2.4 Gastrointestinal Tract Simulation (GITS) Experiments

The frozen bacterial cultures were subjected to GITS experiments, as previously described by Sumeri, Arike, Adamberg, and Paalme (2008). GITS experiments were carried out with 5 separate food matrixes used for the incorporation of *Lactobacillus rhamnosus* GG and 5 different bile concentrations, tested in 3 parallels for each experimental combination - 75 experiments were carried out in total.

The simulations were carried out in a single fermenter, spatially and temporally mimicking human gastric transit. At the beginning of GITS experiment, the adult fasted stomach conditions were reproduced (100 ml of 10 mmol L^{-1} HCl), then 200 ml of bacterial culture was added, thus reaching the working volume (300 ml) of the vessel.

The next step in the simulation was titration to pH 3.0 with 1 mol L^{-1} HCl at a rate of 20 mmol h^{-1} (`gastric phase`). The ´gastric phase´ was followed by neutralization with 1 mol L^{-1} $NaHCO_3$ to pH 6.0 and subsequent addition of bile salts to simulate the passing into the duodenum, followed by a 30 minute bile incubation phase. Different bile salt concentrations (0.5% to 4%) were applied to obtain the final bile salt concentrations in the range from 0.05% to 0.4%, with a 0% bile concentration serving as a reference control.

The îleum phase´ was reproduced with 5,5h dilution (D=0.4 h^{-1}) of the fermenter contents, while the pH was kept constant (= pH 6.5) by $NaHCO_3$ titration.

In preparation for the GITS experiments, 80 g of frozen milk beads were added fresh milk up to 200 ml, mixed till melting and subjected to the GITS experiment. In case of the other food matrixes, titrated apple juice/soymilk/ whey protein powder drink was prepared as previously described and added up to 200 ml to the corresponding beads, mixed till melting and also subjected to GITS experiments.

2.5 Determination of Bacterial Viability in the GITS Experiments

Viability of bacteria was assessed by plate counts. The numbers of colony-forming units (cfu/ml^{-1}) were determined by counting colonies from the serial dilutions plated on M.R.S. agar (LAB M, UK) using a pour-plate method (de Man, Rogosa, & Sharpe, 1960). Viable bacterial counts were determined from the fresh pH auxostat culture, before and after freezing in liquid nitrogen and also from several points during the GIT simulation (inoculum, pH 3, bile, bile 30 min, end of 5,5h dilution phase).

The survival rate of *Lactobacillus rhamnosus* GG was calculated as the difference in the viable bacterial counts (Log (cfu/ml)) at the beginning and the end of the GITS experiments, while the final counts were normalized taking into account the variation of inocula at each experiment.

The viability was calculated as concentration of the bacteria in the fermenter at time t - CFU_t (cfu/ml) as shown in:

$$CFU_t = CFU_{t-1} + (CFU_t * \mu/60) \quad (1)$$

During the dilution GITS phases (after the 30min bile incubation) the correction for the dilution was taken into account according to the specific growth rate μ ($1/h^{-1}$):

$$\mu = D + ((rate(CFU)/CFU_{t+1}) * 60) \quad (2)$$

where rate(CFU) is calculated according to:

$$rate(CFU) = (CFU_{t+1} - CFU_t)/(t_{t+1} - t_t) \quad (3)$$

The viability calculations were carried out with the BioXpert software followed by normalization of data in Excel.

3. Results

3.1 The Effects of Fast Freezing in Different Food Matrixes

During pH-auxostat cultivation the physiologically stable state of the *Lactobacillus rhamnosus* GG cells was achieved and maintained during continuous culture. The constant specific growth rate (D) was reached after flow through of five working volumes (500ml), at which point sample collecting was started.

The experiments were divided into two series – *Lactobacillus rhamnosus* GG was cultivated in a pH-auxostat (viable count prior to collecting samples $1.31 \pm 0.21 * 10^8$) and incorporated into milk (pH = 6.75) for initial observation of the effects of bile variation and then, a second a pH-auxostat was carried out (viable count prior to collecting samples $1.22 \pm 0.12 * 10^8$) with freezing the culture into different commercially available liquid matrixes - UHT milk, soymilk, apple juice, titrated apple juice, whey protein powder drink and M.R.S. Broth as reference.

Immediately after fast freezing the viable counts of *Lactobacillus rhamnosus* GG remained above 10^8 for all the matrixes tested, as seen in Figure 1. In case of soymilk a 0.2 log unit drop was observed in the viable count after fast freezing, also for untitrated apple juice there was a drop of 0.21 log units. For M.R.S broth, whey protein powder drink and titrated apple juice slightly higher viable counts were detected in freshly frozen samples, which can be related to physical occurrences on the cell chains as noted by Foschino, Fiori, and Galli (1996).

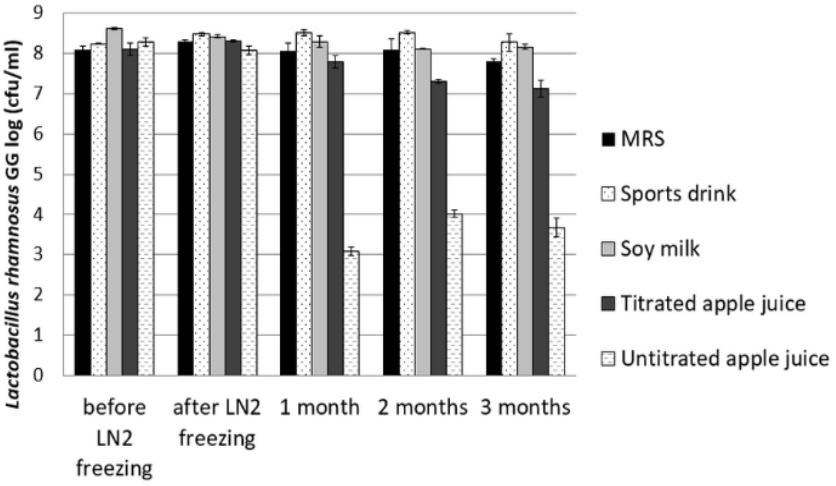

Figure 1. Stability of *Lactobacillus rhamnosus* GG frozen in various matrixes during 3-month storage at -40^{0}C

After 3 months of storage at -40 ^{0}C the viable counts were $1.48 * 10^8$, $1.33 * 10^8$, $3.85* 10^7$ and $1.05 *10^7$ cfu/ml in frozen whey protein powder drink, soymilk, M.R.S broth and titrated apple juice, respectively, hence remaining at the level between 10^6 -10^8 which is usually recommended for probiotics (Cruz, Antunes, Sousa, Faria, & Saad, 2009). The average loss of viability during storage at -40 ^{0}C of *Lactobacillus rhamnosus* GG was 0.99 ± 0.31 log units in titrated apple juice, 0.75 ± 0.12 log units in case of M.R.S broth and only $0.29 \pm 0.0.6$ and 0.11 ± 0.19 in soymilk and whey protein powder drink, demonstrating good viability during storage for the latter matrixes. The storage stability of *Lactobacillus rhamnosus* GG frozen in titrated apple juice was good, remaining above 10^6, however, in untitrated apple juice the viability dropped significantly, to $3.65*10^3$ cfu/ml. The pH was relatively stable during storage (data not shown) for M.R.S broth (remained above pH = 5.50) and soymilk (above pH = 6.30) with a slight drop in case of the whey protein powder drink which had pH 6.45 at the beginning and pH = 6.09 at the end of storage. For untitrated apple juice the pH was low – with an average pH of 3.5. In case of titrated apple juice the pH remained high during storage (about pH = 7), probably due to the titration since the pH of frozen apricot and peach juices and of frozen apple products has been shown to decrease during storage (Elhadad, Alwakdi, Abushita, & Abdulsalam, 2013; Sigita, Krasnova, Seglina, Aboltins & Skrupskis, 2013). Given the low survival during storage untitrated apple juice beads were excluded from further GITS experiments.

3.2 Resistance to Simulated Gastrointestinal Conditions

The GITS experiments with varying bile concentrations were carried out and the results are shown in Figure 2. *Lactobacillus rhamnosus* GG survived well during the `gastric phase` (pH 3) of the experiments, the average loss in viability in M. R. S. Broth was 0.67 ± 0.07 log units; with 0.43 ± 0.08; 0.35 ± 0.09 and 0.25 ± 0.08 log units in

soymilk, apple juice and milk, respectively, with the number of bacteria practically unchanged for whey protein powder drink.

´Food` matrixes	Concentration of bile salts solution (vol/vol, %)				
MRS	**0%**	**0.05%**	**0.10%**	**0.20%**	**0.40%**
pH 3	-0.59 ± 0.02	-0.75 ± 0.07	-0.7 ± 0.006	-0.59 ± 0.02	-0.71 ± 0.04
Bile	-0.61 ± 0.02	-1.04 ± 0.05	-1.21 ± 0.10	-1.7 ± 0.10	-1.78 ± 0.10
Bile 30min	-0.65 ± 0.08	-1.03 ± 0.02	-2.58 ± 0.20	-3.5 ± 0.33	-5.61 ± 0.34
5,5h dilution	0.45 ± 0.05	-0.89 ± 0.10	-1.71 ± 0.10	-2.63 ± 0.26	-4.3 ± 0.12
Milk	**0%**	**0.05%**	**0.10%**	**0.20%**	**0.40%**
pH 3	-0.26 ± 0.01	-0.27 ± 0.01	-0.18 ± 0.01	-0.18 ± 0.02	-0.36 ± 0.03
Bile	-0.26 ± 0.02	-0.28 ± 0.02	-0.27 ± 0.01	-0.2 ± 0.01	-2.28 ± 0.06
Bile 30min	-0.28 ± 0.02	-0.31 ± 0.03	-0.38 ± 0.04	-0.32 ± 0.01	-3.46 ± 0.06
5,5h dilution	0.21 ± 0.02	0.46 ± 0.01	0.38 ± 0.03	0.39 ± 0.04	-2.2 ± 0.20
Apple juice	**0%**	**0.05%**	**0.10%**	**0.20%**	**0.40%**
pH 3	-0.27 ± 0.04	-0.32 ± 0.01	-0.36 ± 0.03	-0.51 ± 0.04	-0.31 ± 0.04
Bile	-0.3 ± 0.10	-0.65 ± 0.02	-0.89 ± 0.02	-4.72 ± 0.38	-5.07 ± 0.10
Bile 30min	-0.44 ± 0.16	-0.75 ± 0.06	-1.23 ± 0.11	-4.74 ± 0.40	-5.02 ± 0.08
5,5h dilution	-0.17 ± 0.09	-1.17 ± 0.02	-1.7 ± 0.17	-4.31 ± 0.20	-5.18 ± 0.33
Whey protein powder drink	**0%**	**0.05%**	**0.10%**	**0.20%**	**0.40%**
pH 3	0.07 ± 0.006	0.03 ± 0.003	0.05 ± 0.004	0.05 ± 0.004	-0.04 ± 0.002
Bile	0.03 ± 0.002	-0.004± 0.0003	-0.04 ± 0.002	-0.02 ± 0.002	-1.93 ± 0.03
Bile 30min	0.01 ± 0.001	-0.05 ± 0.005	-0.05 ± 0.003	-0.07 ± 0.007	-3.85 ± 0.08
5,5h dilution	-0.45 ± 0.01	-0.34 ± 0.02	-0.55 ± 0.03	-0.53 ± 0.03	-3.62 ± 0.10
Soymilk	**0%**	**0.05%**	**0.10%**	**0.20%**	**0.40%**
pH 3	-0.51 ± 0.05	-0.46 ± 0.03	-0.39 ± 0.02	-0.31 ± 0.01	-0.47 ± 0.02
Bile	-0.28 ± 0.03	-0.48 ± 0.01	-0.21 ± 0.01	-0.32 ± 0.03	-3.36 ± 0.02
Bile 30min	-0.3 ± 0.01	-0.36 ± 0.03	-0.4 ± 0.04	-0.42 ± 0.03	-3.5 ± 0.05
5,5h dilution	-0.31 ± 0.02	-0.61 ± 0.06	-0.63 ± 0.06	-0.8 ± 0.08	-3.31 ± 0.02

Figure 2. Heatmap representation of survival (Log (cfu/ml)) of *Lactobacillus rhamnosus* GG in various food matrixes during GITS experiments depending on different bile concentrations. Sampling points correspond to the following GITS stages: pH 3 (endpoint of acid addition or `gastric phase`), Bile (directly after bile addition), Bile 30min (after 30min incubation with bile), 5,5h dilution (endpoint of experiment). Color coding added according to the Excel Conditional Formatting on the Green – White – Red Color Scale, with green showing the smallest reduction, red maximum reduction and white median results. Data are means ± SD, calculated from parallel experiments which were averaged and expressed as one data point

During ´gastric phase` whey protein powder drink minimalized the effects of the acidic conditions with virtually no detrimental effect on viability. In case of milk the average loss in viability was the smallest, followed by similar losses in soymilk and apple juice, with the highest average loss in viability observed in M. R. S. Broth.

The resistance of *Lactobacillus rhamnosus* GG to bile salts was dependant on the type of liquid food applied for incorporation and the concentration of bile. A similar trend in the effect of bile concentration was observed in the reference matrix M.R.S. broth and in apple juice - the effect of increasing bile concentration was directly proportional to the decrease of viability of *Lactobacillus rhamnosus* GG. In case of 0.1% bile for M.R.S and apple juice the decrease in viability by the end of the experiment was about 2 log units and for 0.4% bile the drop was about 4 and 5 log units for M.R.S and apple juice, respectively.

In case of titrated apple juice the impact of bile was much more pronounced starting with the 0.1% bile, when compared with the effect of acid conditions (pH 3). However the bile damage was observed directly upon addition with no significant added effect during incubation. That was in contrast with M.R.S, which also showed a significant decrease starting from 0.1% bile, but also having an increasingly detrimental influence in relation with the incubation time.

The whey protein powder drink showed the best resistance upon direct bile addition and during the 30 minute bile incubation as seen in Figure 2. In case of milk and soymilk small similar losses were observed. Noticeable decrease in viability for direct bile addition and incubation for milk and soymilk was only caused by the highest

bile concentration.

For the highest bile concentration applied (0.4%) the smallest loss in survival by the end of the experiment was observed in case of milk, followed by soymilk and whey protein powder drink. For bile concentrations 0% - 0.2% the decrease by the end of the experiment was less than 0.6 log units for whey protein powder drink and 1 log unit for soymilk.

In case of the control experiment with no bile addition, an increase in viability was noted for M.R.S, however in case of apple juice there was still a small drop in viability by the end of the experiment.

4. Discussion

Traditionally, the beneficial properties of probiotics have been made available by incorporating them into food. Stability of the probiotics therefore depends on the properties of the food matrixes, which also influences the survival of probiotics during gastrointestinal transit. In commercial dairy products the mainly used probiotic strains are *Lactobacillus* spp. and *Bifidobacterium* spp., added as adjunct cultures (Vasiljevic & Shah 2008). In case of non-fermented products probiotics can be added as liquid biomass concentrate after they have been grown in industrial medium and harvested by filtration or centrifugation (Makinen, Berger, Bel-Rhlid, & Ananta, 2012).

The pH-auxostat enables the growth of bacteria at the maximum growth rate in suitable and unstressful conditions. The pH-controlled continuous cultivation was successfully applied in case of *Lb. acidophilus* La-5, *Lb. rhamnosus* GG and *Lb. fermentum* ME-3 with M.R.S broth as growth medium by Sumeri *et al.* (2010). For producing frozen bacterial cultures, the method of making granules by dripping cell suspension into liquid nitrogen (LN_2) through a nozzle or disc with bores has been used in commercial applications (Santivarangkna, Kulozik, & Foerst, 2011). So pH-auxostat cultivation and fast freezing of probiotic bacteria in LN_2 were applied in the present study as well.

In general, *Lb. rhamnosus* GG is less sensitive to storage in higher pH juices than in low pH juice (Sheehan, Ross, & Fitzgerald, 2007). For *Lb. rhamnosus* R0011 stored in a commercial apple–pear–raspberry juice blend good viability at 4^0C was shown (Champagne, Raymond, & Gagnon, 2008). On the other hand, low pH of apple juice (pH 3.4/pH 3.6) has been mentioned to have inhibitory properties on *Lactobacillus rhamnosus* strain E800 (Saarela, Alakomi, Puhakka, & Mättö, 2009), with neutralization suggested to prevent the negative effect of fruit juice on probiotic bacteria (Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Therefore, titration of apple juice was also carried out in the current study.

In our experiments, when incorporated into M.R.S, whey protein powder drink and soy milk the survival of LGG during fast freezing was very good, with a small loss observed in titrated apple juice during storage. However, in case of untitrated apple juice an 1.8-fold average loss in survival over 3 month storage was observed. This significant reduction clearly demonstrates that efficiency of fast freezing is also dependent on the matrix type, pH and chemical composition.

The main gastrointestinal stress factors are considered to be the acidic environment of the stomach and the presence and effect of bile salts in the duodenum (Mills, Stanton, Fitzgerald, & Ross, 2011).

Gorbach and Goldin (1989) showed that *Lactobacillus rhamnosus* GG decreased from 10^8 to 10^6 after 0.5 h at pH 2.5 in normal human gastric juice. During *in vitro* gastric simulation (pH = 3 or pH = 2 for 2h, without pepsin) a drop of 3 and 7 log units, respectively, was observed by Succi *et al.* (2005) when incubation with simulated gastric juice (pH = 2, with pepsin and bile) without added glucose and dilute HCl (pH = 2) for 90 minutes resulted in similar losses (~5.6 log units) in survival (Corcoran, Stanton, Fitzgerald, & Ross, 2005).

However, Papadimitriou *et al.* (2015) have stated that the pH values used for conventional *in vitro* assays for probiotics are unrealistically low, while not taking into consideration the effects of food vehicles and the pre-stressing of probiotics due to food processing and storage. In addition, data from various GIT simulators were found to correlate well with *in vivo* results, with the advantange of employing more realistic pH values. This is well in accordance with the logic behind our simulator profile.

In our GI tract model, the rate of addition for hydrochloric acid was equal to the maximum HCl secretion rate for the human stomach (Ewe & Karbach, 1990). The secretion of human gastric acid is facilitated by gastrin which is stimulated by the presence of food components, *eg* peptides, oligopeptides and aminoacids in the stomach. When pH drops below the pH 3 threshold, the release of gastrin is inhibited and in turn the acid secretion decreases (Ewe & Karbach, 1990). So pH value 3 was chosen as transition point to the small intestine phase. Since the length of `gastric phase` was determined by the time required for lowering the pH of the food matrix, the invidual properties and buffering capacities of different foodstuffs also had an effect. Therefore, in our GIT

simulator the human physiological conditions are more closely mimicked, with the pH profile allowing for a more accurate replication of intestinal conditions.

Dietary fat and cholesterol are well known to stimulate bile secretion. In response to increased dietary fat the total concentration of bile acid is also higher (Reddy, Sanders, Owen, & Thompson, 1998). Also, it has been shown that bile salt synthesis rates can vary significantly among people (Bisschop *et al.*, 2004). So, varying bile salt concentrations were used during our gastrointestinal simulations in order to better reflect interpersonal differences and diet influences. A study by He, Zou, Cho, and Ahn (2012) using mixtures with varying concentrations of taurocholic acid, glycocholic acid, taurodeoxycholic acid, and glycodeoxycholate to test probiotic strains, showed that *Lb. rhamnosus* GG was more susceptible to bile acids in comparison to other probiotic strains. Different types of bile have been employed for bile resistance testing. The inhibitory effect of porcine bile has been shown to be greater than bovine or human bile. However, probiotics are usually resistant to human bile, regardless of their response to other types of bile (Dunne *et al.*, 2001; Rakin, Sekulic, & Mojovic, 2012).

In comparison with M.R.S. and titrated apple juice, a smaller reduction of viability in response to bile addition was observed in case of milk and soymilk in our experiments.

In contrast, Champagne and Gardner (2008), found that *Lb. rhamnosus* LB11 stored at 4^0C in fruit juice blend was not significantly affected when tested separately with 0.3% bile and pancreatic enzymes.

Fat content in food has been shown to improve probiotic viability in relation to bile, giving protection from the membrane dissolving effect of bile salts (Meira *et al.*, 2015; Ranadheera, Evans, Adams, & Baines, 2012). In case of *Lb. rhamnosus* GG originating from a commercial product, higher viability in gastric and intestinal phase was observed for full fat peanut butter (fat content of 50.10 ± 1.16%) when compared to reduced fat peanut butter (Klu & Chen, 2015). In agreement, bile addition and incubation had virtually no effect on the viability of *Lb. rhamnosus* GG in case of whey protein powder drink, which had the highest fat content of the matrixes in our experiments.

5. Conclusion

In conclusion, the effects of various food matrixes on the survival of the probiotic bacterium *Lactobacillus rhamnosus* GG during the storage following flash freezing in liquid nitrogen and in simulated gastrointestinal conditions were explored. Since it was shown that similarly to milk, soymilk and whey protein powder drink convey protection against gastrointestinal stresses, the latter are suitable candidates for the incorporation of probiotics. Also, due to the stability during flash freezing and storage, a chance for product development arises – for example, probiotic frozen desserts, puddings, sorbets *etc.* can be formulated.

It was demonstrated that lower bile concentrations also caused a decrease in viability for LGG incorporated into apple juice and M.R.S, so it cannot be assumed that foods with low fat content and therefore stimulating less bile secretion would be better for probiotic delivery.

Since apple juice did not prove to be a good candidate for probiotic incorporation, then perhaps protein or fat addition could give improved viability for this matrix, so that could be an avenue for study. Also, a further characterization of specific components responsible for improved viability is required.

To sum up, food matrixes with certain characteristics, such as suitable fat and protein content are better for the formulation of novel probiotic products in the food industry.

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