Impact of mode of assumption and food matrix on probiotic viability.

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Abstract

The ability of probiotics to survive the gastric transit to the low pH value of the stomach represents one of the key features associated to their effectiveness. Two strains of *L. rhamnosus* were evaluated for their ability, when supplied through diverse food matrices, to survive the exposition to four different simulated gastric juices. Probiotics were cultured in MRS broth medium, used as conventional control, as well as in carrot juice, rice cream and cow's milk in order to define the protecting role of the food matrix to the harsh gastric conditions. Fermented cow's milk was chosen as a reference for its well-known protective role towards probiotics. Matrices of vegetable origin were evaluated for their potential in preserving probiotic viability following ingestion. Results obtained were promising since fermented carrot juice should be considered as a potential alternative to dairy-based products. On the contrary, rice cream offered less protection to probiotics bacteria when exposed to simulated gastric juices and its effect was strain-dependent. Our preliminary work offers new insights to elucidate the role of food matrix in protecting probiotics when exposed to challenging conditions with particular reference to special dietary conditions like deprivation of dairy-based products and/or vegetarian/vegan regimens.

Keywords: Probiotics, Gastric juice, Food matrix, Viability, Survival.

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Introduction

In 2001 an expert panel of the International Scientific Association for Probiotics and Prebiotics revised and defined the term 'probiotics' as 'live microorganisms that, when administered in adequate amounts, confer a health benefit to the host' [1]. The consumption of probiotics often occurred in the form of fermented foods, and the type of matrix that vehicles those beneficial bacteria may reduce their viability or modify the beneficial properties of a product [2-5]. Probiotic bacteria incorporated into foods should be able to survive gastric transit and reach the small intestine in sufficient numbers of viable cells. Hence, to exert some health benefits to the host, probiotic microorganisms should preserve a certain viability level estimated ranging around 10^6–10^7 CFU/mL or g of carrier food product [4]. Furthermore, probiotics should survive to harsh acid gastric and intestinal conditions and be able to adhere to the enteric mucosa.

During the digestive process food remains in the stomach up to 4 h, even if it has been estimated that the average time for the gastric emptying is around one hour [5] and then it transits through the small intestine over 1 to 4 h. The main negative factors that affect the survival of probiotics in the stomach are represented by the low gastric pH and the presence of pepsin. The physiological gastric pH ranges from 2.5 to 3.5, but it can be as low as 1.5 or increase to 6 after food intake. Under fasted conditions, the human stomach shows a pH range between 1 and 3, whereas as a result of food ingestion the gastric conditions change during the stay of food, passing from a rapid peak to pH 6, 7 to a gradual reduction towards values of 1-3 within a couple of hours. However, this time of gastric emptying varies considerably from individual to individual and depending on the degree of filling of the stomach and the volume of the same (average volume 1.5 liters). The average emptying times were estimated in approx. 80.5 ± 22.1 min under fasting conditions at 127 min under full stomach conditions, with faster times for liquid than solid foods [6-8]. Furthermore, considerable variability was found in the composition of gastric juice, with particular reference to bile salts and proteolytic enzymes.

The tolerance of probiotic bacteria to gastric and small intestine conditions seems to be influenced by the carrier. At present, several probiotic products, such as capsules or sticks, have been developed and commercialized in many countries, although traditional yogurts and fermented milks still remain the most common food matrix for the probiotic consumption. Recently non-dairy based fermented foods have been launched on the market, for instance, soy and cereal products, fruit and vegetable juices, and fermented meat and fish [9-11]. It is known that the addition of probiotics into dairy foods may improve their tolerance to the low gastro-intestinal pH level. Briefly, the protecting action exerted by the whole cow's milk, as well as its fat component, reduces the direct exposure of probiotics to gastric conditions. Unfortunately, the widespread of lactose intolerance among adult population represents a significant downside to dairy-based foods consumption. Recent works reported that the physical structure of some carrier foods, such as vegetables like artichokes and olives, should be considered for the protection of probiotics against gastric juices. Other foods like sausage have also shown a potential in retaining the viability of probiotics through gastrointestinal

transit. Anyway, probiotic food preparations still represent a huge challenge since different probiotic species possess dissimilar resistance towards the acidity of the substrate and GIT conditions [12-15].

The aim of this study was to assess the *in vitro* gastrointestinal tolerance of two strains of *Lactobacillus rhamnosus* strains namely ATCC 53103 and a new environmental-isolated microorganism LMG S-29885 when cultured in their election laboratory medium as well as in three different food carriers, in order to evaluate the potential of food matrix to preserve the survival of probiotics following ingestion [16-20].

The evaluation of the gastric resistance was assessed by means of four different gastric juices previously described in literature. Gastric juices recipes were derived from different authors: Charteris et al. [16] (juice 1), Fredua-Agyeman et al. [17] (juice 2, 3), Corcoran et al. [18] (juice 4). Briefly simulated gastric juices are composed by a saline or mineral-enriched solution with a pH correction ranging from 1.6 to 3.4. The four different simulated gastric juices are characterized by different composition, in particular, juice 1 contains high levels of pepsin, juice 2 is only a saline solution, juice 3 is a saline solution added with sodium taurocholate, lecithin, and pepsin, juice 4 contains glucose other than ox-bile, pepsin and lysozyme. Simulated gastric juices considered in this research work have been corrected to pH 2.0 in order to standardize pH conditions and compare the survival results, in the awareness that this very low pH value strongly challenged probiotic strains. Despite their differences in chemical composition these simulated juices were considered to test the gastric tolerance of probiotics grown in laboratory media. The innovative content of our study is the introduction of the concept that the food matrix, used as substrate for the growth of probiotics, can affect their survival following exposition to gastric juice. This could offer significant indications about the mode of delivery of probiotics and the possibility to maximize their effectiveness through fermented foods [21-24].

Materials and Methods

Two different *Lactobacillus rhamnosus* strains were used to perform the experiments of gastric tolerance at pH 2.0 using different simulated gastric juices. One of the two *L. rhamnosus* strains, LMG S-29885, was isolated by AAT from weeds and deposited to the Belgian permanent culture collection BCCM. *L. rhamnosus* ATCC 53103 was used as control in all the experiments. Bacterial strains were routinely cultured in MRS broth (BD Difco, NJ, USA) under microaerophilic conditions or in different food matrices intended for assaying the gastric juices tolerance.

Skim milk (BD Difco, NJ, USA), commercial rice cream for weaning and a home-made carrot juice were fermented by both *L. rhamnosus* strains and tested under simulated gastric juices. Food matrices were prepared as follow: skim milk powder was renatured following the manufacturer instruction. Rice cream was prepared by weighting 10% w/v of rice flour in demineralized water and carrot juice was obtained by a vegetable extractor. All food matrices were sterilized by autoclaving for 30 minutes at 109°C.

Recipes of gastric juices were derived from the literature and the pH was corrected to 2.0 ± 0.1 in order to analyse data at the pH uniformity. Four different gastric juices previously described were used (juice 1 [16] and 2,3 [17], and 4 [18]). Juices 1 and 2 were respectively simulating the empty stomach condition for the presence of pepsin and taurocholate acid and lecithin. Juices 3 and 4 were set to mimic the full stomach condition. In particular, juice 4 was characterized by the presence of glucose. pH was corrected to 2.0 for all the juices, all reagents were supplied by Sigma-Aldrich (MO, USA).

L. rhamnosus ATCC 53103 and LMG-S 29885 were grown for 18 hours in MRS under microaerophilic conditions at 37°C. Bacteria cultures were washed twice with water and adjusted to OD6000, 7. 100 µl of these preparations were inoculated into 10 ml of MRS, skim milk, rice cream and carrot juice. All tubes were incubated under microaerophilic conditions for 18 h at 37°C. 1 ml of the two *L. rhamnosus* strains grown in MRS as well as in the fermented food matrices was mixed with 9 ml of the four simulated gastric juices and vital counts were recorded at T0, T15, T30 and T60 minutes. All tubes were incubated at 37°C under shaking for one hour.

Decimal serial dilutions were performed to estimate vital counts and logarithmic decrease during the incubation under simulated gastric juices. Plates were spread from the -2 to the -7 with 100 µl of the diluted solution and incubated in anaerobic jar at 37°C for 72 h.

Results

The protection towards probiotic viability exerted by the delivery matrix was evaluated by means of three different foods and four different simulated gastric juices identified in the literature. MRS broth laboratory medium was included in the analysis as a reference, in order to assess the tolerance of the two strains to the simulated gastric juice when they were grown in their election medium. Strains were therefore grown in the selected matrices and then put in contact with simulated juices [25-28].

The concentration in viable cells following *in vitro* exposure to simulated gastric juices was evaluated by means of plate counts of serial decimal dilutions at different time points. Results were then converted in log10 CFUs in order to normalize the values. The impact of food matrices as growth media in the presence of the 4 simulated juices was measured for both *L. rhamnosus* strains. The loss in probiotic viability was calculated as difference between T0 log10 count and the corresponding value after 15, 30 and 60 minutes exposure. The following tables report the loss in probiotic viability obtained for the 3 considered food matrices as well for the laboratory reference MRS (Table 1). In this case, both strains showed a significant reduction in viability when incubate with juices 1 and 2, leading to total cell death following 60 minutes of exposure. After contact with juice 3, the 2 strains displayed a slightly different behavior, with a stronger resistance shown by

strain LMG S-29885 (about 4.4 log10 loss against 7.1 for ATCC53013).

Table 1. *Loss of viability (in log10 CFUs) of lactobacilli, grown in laboratory medium MRS, at different time points following exposure to simulated gastric juices.*

	ATCC53103			LMG S-29885		
Simulated gastric juice	15 min	30 min	60 min	15 min	30 min	60 min
1	2,88	8,09	8,09	0,63	5,82	8,23
2	2,52	5,42	6,82	1,67	6,30	7,86
3	0,02	5,20	7,07	0,00	1,46	4,35
4	0,00	0,06	0,11	0,01	0,26	0,30

On the contrary, the high-glucose content of juice 4 was able to maintain the original viability of both strains with less than 0.5 log10 reduction in 60 minutes. Table 2 reports the same type of information (loss of probiotic viability in log10 CFUs) referred to the growth of probiotics in cow's skimmed milk. The overall results confirmed what already observed and reported in the literature about a significant protective effect exerted by dairy matrix on bacterial cells, especially following the exposure to detrimental conditions such as osmotic and thermal stress. Both strains showed in fact high tolerance to the four simulated gastric juices when cultured in the milk with a maximal reduction of vitality in between 0.2-0.3 log10 CFUs. Very poor information is retrievable from the literature about the impact of food, other than dairy-based, on the protection of probiotics challenged by adverse environmental conditions. Much of this knowledge was applied to the maximization of probiotic survival following dehydration such as lyophilization and spray-drying, by studying the use of alternative matrices to obtain stable bacterial powders [19,20]. Conversely, the effect of the growth matrix on the survival of probiotics following ingestion, with special reference to the type of food in which the probiotic in included, or with which the probiotic is co-administered, and to the time of consumption during the day, has been poorly considered. In order to partially fill this gap, our research work was focused on the impact of cereals and vegetables, such as rice and carrot, on the ability of probiotics to survive the contact with gastric juice. Rice cream (Table 3) offered a significantly lower protection to probiotic than milk since both *L. rhamnosus* strains showed an almost total cell death when exposed to juice 1 for 60 minutes. Strain ATCC 53103 strongly decreased its viability already at 30 minutes while strain LMG S-29885 displayed a more gradual decrease in viable cell concentration. A similar discrepancy between the two tested strains was confirmed also for simulated juices 2 and 3, indicating a stronger sensitivity of strain ATCC 53103 to harsh environmental conditions when assumed with cereal-based food such as rice. Strain LMG S-29885 was not disturbed by the contact with gastric juices when grown in rice (loss of less than 0.1 log10 CFUs). The exposure to simulated juice 4 was confirmed to induce only a 0.4-0.5 log10 decrease in viability, indicating a significant contribution of glucose in the juice to the resistance to pH stress. Vegetable juices were tested, with special reference to carrot mainly due to the easy handling for home-made preparation of a stable extract and for its significant content in carbohydrates. Table 4 summarizes the loss of viability of the two *L. rhamnosus* strains grown in carrot juice and exposed to the simulated gastric juices for 15, 30 and 60 minutes. The major performances, in terms of higher resistance, were confirmed for strain LMG S-29885 (about 0.1 log10 after 60 minutes) compared to ATCC 53103 (range 0.3-0.7 log10 at T60). Carrot juice was therefore observed to be a good vehicle for probiotics independently from the time of consumption and the state of emptying of the stomach. Some strains could perform better than others with a food type-dependent behaviour.

Table 2. *Loss of viability (in log10 CFUs) of lactobacilli grown in skimmed cow's milk at different time points following exposure to simulated gastric juices.*

	ATCC53103			LMG S-29885		
Simulated gastric juice	15 min	30 min	60 min	15 min	30 min	60 min
1	0,01	0,01	0,23	0,01	0,08	0,15
2	0,00	0,00	0,02	0,24	0,27	0,28
3	0,11	0,13	0,19	0,17	0,17	0,23
4	0,14	0,15	0,16	0,13	0,15	0,16

Table 3. *Loss of viability (in log10 CFUs) of lactobacilli grown in rice cream at different time points following exposure to simulated gastric juices.*

	ATCC53103			LMG S-29885		
Simulated gastric juice	15 min	30 min	60 min	15 min	30 min	60 min
1	1,91	7,09	7,09	1,18	3,31	7,26
2	0,24	1,36	2,33	0,00	0,04	0,05
3	0,17	1,71	3,07	0,04	0,07	0,09
4	0,28	0,39	0,58	0,02	0,35	0,41

Table 4. *Loss of viability (in log10 CFUs) of lactobacilli grown in carrot juice at different time points following exposure to simulated gastric juices.*

	ATCC53103			LMG S-29885		
Simulated gastric juice	15 min	30 min	60 min	15 min	30 min	60 min
1	0,10	0,27	0,44	0,01	0,05	0,07
2	0,06	0,21	0,29	0,01	0,05	0,08
3	0,07	0,12	0,36	0,00	0,06	0,08
4	0,26	0,56	0,71	0,02	0,06	0,13

A summary of the survival of probiotics, expressed as % of survived cells, in the different tested conditions is provided in Figure 1. Laboratory medium MRS was confirmed not to exert any protective effect on probiotics, except that in case of simulated juice 4 for which the presence of glucose seems to

be able to guarantee a very significant survival for the strains, independently from the food matrix. Cow's skimmed milk confirmed its protective role for probiotics, since they were significantly stable in viability despite the tested gastric juice. Nevertheless, milk and dairy-based substrates are not welcome to all consumers and are forbidden to those suffering from intolerance and allergies. For this reason, the use of alternative matrices to provide probiotics, as fermented foods and/or in co-administration with food, is of great interest. Rice cream was not able to provide the appropriate protection to probiotics when pepsin and bile salts were included in the simulated gastric juice (no. 1). A significant strain-dependent dynamic was observed with juices 2 and 3 because strain LMG S-29885 was found to express an higher % of survival, close to 100%, if supplied as fermented rice-based food, compared to ATCC 53103 which survival was around 60%.

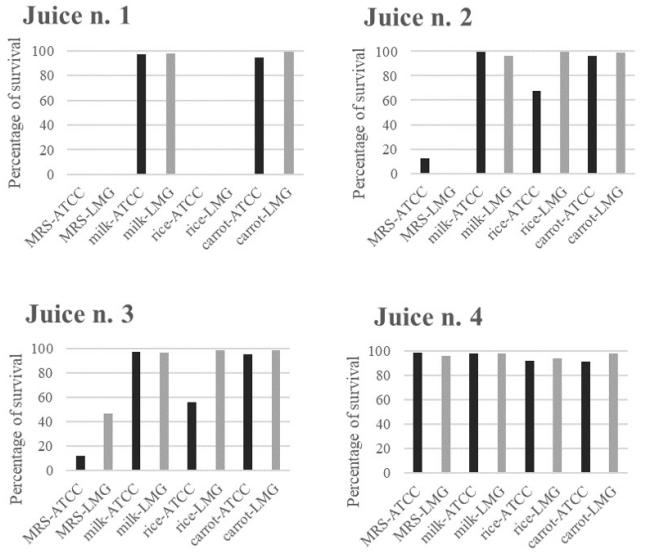

Figure 1. *Presents the two strains in different colour which are labelled in the vertical axis.*

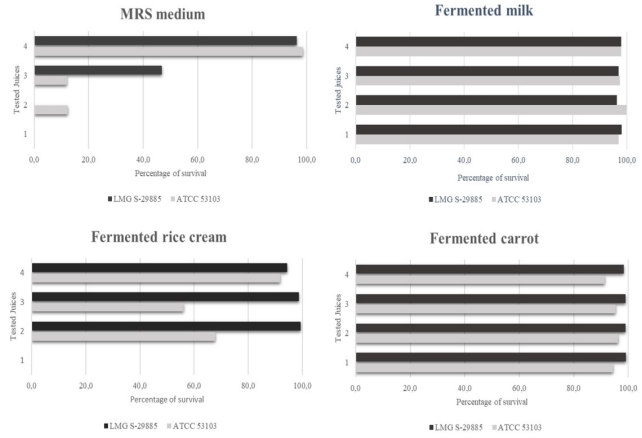

Figure 2. *Presents the percentage of survival of the two strains by clustering data for type of matrix instead of type of juice.*

Another substrate if vegetable origin such as carrot, was found to protect probiotics independently from the state of emptying of the stomach, with a slight difference between the tested strains, since the 98-99% of the population of *L. rhamnosus* LMG S-29885 survived 60 minutes contact with the juices and ATCC 53103 survived in the range 91-96% (Figures 1 and 2) [29-32].

Discussion

The aim of the present work was to assess the protection features of fermented matrices in the survival of two *L. rhamnosus* (ATCC 53103 e LMG S-29885) strains when exposed to different types of simulated gastric juices. Food matrices were selected by taking into consideration some previously published data obtained with dairy products that were evaluated for their positive protective properties [4]. Despite this useful feature, dairy fermented products were reconsidered during the last decades due to the frequent allergies and intolerance out comings [13].

Our work intended to reveal if alternative fermented matrices, such as cereal and vegetables, could potentially offer protection to probiotic strains during gastric transit. The fermented milk was chosen as reference to compare results obtained with non-dairy fermented matrices.

Recent literature [21,22] showed the *Lactobacillus* spp. abilities of successfully fermenting pure carrot juice or blended with blueberry and beetroot juice. Aboulfazli et al. reported that vegetable matrices could improve probiotics vitality during the gastric transit [23]. In this work, probiotic microorganisms were incorporated to ice-cream prepared in variable proportions with cow, soy and coconut milks. As final result authors found that soya milk exerted a protective role towards probiotic bacteria but concomitantly altered unacceptably the total taste. Instead, other studies revealed that the consumption of cereals, cow milk and probiotics guarantee a high survival probiotic rate compared to vegetable matrices such as apple juice [24].

Our results support the idea that the fermentation of food matrices could represent a strategy to promote the pH acidity tolerance during the digestive process. Tested strains ATCC 53103 and LMG S-29885 were able to ferment carrot juice and to survive for the time of the analysis losing less than half a logarithm within 60 minutes exposure at pH 2.0. LMG S-29885 showed the ability to take more advantage than ATCC 53103, when grown in vegetable-based matrices, following exposure to gastric juice. Rice cream offered minor protection during the exposure to simulated gastric juices compared to fermented carrot juice. Strain LMG S-29885 exhibited a greater vital rate compared to the other strain, losing less than 0.5 log10 viability when exposed to juices 2 and 3 in comparison to ATCC 53103 that lost almost three logarithms. At the end of one-hour incubation in the presence of the juice 1, both strains were completely deprived of their viability. Those results may be attributable to the environmental origin of isolation of LMG-S-29885 from weeds, the strain likely displayed an increased adaptation to vegetable matrices even when exposed to acid harsh conditions.

The reference fermented milk gave the expected results in line of those available in literature confirming the good protection exerted by dairy matrices against simulated gastric juices. The

maximal reduction of vitality was assessed for LMG S-29885 co-incubated with juice 2 around one third of logarithm respect the T0 count. Starting from these results it is possible to underline the strong sensitivity of the ATCC 53103 to the pepsin, since juice 1 was prepared with 3 gr/l of porcine pepsin.

Juice 4, containing 3, 5 gr/l of glucose, sustained and protected strains from the low pH acidity independently of the fermented food matrix tested. A possible speculation related to high probiotics survival could be attributable to the digestion of complex sugar macromolecules into small sugars as glucose. In fact, Corcoran et al. demonstrated that the presence of glucose improve probiotic viability when exposed to low pH solutions, and our data obtained for the election MRS medium confirmed this finding [18].

The presence of the food matrices, with special reference to cow's milk and carrot juice, preserved probiotic viability following exposition to all of the tested gastric juices, mimicking the conditions of fasted and full stomach. Conversely, both strains grown in MRS and tested under the four different conditions showed improved tolerance in the presence of juices 3 and 4 that mimic full stomach conditions. Considering our overall results and according to previous literature the assumption of probiotic should be possibly associated to food consumption, preferably up to 30 minutes from the end of the meal [17].

Our work identified in the fermented carrot juice a possible and alternative food matrix to the classical dairy based one. Results obtained were in line to those of Aboulfazli et al. that identified vegetables matrices as a valuable source of protection for probiotic under simulated gastric juices [23]. All data taken together suggested that the food fermented matrices offered a protection against the low pH values and/or the presence of digestive enzymes, however other investigati ons should be assessed in order to understand the presumptive role of food macro-components in protection of probiotic living cells. Our study presents several limitations that must be acknowledged, first of all the limited number of food matrices analysed and the single pH value tested. The simulation of the dynamic digestive process, comprising the pH slow decrease, may offer some different results and it should be evaluated with the intent of supply reliable results that better mimic *in vivo* conditions. Furthermore some *in vivo* experiments will provide reliable data to support the insights provided by our preliminary work.

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