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Viability of *Lactobacillus delbrueckii* Under Human Gastrointestinal Conditions Simulated *In Vitro*

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Abstract: Problem statement: *Lactobacillus delbrueckii* subsp. *bulgaricus* is a lactic bacteria mostly used in the production of yoghurt and it has an important probiotic activity that brings benefits to the human body. However, the gastrointestinal tract has aggressive conditions, such as the acid pH in the stomach and the bile in the duodenum, that reduce the viability of this bacteria. **Approach:** In order to evaluate the effect of the human gastrointestinal conditions on *Lactobacillus delbrueckii*'s viability, a simulated *in vitro* gastrointestinal system was designed, which consisted of two reactors where stomach and human small intestine conditions were simulated. **Results:** *Lactobacillus delbrueckii* cells were treated in human gastric conditions simulated *in vitro* (gastric juice adjusted to pH 2, 37°C, 90 min and 50 rpm) and in intestinal conditions simulated *in vitro* (pancreatic juice adjusted to pH 6.8, 37°C, 150 min and 50 rpm) and in presence of a sample of food or beverages. A sample of typical Mexican food was added and at the end of the treatment 73% of the cells remained viable. This means 36.5 times more viability with respect to the cells treated under the same conditions in presence of a sample of milk with 8% starch. At the end of the treatment, the viability of cells treated in simulated *in vitro* gastrointestinal juices without sample of food or beverage (blank) was 1%. **Conclusion:** The results indicated that the *in vitro* simulated human gastrointestinal conditions were aggressive to the *Lactobacillus delbrueckii*'s viability. To minimize this negative effect it is suggested that probiotics be consumed with some food because this could increase the probability that the bacteria reach the human colon in a large number and carry out their probiotic effect.

Key words: Probiotics, functional food, viability

INTRODUCTION

The interest to know the role of probiotic lactic bacteria in human's health has (Chandramouli *et al*., 2004) been increasing for the last 20 years (Marteau *et al*., 1997). Nowadays, the dairy industry has incorporated lactic bacteria not just as fundamental part in the production of lactic fermented food, but as an active ingredient of the well known "functional foods" like yoghurt and fermented milks, that have probiotics effects in the human organism and are intended to solve gastrointestinal problems as lactose intolerance and constipation (Sanders and Klaenhammer, 2001; Walker and Duffy, 1998). In recent years it has been proved that probiotic lactic bacteria brings some protection against pathogen microorganisms (Casas and Dobrogosz, 2000; Hamilton-Miller *et al*., 1999), stimulates the immune system (Aattouri *et al*., 2002) and it even contributes to the lessening of colon cancer. Probiotic lactic bacteria belong principally to the genus *Lactobacillus* and *Bifidobacterium* and yield their benefits in the human large intestine. To accomplish the probiotic effect, lactic bacteria must survive viable and at a large number to the colon.

Some authors mention that lactic bacteria in yoghurt must be active and not below 10^7 Colony Forming Units (CFU) per gram of product concentration (Ouwehand and Salminen, 1998). However, there are several studies that show that lactic bacteria contained in the yoghurt might lose their viability during the stage of storage in refrigeration and during the path along the upper gastrointestinal tract of the consumer (Ouwehand and Salminen, 1998; Kailasapathy and Rybka, 1997; Dave and Shah, 1998).

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The main factors in the gastrointestinal human tract that can affect the bacterial viability before the lactic bacteria reach to the human colon are the acid pH in the stomach and the bile in the duodenum.

Several systems have been developed to simulate the physicochemical conditions *in vitro* present in the gastrointestinal human tract and to allow the study of lactic bacteria viability. The simulation systems range from simple ones where the lactic bacteria is treated in solutions of acid medium and solutions of hepatic bile (Fávaro-Trindade and Grosso, 2002; Huang and Adams, 2004), to more complex systems that simulate the human gastrointestinal tract to study the probiotic lactic bacteria interactions within the intestinal microbial environment or determine the effect of probiotic lactic bacteria and symbiotic products in the human intestinal microbiota (De Boever *et al*., 2000; Mainville *et al*., 2005). Minekus *et al*. (1995) proposed an intestinal human tract model that contained four chambers to simulate the stomach, duodenum, jejunum and ileum. Other viability studies of lactic bacteria have been performed *in vivo* in pig intestine (Iyer *et al*., 2005).

In this research, we studied the viability of *Lactobacillus delbrueckii* subsp. *bulgaricus.* First it was treated in acid medium with addition of enzymes such as pepsin and mucin to simulate *in vitro* the human stomach conditions and after that, in pancreatic juice that contained pancreatin, bile and mucin, thus simulating the human small intestine conditions *in vitro*. Also, the effect of samples of meal and beverages (stimulant and alcoholic) on bacterial viability was evaluated.

MATERIALS AND METHODS

Microorganism: The *Lactobacillus delbrueckii* subsp. *bulgaricus* NRRL-734 strain was obtained from the United States Department of Agriculture and it was preserved in MRS agar contained in Petri plates (Man and Rogosa, 1960).

Design of a *in vitro* simulation system of the human gastrointestinal conditions: The gastrointestinal simulation system *in vitro* consisted of 2 jacketed bioreactors with removable lids and a capacity of 500 mL, made of borosilicate glass. The bioreactors lids had 5 entries to supply HCl, NaOH solutions, to take the samples and to place the pH electrode and the thermometer.

The bioreactors were connected in sequence to a bath with controlled temperature, which maintained them to 37°C to the physiological temperature of the human body. In the same way, the bioreactors were put onto stir plates to keep them in constant shaking at 50 rpm. The system was installed into a laminar flow cabinet to maintain the sterility conditions. The first reactor contained the human stomach simulation *in vitro* and second one hold the human small intestine simulation *in vitro*, which was connected to a flexible tubing of 1 cm internal diameter to simulate the intestinal peristalsis by means of a peristaltic pump that worked at 50 rpm.

Food and beverage samples design (stimulant and alcoholic) used to evaluate the effect on *Lactobacillus delbrueckii's* viability: With the purpose of evaluating the effect of other kinds of food with the functional food that contains lactic probiotic bacteria and to simulate the nutritional habits of people that consume functional food, three samples of food and two beverages (stimulant and alcoholic) samples were tested. The food samples designed were: a 8% starch solution, cow milk with 8% starch and a typical Mexican food like "chilaquiles". The samples of cow milk with starch were designed with a texture similar to that of yoghurt. At the end of the sterilization process, this sample kept its viscous consistency with no precipitation of the milk proteins. The typical Mexican food was designed with the following composition: 75 g of tortilla, 20 g of chicken and 5 g of serrano hot pepper.

On the other hand, the sample of stimulant beverage consisted of 1% sterile black coffee solution, while the alcoholic beverage sample consisted of beer with 4.5% alcohol. All the samples were evaluated in separated treatments.

Cellular viability technique: To determine the viable counts of *Lactobacillus delbrueckii*, 1 mL of broth culture was taken and it was diluted with 9 mL of 0.9% (p/v) sterile saline solution. From this solution several dilutions were made and 0.1 mL of each one was inoculated by extension in Petri plates with MRS agar (2% p/v). The plates were incubated at 38°C for 48 h; at the end of the incubation the *Lactobacillus delbrueckii* colonies were counted and the results were reported as percentage of bacterial viability.

Viability of *Lactobacillus delbrueckii* under *in vitro* simulated human gastrointestinal conditions: Kinetics of viability loss of *Lactobacillus delbrueckii* cells were made. For 90 min the cells were treated in simulated gastric conditions *in vitro* (3% pepsin SIGMA-Aldrich P7000, 4% mucin SIGMA-Aldrich M2378, 0.5% NaCl adjusting the pH to 2.0 with 5 M

HCl) with 100 mL of food or beverage at 37°C with continuous shaking at 50 rpm. Subsequently, the cells were treated for 150 min under intestinal conditions *in vitro* (1% pancreatin SIGMA-Aldrich P1500, 4% mucin, 0.3% bilis Oxgall Difco 212820 and 0.5% NaCl adjusting the pH to 6.8 with 1.5 M NaOH) at the same temperature and also with constant shaking conditions. At this stage the peristaltic movement in the human small intestine was simulated with a peristaltic pump that worked at 50 rpm. During the kinetics 1 mL of sample was taken every 30 min to determine the bacterial viability by plate count using MRS agar. The results were expressed in a percentage of viability and graphics were made in order to determine the rate of viability loss which was expressed in % viability loss/min^{-1}. The blank was a sample of *Lactobacillus delbrueckii* cells exposed to simulated gastrointestinal conditions *in vitro*.

Statistical analysis: The experiments were made by triplicate and the results expressed in percentage of viability. The program SPSS version 12.0 was used to do the statistical analysis; an ANOVA of repeated measurements was applied to determine the significant statistical differences between the treatments. The Duncan multiple range test was used to identify those treatments in which differences were found. The data was analyzed with a 5% of significance level.

RESULTS

Viability of *Lactobacillus delbrueckii* under *in vitro* simulated human gastrointestinal conditions: The Table 1 and Fig. 1 show the results of viability of *Lactobacillus delbrueckii* cells treated in gastric and in intestinal conditions *in vitro* and in presence of the follow samples: starch solution, cow milk with starch, typical Mexican food (chilaquiles), black coffee and beer; the blank sample was *Lactobacillus delbrueckii* cells.

At the beginning of the kinetics of viability loss of *Lactobacillus delbrueckii*, the cellular concentration of the starch solution was 1.96×10^9; 1.27×10^9 and 1.25×10^9 UFC mL^{-1} for the starch with cow milk and the black coffee, respectively. In the case of the typical Mexican food, the concentration of viable cells corresponds to 1.92×10^9 and 5.38×10^9 UFC mL^{-1} to the blank sample. At the end of the treatment in simulated gastric conditions *in vitro* (90 min) 40% (5×10^8 UFC mL^{-1}) and 29% (6.18×10^8 UFC mL^{-1}) of the cells treated in presence of cow milk with starch and a starch solution kept their viability, while 28% (1.43×10^9 UFC mL^{-1}) of the cells of the blank remained viable; this means 1.4 times less viability than the cells treated with a sample of cow milk with starch. When the *Lactobacillus delbrueckii* cells were mixed with a sample of black coffee and typical Mexican food they preserved 67% (8.45×10^8 UFC mL^{-1}) and 80% (1.5×10^9 UFC mL^{-1}) of their viability, respectively. At the end of the treatment in intestinal conditions *in vitro*, the cells treated with a starch solution and the cells of the blank preserved 1% of their viability, while the cells treated with a sample of cow milk with starch and black coffee, kept 2 and 6.5 times more viability that the blank. With the addition of a sample of "chilaquiles", 73% (1.43×10^9 UFC mL^{-1}) of *Lactobacillus delbrueckii* cells remained viable at the end of the kinetic. This means 73 times more viability than the blank and 36.5 times more viability than the cells treated with the addition of a sample of cow milk with starch, at the same simulated gastrointestinal conditions *in vitro.* Nevertheless, *Lactobacillus delbrueckii* cells completely lost their viability when they were treated with a sample of beer and in gastric juice *in vitro* (these results are not presented in the Fig. 1).

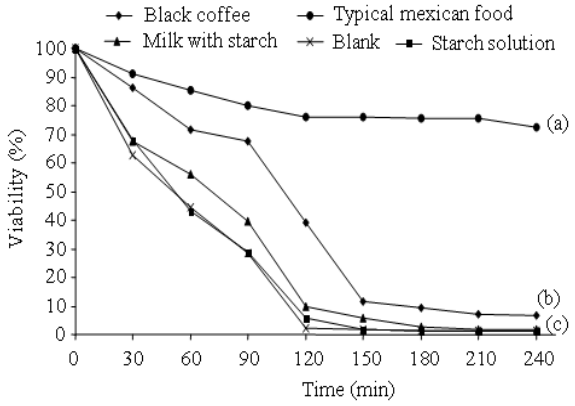

Fig. 1: Viability of *Lactobacillus delbrueckii* exposed to *in vitro* simulated human gastrointestinal conditions and in presence of several samples of food and beverages

Note: n = 3, the letters (a, b, c) indicate that there are significant statistical difference between the viability of the several treatments, Duncan ($p<0.05$). Treatments: starch solution, cow milk with starch and the blank did not reflect any significant statistical difference (a). Treatments in presence of 1% black coffee (b) and in presence of typical Mexican food (c), reflected significant statistical difference

Table 1: Viability of *Lactobacillus delbrueckii* cells exposed to *in vitro* simulated human gastrointestinal conditions

Time (min)	Blank[a] (%)	Starch solution[a] (%)	Starch/cow milk[a] (%)	Typical mexican food[c] (%)	Black coffee[b] (%)
0	100±00.0	100±0.00	100±0.00	100±0.00	100±00.0
30	63±10.9	68±6.99	68±5.35	91±5.62	86±17.8
60	45±05.2	43±8.16	56±3.01	85±7.49	72±16.0
90	28±09.7	29±7.13	40±2.72	80±10.85	67±17.1
120	02±00.5	06±3.58	10±0.86	76±8.52	39±11.1
150	02±00.2	02±0.80	06±1.88	76±8.52	11±07.5
180	01±00.2	01±0.45	02±1.31	75±8.58	09±06.8
210	01±00.1	01±0.33	02±0.30	75±8.58	07±04.3
240	01±00.0	01±0.20	02±0.30	73±10.69	07±03.5

Note: The results presented are the average of 3 repetitions ± DE; The treatments marked with [a, b, c] show that there were significant statistical differences of viability. Duncan ($p<0.05$)

Table 2: Rate of viability loss of *Lactobacillus delbrueckii* cells under *in vitro* simulated human gastrointestinal conditions

	(Percentage of viability loss/min^{-1})				
Period (min)	Blank	Starch solution	Cow milk/starch	Typical mexican food	Black coffee
0-30	1.247	1.072	1.080	0.291	0.457
30-60	0.605	0.818	0.393	0.204	0.485
60-90	0.148	0.485	0.536	0.177	0.144
90-120	0.865	0.768	0.997	0.124	0.947
120-150	0.024	0.136	0.136	0.000	0.921
150-180	0.008	0.012	0.109	0.021	0.070
180-210	0.002	0.003	0.028	0.000	0.074

Note: The results showed the average of 3 repetitions ± DE

The ANOVA of repeated measurements indicated that there existed significant statistical difference between the viability of *Lactobacillus delbrueckii* cells treated in simulated gastrointestinal conditions *in vitro* ($F_{1,4}$ = 102.615, $p<0.0001$). In the same way, the Duncan test proved that there were not significant statistical differences between the viability of the *Lactobacillus delbrueckii* cells exposed to gastrointestinal juices (blank), starch solution and cow milk with starch. However, there were significant statistical differences in reference to the viability of cells treated in presence of black coffee and typical Mexican food (Duncan, $p\leq0.05$).

The estimated rates of viability loss for the different treatments of *Lactobacillus delbrueckii* cells treated in simulated gastrointestinal conditions *in vitro* were higher during two periods: At the beginning of the stage in simulated gastric conditions *in vitro* from 0 to 30 min and at the beginning of the stage in simulated intestinal conditions from 90-120 min (Table 2).

DISCUSSION

Lactobacillus delbrueckii lost its viability quickly when it was treated in simulated gastric conditions (simulated stomach *in vitro*) in presence of a starch solution sample. A similar effect was observed in the blank treated in the same gastric conditions *in vitro*. With the addition of a sample of cow milk and starch, the viability of *Lactobacillus delbrueckii* cells was higher than the viability of cells of the blank and the viability of cells treated in presence of starch solution. With a sample of typical Mexican food, the viability was 36.5 times more than the viability of *Lactobacillus delbrueckii* cells treated in presence of a sample of starch solution and in simulated gastrointestinal conditions *in vitro*. During the first 30 min of the stage in simulated small intestine conditions *in vitro*, the *Lactobacillus delbrueckii* cells were treated in intestinal juices and in presence of a sample of starch solution and cow milk with starch, quickly loosing their viability, falling from 29% to 6% and from 40% to10% respectively; as for the blank, the viability fell from 28% to 2%. In the case of the sample of cells treated with addition of black coffee, the most aggressive effect over bacterial viability appeared between the 90 and 150 min of the kinetic (intestinal conditions *in vitro*).

This study showed that when a sample of typical Mexican food like “chilaquiles” was added to the simulation system, the aggressive effect of the simulated intestinal conditions on the *Lactobacillus delbrueckii* viability diminished, thus at the end of the treatment, the bacterial viability was significantly higher compared with the cells viability in the other treatments.

The fast loss of bacterial viability during the first 30 min of the stages of simulated stomach and the small intestine *in vitro* could be due to the fact that the

Lactobacillus delbrueckii cells were treated first in an acid media (pH 2) and when the cells showed possible phase of adjustment they were exposed to more aggressive conditions (addition of hepatic bile and pancreatin, pH 6.8).

On the other hand, the protective effect of the sample of typical Mexican food could be attributed mainly to the slightly viscous consistency of the mixture of tortilla, chicken and hot pepper with the simulated gastrointestinal juices *in vitro.* This mixture interacted with the *Lactobacillus delbrueckii* cells and brought some physical protection to the cells from the aggressive effect of simulated gastrointestinal conditions *in vitro* evaluated in this study. Some authors mentioned that the matrix food might protect the lactic bacteria, such as the *Bifidobacterium*, of the acid pH present in the human stomach (Walker and Duffy, 1998). The protective effect could be explained by the fact that diffusion processes had a less effect between the reaction medium and the *Lactobacillus delbrueckii* cells, resulting in the high bacterial viability at the end of the treatments.

When a sample of 1% black coffee or cow milk with starch were added to the simulation system, the *Lactobacillus delbrueckii* viability was significantly lower in reference to the bacterial viability in presence of a sample of typical Mexican food. The mixture of gastrointestinal juices with the black coffee solution and the mixture with cow milk and starch did not have a viscous consistency such as in the "chilaquiles" treatment. Therefore, in these treatments possibly the diffusion processes were easier and then more interaction occurred between the environment and the *Lactobacillus delbrueckii* cells. When a sample of beer was added to the medium (simulated gastrointestinal juices *in vitro*), *Lactobacillus delbrueckii* lost its viability completely and this could be attributed to the presence of ethylic alcohol in the beer.

Other authors have observed similar behaviors of viability loss in different strains of lactic bacteria treated in simulated gastrointestinal conditions *in vitro*. Authors such as Mainville *et al*., (2005) reported that *Bifidobacterium animalis* treated in acid medium (pH 2) for 90 min conserved 52% of its viability. Strains like *Lactobacillus rhamnosus, Lactobacillus kefir, Bifidobacterium longum* and *Bifidobacterium infantis* totally lost their viability at the end of 15 min in acid environment (pH 2.0). Mainville *et al*., (2005) also reported that *Lactobacillus kefirgranum* and *Bifidobacterium infantis* kept 4% and 1% of its viability respectively, at the end of a treatment of 90 min in environment with 0.3% bile Oxgall, whereas *Lactobacillus kefir* kept 78% of its viability treated under the same conditions. Marteau *et al*., (1997) reported that *Lactobacillus delbrueckii* subsp. *bulgaricus* kept approximately 30% of its viability after 70 min in simulated gastric juice *in vitro* (pH 2.1) with addition of pepsinogen (370 U mL^{-1}).. They reported the total loss of *Lactobacillus delbrueckii* viability after 110 min in simulated gastric juice *in vitro* (pH 1.8) with the addition of pepsinogen.

CONCLUSION

The *in vitro* simulation system designed in this study made possible to study the principal factors that may affect *Lactobacillus delbrueckii* subsp. *bulgaricus* NRRL-734. Some of these factors are the acid pH in the stomach and the hepatic bile in the small intestine, also, the presence of some important digestive enzymes such as pepsin and pancreatin. It was possible to suggest that *Lactobacillus delbrueckii* could survive in a high number and reach the colon when it is consumed together with similar food like those evaluated in this study. Future research on the same lines to determine the effects of typical food on the viability of the probiotics that are contained in functional foods will make it possible to establish recommendations to the consumer on how to consume the functional food, thereby allowing the survival of a higher quantity of viable probiotic lactic bacteria and at the same time on how to obtain higher benefits for probiotics consumers.

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