ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2012, Vol 19, No 1, 99-102
www.aaem.pl

The viability and intestinal epithelial cell adhesion of probiotic strain combination – *in vitro* study

Jacek Piątek[1,2], Magdalena Gibas-Dorna[2], Anna Olejnik[3], Hanna Krauss[2], Krzysztof Wierzbicki[2], Wioletta Żukiewicz-Sobczak[1], Maciej Głowacki[4]

[1] *Department of Allergology and Environmental Hazards, Institute of Rural Health, Lublin, Poland*
[2] *Department of Physiology, University of Medical Sciences, Poznan, Poland*
[3] *Department of Biotechnology and Microbiology of Food, University of Life Sciences, Poznan, Poland*
[4] *Department of Pediatric Orthopaedics, University of Medical Sciences, Poznan, Poland*

Piątek J, Gibas-Dorna M, Olejnik A, Krauss H, Wierzbicki K, Żukiewicz-Sobczak W, Głowacki M. The viability and intestinal epithelial cell adhesion of probiotic strain combination – *in vitro* study. Ann Agric Environ Med. 2012; 19(1): 99-102.

Abstract

To be effective, probiotic bacteria must exhibit a number of functional characteristics, including the resistance to gastric acidity and the ability to adhere to the intestinal epithelium. In this study, we examined *in vitro* the viability of lactic acid bacteria (LAB) combination after exposure to low pH, and the adhesion of LAB to Caco-2 cells during coincubation of 9 bacterial strains. To test bacterial viability, 6 commercially available products were incubated in 0.1 N HCl at pH 1.2 for 60 min. The greatest growth inhibition was noted for the non-capsulated product containing the Lactobacillus rhamnosus strain (log reduction of CFU = 6.4), and the best survival observed for the product containing 9 bacterial strains, equipped with a modern capsule made according to the Multi-Resistant Encapsulation technology (log reduction of CFU = 0.1). In the adhesion experiment, the combination of 9 bacterial strains was added to 17-day-old Caco-2 cell culture for 90 min. The greatest efficiency of adhesion was observed for the inoculum containing 5.5×10^8 CFU/mL/9.6 cm^2 of Caco-2 and the dose of probiotic bacteria of 190 cells per one Caco-2 cell. As a result, approximately 157 bacterial cells adhered to one Caco-2 cell. The results indicate that the combination of 9 bacterial strains in the examined product is characterized as highly adhesive.

Key words

probiotics, Lactic acid bacteria, bacterial viability, adhesion, encapsulation

INTRODUCTION

According to the generally accepted definition, probiotics are live microorganisms beneficially affecting the health of a host animal [1]. Commercially available products containing probiotics are in the form of fermented dairy products and dietary supplements. Before reaching the intestinal tract, probiotic bacteria must first survive in the acidic and protease-rich conditions of the gastric juice, and then resist the toxic effects of bile acids. The normal exposure to hydrochloric acid takes approximately 60 min. Several reports have indicated that the survival and viability of bacteria from most probiotic products in gastric juice is rather poor, and only few of them exhibit stability and resistance to adverse environmental conditions [2, 3]. Therefore, to improve the survival of these bacteria in the human gastro-intestinal system, new technological strategies were developed. Microencapsulation of bacterial cells is the method that tends to stabilize cells, potentially enhancing their viability and stability in the production, storage and handling of lactic cultures [4].

Another very important characteristics affecting the beneficial activity of probiotic strains *in vivo* are their intestinal epithelial adhesion properties [5, 6]. In fact, bacterial adhesion is considered to be one of most preferable characteristic of probiotic strains. The ability of probiotic bacteria to adhere to mucus and/or intestinal epithelial cells is one of the mechanisms protecting the host organism from pathogen invasion and adhesion. The effect is observed even if the bacterial adhesion is transient and does not lead to permanent intestinal colonization [7, 8]. Probiotic strains of high adherence capacity effectively prevent diarrhea, shorten the duration of diarrheal episodes, and alleviate inflammatory responses [9]. This illustrates the necessity for studies on factors determining the stability of the characteristics that give rise to the probiotic health effects, and to satisfy the increasing market request to obtain products in which probiotic cultures are more active [10, 11, 12, 13].

The aim of the presented study was to investigate both the resistance of 9 probiotic strains from one tested product to adverse environmental conditions, such as low pH, and their ability to adhere to the intestinal epithelium.

MATERIAL AND METHODS

The bacterial viability. The bacterial viability of 6 commercially available products were tested according to the method by Sorensen [14]. Experimental *in vitro* conditions mimicked the acidic conditions of the gastric juice.

Address for correspondence: Jacek Piątek MD, PhD, Department of Physiology, University of Medical Sciences, Święcicki 6, 60-781 Poznań, Poland.
E-mail: drpiatek@interia.eu

Received: 20 November 2011; accepted: 09 March 2012

To count the encapsulated bacteria incorporated into the tested product, 1-10 capsules with probiotics were homogenized and the CFU value estimated. Next, the entrapped bacteria were incubated in 0.1 N HCl at pH 1.2 - incubation time 60 min. After incubation, the material was suspended in the phosphate buffer at pH 6.8 until the absolute breakdown of the capsules, but for no longer than 60 min. The experiment was conducted at 37°C.

Content of the tested material is presented in Table 1.

Table 1. Tested products

Tested products	No.	Types of bacterial strains in tested products
Product A; 9 strains encapsulated	010211	Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus plantarum Lactobacillus Helvetius Lactococcus lactis Bifidobacterium longum Bifidobacterium bifidum Bifidobacterium breve Streptococcus thermophilus
Product B; 9 strains without capsulation	010210	Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus plantarum Lactobacillus Helvetius Lactococcus lactis Bifidobacterium longum Bifidobacterium bifidum Bifidobacterium breve Streptococcus thermophilus
Product C; 1 strain without capsulation	018410	Lactobacillus rhamnosus
Product D; 3 strains encapsulated	121109413	Lactobacillus acidophilus Lactobacillus bulgarius Bifidobacterium animalis
Product E; 2 strains encapsulated	12032	Lactobacillus acidophilus Lactobacillus rhamnosus
Product F; 4 strains encapsulated	01748	Lactobacillus bulgaricus Lactobacillus acidophilus Lactobacillus rhamnosus Bifidobacterium bifidum

THE INTESTINAL EPITHELIAL CELL ADHESION

Preparation of Caco-2 cell cultures. For the adhesion assay, a 17-day-old (well-differentiated) Caco-2 cell (ECACC 86010202) culture was used. Caco-2 cells were placed in Dulbecco's modified Eagle's minimal essential medium DMEM (Sigma, USA), supplemented with 10% foetal bovine serum (Gibco, USA), and 1% nonessential aminoacids solution (Sigma, USA) and gentamicin (Gibco, USA). The Caco-2 cells were grown under standard conditions (37°C, 5% CO_2, 95% humidity) in a tissue culture flask with a 25 square centimeter growth surface. The culture was performed until 80-90% of the surface area was covered. The cells then were detached from the cover glass and dispersed using trypsin-EDTA solution (0.25% porcine trypsin and 0.2% EDTA in PBS, Sigma, USA), and replaced in a new culture flask. The passage was performed twice within 7 days.

Prepared Caco-2 cells were transferred to a flask with a 75 square centimeter growth surface, and the cultivation conducted as described above. After 4 days of cultivation, the cellular layer covered 80% of the surface area. The cells were detached from the cover glass using trypsin-EDTA solution and counted in a Neubauer hematocytometer chamber. Viability was estimated using trypan blue staining.

Obtained Caco-2 cells were cultured for adhesion assay on Falcon culture plates (growth area 9.6 square cm). The initial cell density was 3.4×10^4 cells per square centimeter. Caco-2 cultures were incubated for 17 days and the medium replaced by a new one every 24-48 hours, depending on the differentiation stage. Cultivation was performed under standard conditions, as described above. After 17 days and before adhesion assay, the cells were washed in PBS buffer (Sigma, USA) and counted using a hematocytometer chamber for the 3 different cultures.

Preparation of bacteria for the adhesion process and estimation of adhesive properties. The content of Product B was suspended in 0.9% NaCl and 100 µL was placed in 10 mL of MRS broth (de Man, Rogosa, Sharpe). The culture was incubated under anaerobic conditions at 37°C for 20 hours. After incubation, the microbial culture was centrifuged at 4,500 rpm for 10 min. and the supernatant washed twice with 0.9% NaCl. Bacterial cells (5×10^6, 5×10^7, and 5×10^8) were suspended in 1mL of DMEM without antibiotics and foetal bovine serum. The bacteria were then incubated with Caco-2 cells for 90 min. under standard conditions.

Non-adhering bacteria were removed and the epithelial cells rinsed 3 times with PBS buffer. To release attached bacterial cells, the Caco-2 monolayer was treated with a solution of 1% Triton X-100 detergent (Sigma, USA) mixed with PBS buffer. The lysis was carried out on ice for 10 min. Then the lysates were centrifuged at 4,500 rpm for 10 min. The supernatant was washed twice with PBS. Finally, the supernatant was suspended in 1 mL of 0.9% NaCl, inoculated, and the number of adhered bacteria quantified according to the serial dilution method of Koch.

According to the Koch method, serial decimal dilutions ranging from 10^{-1} - 10^{-5} CFU per mL were prepared. Material from each dilution was inoculated into Petri dishes by the pour method, using MRS broth. Bacterial cultures were incubated under anaerobic conditions at 37°C for 48 hours. The bacterial cell density from the stock culture used for the adhesion assay was also estimated. After incubation, the number of adhered bacteria was quantified. Based on the obtained results, the dose of bacteria used for adhesion process, the number of adhering bacteria for used inoculum (%), and the number of adhering bacteria per one Caco-2 epithelial cell were calculated.

STATISTICS

Statistical analysis of data was performed using the STATISTICA statistical package programme, version 9. For the viability study, significant differences in the effect of the different products were tested using two-way analysis of variance (ANOVA). Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Worldwide acceptable criteria for the *in vitro* selection of probiotic bacteria include isolation of strains capable of

performing effectively in the gastrointestinal tract. To be effective, the bacteria must posses a number of functional characteristics, including nonpathogenic behaviour, resistance to technologic processes, resistance to gastric acidity and bile toxicity (viability), high properties to adhere to gut epithelium, ability to persist within the gastrointestinal tract, production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities [15]. Our study focuses on 2 important characteristics: bacterial viability after *in vitro* exposition to low pH, and the adhesive properties of 9 bacterial strains from the tested product. Products were analyzed as a whole, without preparation of individual components, which means in a form containing a probiotic bacteria combination and ready to be used by the consumer.

Bacterial viability. All ingested products that enter the gastrointestinal tract must transit the acidic environment of the stomach. The exposition to gastric HCl takes approximately 60-90 min. and serves for digestion and disinfection. Therefore, studies on providing probiotic-based food products with high survival properties in acidic conditions are an approach currently receiving considerable interest. At present, microencapsulation appears to be the best method protecting good bacteria from the gastric primary defence system [16]. The results obtained describe the bacterial viability of a probiotic product containing 9 bacterial strains, and compare survival at low pH of 6 encapsulated and non-encapsulated products containing different bacterial strains and quantities. The drop in quantity of encapsulated bacteria (Products A, D, E, F) was lower in comparison with the growth inhibition of free bacteria (Product C) grown under identical culturing conditions after 60 min. exposure to low pH (Tab. 2). Significant difference was also observed when comparing the same combination of bacterial strains with and without encapsulation (Product A vs. Product B). A better survival of the coated bacteria was observed. The highest growth inhibition at low pH was noted for the product containing Lactobacillus rhamnosus strain (Product C) without capsule, while the best survival of bacteria was observed for Product A which contained 9 bacterial strains, equipped with a modern capsule made according to the MURE technology (Multi-Resistant Encapsulation). Acidity reduction of the CFU reached 72% for Product C and 8% for Product A. Products D, E, and F, coated with traditional capsules, exhibited a significantly percentage in acidity reduction of the CFU, compared with Product A (values for Products D, E, and F were 21%, 25% and 32%, respectively). The results indicating high bacterial viability in the encapsulated products correspond to the results found in the available literature [17, 18, 19]. In conclusion, encapsulation increases the resistance of the bacteria to low pH of medium environment, and the resistance is greater when the bacteria are encapsulated according to the MURE technology, compared with traditional coating.

Table 2. The number of lactic acid bacteria before and after 60 min exposure to 0.1 N HCl at pH 1.2.

Tested products	0hr $\log_{10}$ CFU ml^{-1}	1hr $\log_{10}$ CFU ml^{-1}	% acidity reduction
Product A; 9 strains encapsulated	9.43±0.04	8.67±0.03	8
Product B; 9 strains without capsulation	9.52±0.04	8,12±0.04	15
Product C; 1 strain without capsulation	8.86±0.03	2.46±0.03	72
Product D; 3 strains encapsulated	8.68±0.05	6.88±0.02	21
Product E; 2 strains encapsulated	9.58±0.03	7.18±0.04	25
Product F; 4 strains encapsulated	8.88±0.05	6.08±0.04	32

Data are expressed as means±SD of three replications

Adhesive properties of 9 bacterial strains from selected probiotic products. The most studied probiotics are the lactic acid (LA) bacteria, particularly Lactobacillus and Bifidobacterium. We studied the adhesion of LA bacteria to the epithelial cells during coincubation of 9 bacterial strains from one probiotic product (Product B) with Caco-2 cells (Tab. 2-3).

Table 3. The dose of bacteria used for adhesion and the number of adhering bacteria

Dose of bacteria CFU/mL	Dose of bacteria CFU/1 Caco-2 cell	Number of adhering bacteria	Adhesion %
5.5×10^6	1.9	$(2.48\pm0.03)\times10^6$	45.5 %
5.5×10^7	19	$(3.28\pm0.02)\times10^7$	60.2 %
5.5×10^8	190	$(4.53\pm0.02)\times10^8$	83.1 %

Adhesion is expressed as the percentage of bacterial counts adhered to Caco-2 cells to the total number of bacteria added.
The means of three independent determinations ±SD are presented

The adhesive properties of particular selected strains found in Product B are described well in the available literature, and vary one from another. Depending on the origin and the dose, different types of bacteria represent different adhesive properties. For example, there are data indicating the adhesive properties of Lactobacillus rhamnosus at the level of 7.2-14.4% [20] and at the level of 20% [21]. Other reports present quite different results, describing Lactobacillus rhamnosus as a strain with low ability to adhere to the epithelial cells (0.2% adhering cells, which means 2 adhered cells per one Caco-2 cell) [22]. For the isolated Lactobacillus Plantarum strain, the adhesion properties are also within a wide range of values. Tuomola et al. obtained an adhesion percentage at the level of 6.7%, while Moussavi et al. at the level of 80%. As reported by Candela et al. and Gagnon et al., similar adhesion was described for Bifidobacterum longum and Bifidobacterum lactis, and potentially low abilities to adhere to the ephithelial cells are characteristic for Lactobacillus acidophilus (approx. 5 bacterial cells per one Caco-2 cell) [23, 24].

The above data, however, do not include the possible interactions between strains in a mixture of probiotic bacteria normally found in market products. A combination of bacterial strains and the final adhesive properties might be of particular importance since probiotic health products usually contain more than one bacterial strain. Our study has shown that when probiotics are in combination, the adhesive properties of the tested products are very strong. The number of adhering bacteria depends on the dose of the tested product used for adhesion assay. The greatest efficiency of adhesion was observed for the inoculum containing 5.5×10^8 CFU/mL/9.6 cm^2 of Caco-2, and the dose of probiotic bacteria of 190 cells per one Caco-2 cell. As a result, approximately 83% of bacterial cells adhered to one Caco-2 cell.

Candela et al. classified microorganisms according to their adhesive properties into 3 categories: 1) non-adhesive

strains, when less than 5 cells adhere to Caco-2, 2) adhesive strains, when the effectiveness of adhesion means 5-40 cells adhered to one Caco-2 cell, and 3) highly adhesive strains, when the level of adhesion exceeds 40 cells per one epithelial cell [23]. Based on these criteria, it may concluded that the combination of 9 bacterial strains in the product we examined is characterized as highly adhesive.

Our results are supported by other studies showing increased adhesive properties with combinations of probiotic strains. Ouwehand et al. described enhanced adhesion for Bifidobacterium lactis Bb12 in combination with L. rhamnosus 66 or L. delbrueckii ssp. bulgaricus [25]. Collado et al. observed that specific probiotic combinations are able to enhance the inhibition percentages of pathogens adhesion to intestinal mucus when compared to individual strains [26, 27].

Although the presented study does not explain the possible mechanisms involved in interactions between particular bacterial strains, it strongly indicates the importance of examining new probiotic combinations exhibiting high adhesive properties for the market products, which may provide a rational for selecting a combination of probiotic strains for further studies *in vivo*.

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