

Article

Stress effect of food matrices on viability of probiotic cells during model digestion

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Abstract: The aim was to evaluate the influence of model (alcohol, sugar, salt, protein and acid) and real foods and beverages on the viability of probiotics during incubation and artificial digestion. Viability of monocultures *Lactobacillus acidophilus* CCM4833 and *Bifidobacterium breve* CCM7825T and commercial mixture of 9 probiotic bacterial strains were tested by cultivation assay and flow cytometry. In model foods, the best viability was determined in the presence of 0.2 g/L glucose, 10% albumin and 10% ethanol. As the most suitable real food for probiotic survival complex protein and carbohydrate substrates were found, such as beef broth, potato salad with pork, chicken with rice, chocolate spread, porridge and yoghurt. The best liquid was milk and meat broth, followed by coca-cola, beer and coffee. Viability of probiotics was higher when consumed with meals than with beverages only. Addition of prebiotics increased the viability of probiotics especially in presence of instant and fast foods. Generally, the highest viability of probiotics during artificial digestion was observed in mixed culture in presence of protein, sugar and fat or their combination. The increase of cell viability observed in such foods during model digestion may further contribute to the positive effect of probiotics on human health.

Keywords: Probiotics; food matrices; food stress; cell viability; model digestion;

1. Introduction

Probiotics are living microorganisms that, when administered in sufficient quantity (around 10^6 – 10^7 CFU/mL or g of carrier food product) in provide a health benefit to the host, in particular through a replacement or inclusion process beneficial bacteria in the gastrointestinal tract [1,2].

In many studies the effects of probiotics and prebiotics on various diseases was observed. Some studies confirmed that dietary fiber and probiotics have positive effects on infectious diseases [3]. Probiotics have also shown positive responses to clinical treatment against several diseases and disorders, such as constipation and diarrhoea, food allergies, inflammatory bowel disease, preventing and treatment of diabetes, obesity and cancer and diseases related to pathogenic microbes [4-6]. The antimicrobial activity of probiotics occurs through i) reduced pH due to production of acetic and lactic acids, ii) bacteriocins accumulation and iii) compounds blocking bacterial adhesion to the epithelial cells and consequently reducing pathogen toxins production [7]. Probiotic bacteria are crucial for the maturation of immune cells too. This intestinal microbiota stimulates the maturation and functionality of the immune cells through their metabolites [5]. Probiotics are involved in regulation of intestinal health, improved lactose digestion and maintaining bone health and they make functional components such as antioxidants and anti-hypertensive [7]. Further, recent

evidence and ongoing studies suggest that intestinal microbiota has a bidirectional effect on mood disorders and can thus effect on stress and anxiety [4,8]. Research on human diseases is revealing the vital roles played by the gut microbiota. Understanding the impact of the gut microbiota on the host health is essential to design strategies focused in probiotics manipulation. So, other knowledge on options which are responsible for increase of viability of probiotic will allow us to design new strategies to improve the health of the consumer [9].

There are several mechanisms, by which probiotics may benefit human, including production of antimicrobial substances, strengthening of intestinal barrier, modulation of immune response, and antagonism of pathogenic microorganisms either by production of antimicrobial agents or by competition for binding sites, nutrients, and growth factors [4,10,11]. More precisely, the interaction between probiotics and pathogens can be divided into three steps: the physical interaction between the probiotic and the epithelium, the interaction between probiotics and the immune system and, finally, direct interaction between probiotics and pathogens [12]. The interaction between probiotics and pathogens may be observed in the hosts, but also in foods where can have a positive effect too and incorporating of probiotics in food matrices can be new option for food safety [11,12]. Moreover, probiotic bacteria incorporated into foods should be able to survive gastric transit and reach the small intestine in sufficient numbers of viable cells [2,11].

In food-delivered probiotics, viability of probiotics is essential to achieve the health benefits associated to their consumption. Except technological stress during food processing followed by stress connected with stabilization, packaging and storage, probiotics should undergo additional stress during consumption. The passage through the unique environment of gastrointestinal tract is a source of high gastric acidity, oxygen stress induced by ROS released from mucosal surfaces, bile salt stress, osmotic stress and many other effects [2]. Thus, food substrate used to vehicle probiotics can be considered as one of the major factors in regulating colonization of probiotics in gastrointestinal tract. Food helps to buffer the bacteria through the stomach and may contain other functional ingredients, for example prebiotics, that can influence growth, viability and survival, acid and bile tolerance, adhesion of probiotics to intestinal cells [11] and different functionality of probiotics that determine their efficacy in gastrointestinal tract [13].

A way of increasing the efficacy of probiotic preparations may be the combination of both probiotics and prebiotics as synbiotics, which provide an improved survival during the passage of the probiotic bacteria through the intestinal tract [14]. Development of functional foods also may to modulate gut microbiota and convey health effects. Major challenge in this area can be the incorporation of probiotics in foods, selection of the prebiotic candidate or selection of bacterial strains and encapsulation of probiotic bacteria [8]. However, the addition of prebiotics to products may negatively influence the product. Probiotics may also change the sensory quality of product, because some strains may grow in the food matrix and producing metabolites, which interact with the food. The encapsulation of the probiotic may also affect the food texture [15]. On the other hand, dietary supplements in the form such as capsules, tablets and other formats may be used. Nevertheless, a current study on probiotics does not present a definitive answer as to whether there is superiority or equivalence on delivery of probiotics in foods or in the form of supplement [10].

However, probiotics used as supplements may reduce functional efficacy of probiotics due to exclusion of the potential synergistic effect of the food if they did not serve together with appropriate food [13]. Many studies have focused on testing the effect of food matrix on probiotics during storage. Regarding the incorporation of probiotics into food products, the food matrix should meet these

requirements: low aw; neutral or slightly acidic pH, presence of fiber or prebiotic compounds and a high buffering capacity (for example a high fat content). For incorporation of probiotic strains into the product is also important the strain selection [16]. However, the effect of food on probiotics during digestion should be tested as well, because the ability to survive in the gastrointestinal environment is recognized as a fundamental requisite for probiotics [17].

The main goal of this work was a study the influence of different types of real food and beverages in on the viability and growth of probiotic bacteria during simulated passing through the gastrointestinal tract. The main goal of this study was to mimic the most common conditions and environments used during probiotics consumption in broad population, such as effect of diverse real foods including some homemade products, instant foods, snacks, fast food products and some beverages to find practical recommendation of dietary regime to optimum probiotics intake.

2. Materials and Methods

2.1 Material

2.1.1 Probiotic strains

Bacterial strains *Lactobacillus acidophilus* CCM 4833 and *Bifidobacterium breve* CCM 7825T used in this study were purchased from Czech Collection of Microorganisms in Brno, Czech Republic. The commercial preparation Biopron 9 (Walmart, Ltd.) was selected as an optimum source of mixed probiotic strains. This food supplement contained a mixture of 9 bacterial cultures of *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus lactis* and *Streptococcus thermophilus*, 9 x 10⁹ CFU (colony-forming units) in daily dose; additionally, the capsule contains 240 mg of fructo-oligosaccharides.

2.1.2 Food matrices

All used samples of real foods are summarized in **Table 1**. The set of real samples should represent typical foods and beverages consumed daily by most of population. Beverages, instant foods (instant soups; pasta with cream sauce; porridge) and snacks (yoghurt, pudding, chocolate spread, pastry, poppy seed cake, chips; potato salad) were obtained from local retail. Instant foods were mixed with hot water or milk according to producer’s recommendations. Home made products (mixed vegetable broth; chicken broth; beef broth; boiled chicken meat with rice, potato salad with fried pork) were prepared immediately before experiments from natural materials (300 g) boiled in water (1 L) with 3 g of salt. Hamburger was received from fast food retail. Fruits and vegetables were used as mixtures of tomato, cucumber and pepper with the ratio of 1:1:1.

Table 1. Overview of tested real foods and beverages

Beverages	Soups	Main courses	snacks	Fruit, vegetables,
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water	Beef broth instant	Hamburger – fast food	Yoghurt chocolate	
black tea	pea instant soup with croutons	Pasta with cream sauce - instant	Pudding with whipped cream	apple with banana
black coffee	instant soup with liver dumplings	Chicken with rice - boiled	Chocolate spread	tomato, cucumber and white pepper
beer (4.9 % alcohol)	vegetable broth - homemade	Potato salad with fried pork	porridge	
juice orange	chicken broth - homemade		pastry	
milk (3.5% fat)	beef broth - homemade		Poppy seed cake	
Coca-cola			Chips - salted	

2.2 Methods

2.2.1 Cultivation of probiotic bacteria

The strains were inoculated into MRS broth (HiMedia, India) and incubated for 48 hours at 37 °C. The cells were harvested by centrifugation at 6000 rpm for 4 °C for 10 min (Hermle Z36 HK, Hermle, Germany), and washed with distilled water. Afterwards, cells were used for inoculation and after incubation and cultivation in different environment to viability analysis.

Before use, the probiotic strains from the commercial Biopron capsule were hydrated by sterile water for 20 minutes and then used for inoculation and cultivation.

2.2.2 Model food matrices

As model food matrices, solutions with various concentrations of alcohol (ethanol 5–40 %), sugar (0.2–20 g/L of glucose), salt (0.4–10 g/L of NaCl), protein (bovine serum albumin 5–20 g/L) in solutions of different pH (pH 7, pH 3) were prepared. Standard and pure chemicals were purchased from Sigma Aldrich (Germany). All used model foods are summarized in **Table 2**.

Table 2. Overview of tested model foods

	1	2	3	4
Sterile distiller water	pH 7	pH 3 (with HCl)		
Protein (Bovine Albumin)	5 g/L	10 g/L	20 g/L	
Saccharide (Glucose)	0.2 g/L	2 g/L	10 g/L	
Salt (NaCl)	0.4 g/L	1.2 g/L	4 g/L	10 g/L
Alcohol (Ethanol)	5%	10%	20%	40%

2.3 Model digestion

Artificial stomach juice was prepared from 0.25 g of pepsin dissolved in 100 mL of distilled water. To this solution 0.84 mL of 35 % hydrochloric acid was added. Final pH was adjusted to 0.9. Artificial

pancreatic fluid was prepared with 0.25 g of pancreatin and 1.5 g of sodium hydrogen carbonate in 100 mL of water (pH = 8.9). Bile fluid was composed of 0.4 g of bile acid salts dissolved in 100 mL of phosphate buffer. Probiotics were added to 300 g of beverage, liquid food or solid food homogenized in appropriate water volume. A mixture of probiotic cells (1.10^{10} CFU) and food/beverage was first incubated at 37 °C for 15 min to reach starting values of cell numbers. Then, the mixture of probiotics and food matrix was mixed with stomach juice (in the ratio of 3:1) and incubated 20 min at 37°C. Additionally, intestinal fluids formed by mixture of pancreatic fluid and bile salts in the ration of 1:1 (v/v) were added to final ratio of food and all digestion juices 3:2, similarly to GIT conditions. Model digestion was set as a shortened continuous process.

2.4 Determination of cell viability by cultivation method and flow cytometry

The viability of probiotic bacteria was tested by cultivation assay on Petri dishes with MRS medium by overflow method. After 48 hours of cultivation colony forming units, e.g. bacterial colonies (CFU/mL) were counted. Cultivation method was used for evaluation of all types of foods and beverages including model foods.

The viability of probiotic bacteria exposed to model and real liquid foods and beverages was followed also by direct analysis of cell viability using flow cytometry (Apogee Flow Systems, Hemel Hempstead, UK). As a fluorescent probe propidium iodide (Sigma-Aldrich, Germany) was used. For analysis, 100 µl of liquid sample were taken, diluted to 1 ml and added propidium jodide. After 5 minutes of incubation in darkness was measured viability of probiotic cells in clear liquid model and real food matrixes in each stage of digestion.

2.5 Viability of monocultures and mixed probiotic cultures in model and real foods and beverages in conditions of model digestion

Probiotic bacterial cultures of *Lactobacillus acidophilus*, *Bifidobacterium breve*, or a commercial mixture of probiotic cells (1 capsule of Biopron 9; about 9.10^9 CFU) were added to the 300 g of model or real food/beverages in the amount of 1.10^{10} CFU. Each of tested probiotics sample was first mixed with food/beverage and incubated for 15 minutes at 37°C. Then, the samples were exposed to model digestion according to paragraph 2.3. In regular intervals (after or before incubation), the amount of living cells was measured using cultivation methods and flow cytometry. The evaluation of viability, CFU number and, thus, influence of individual model and real foods is complicated by continuous growth of probiotic cells in any environment. Thus, number of viable cells was measured gradually at the beginning of each stage of continuously performed model digestion and after its finishing. First evaluation was done after incubation of probiotics with food matrix as the response to dilution and food stress environment. This measurement was followed by evaluation after acidification at the beginning of stomach digestion, then after 20 min incubation in stomach juice (pH 2.1), followed by cell counting after alkalization and mixing with the mixture of pancreatic and bile juices and at the end of digestion process (pH 7.9; 2 hours). Acidity of environment (pH) was checked during the whole digestion process.

2.5 Viability of mixture of probiotic cells in different types of foods and beverages with/without addition of prebiotics

Probiotics from commercial capsule were re-hydrated for 20 min, and solubilized in sterile distilled water. Then, cells were mixed with real food matrix and with or without the addition of a prebiotics (300 mg of inulin; Sigma-Aldrich, Germany). Recommended daily dose of probiotics mixture (1 capsule; 1.10^{10} CFU) was suspended in 300 g of homogenized food or beverage. This prepared mixture was then incubated at 37°C for 15 minutes. Then, samples were taken for analysis of number of viable cells at the beginning of digestion process (1 ml). Because of heterogenic character of samples, viability of probiotic cells was determined using cultivation techniques. Determining of the number of cells in the samples taken was performed after 48 hour of cultivation at MRS agar.

2.6. Viability of monocultures and mixed probiotic cultures in real foods and beverages in conditions of model digestion

Probiotic bacterial cultures of *Lactobacillus acidophilus*, *Bifidobacterium breve*, or a commercial mixture of probiotic cells (content of capsule Biopron 9) were added to the 300 g of model or real food/beverages in the amount of 1.10^{10} CFU. Each of tested probiotics sample was first mixed with food/beverage and incubated for 15 minutes. Then, the samples were exposed to model digestion according to paragraph 2.3. In regular intervals (after or during incubation), the amount of living cells was measured. The viability of probiotics during simulated gastrointestinal conditions were performed using cultivation methods and flow cytometry (liquid samples). Cultivation methods were used for all heterogenic samples, homogenous solutions was possible to determine directly by FC. The evaluation of viability and CFU in individual model and real foods are complicated by continuous growth of probiotic cells in any environment. Thus, the viability after digestion (or individual stages) was compared to the value of CFU at the beginning of model digestion, after short-time incubation with the food.

3. Results

3.1. Viability of probiotics in model food matrices

The aim of present study was to evaluate viability of tested probiotic cultures in the presence of selected model and real foods and beverages in model conditions of digestive tract. As a model food, solutions with various concentrations of alcohol, sugar, salt, protein and acetic acid were prepared. Real foods were selected according to most common dietary preferences in population.

In all model foods except acidic food, the pH value was set to pH 7, model acid food is of pH 3. In **Table 3** are presented pH values in tested real beverages and foods. With exception to juice (pH 3) and beer (pH 5.1), all tested beverages and foods exhibited pH in the range of 6.0 -7.3. Thus, we cannot expect some dramatic influence of pH of tested food matrixes on viability of probiotic cells. However, more acidic pH (approx. 5.5 – 6.5) will be probably more suitable for probiotic bacteria growth.

3.1.1. Growth of mixed probiotic culture in model foods exposed to artificial digestion

The first part of this study the influence of basic model foods on concentration and viability of probiotic cells during artificial digestion was studied (**Figure 1A-F**). As the probiotic culture, mixed commercial preparative was used. The measurement was performed using a flow cytometry and as model foods water (water-based food), acetic acid solution of pH 3 (acid food), glucose (sweet food),

protein (protein-based food), sodium chloride (salted food) and ethanol (alcohol-based food) at different concentrations (see **Table 2**) were used. The evaluation of viability and CFU in individual model and real foods is complicated by continuous growth of probiotic cells in any environment, thus, in some samples, an increase of CFU after model digestion was observed. It is necessary to note that number of CFU determined by flow cytometry is related to 1 µl of sample because of extreme sensitivity of this method when compared with cultivation techniques.

Table 3: pH values in real beverages and foods

Beverage/liquid	pH	Food	pH
water	7.0	Pasta with cream sauce - instant	7.1
black tea	6.9	Chicken with rice - boiled	7.2
black coffee	6.0	Potato salad with fried pork	6.7
beer (4.9 % alcohol)	5.1	Hamburger – fast food	7.1
juice orange	3.2	Yoghurt chocolate	6.2
milk (3.5% fat)	7.1	Pudding with whipped cream	7.3
Coca-cola	5.6	Chocolate spread	6.3
Beef broth	6.2	porridge	5.2
Vegetable broth	6.0	pastry	7.0
Chicken broth	6.3	Poppy seed cake	6.2
Beef broth instant	6.2	Chips - salted	7.1
Pea instant soup with croutons	6.4	Fruits (apple + banana)	6.7
instant soup with liver dumplings	6.5	Vegetables mix	5.8

First, model digestion using the probiotic mixture without addition of food matrix was measured. Probiotic capsule content was re-hydrated in water and placed directly into stomach and, then, to intestine fluids. This experiment simulated a model swallowing an intake of dry probiotic capsule without addition of any food or beverage. A significant decrease of the number of living cells was observed after model digestion without the presence of some food matrix. After drastic pH changes, a dramatic decrease (approx. 17.5x) of the number of originally present cells was recorded at the beginning of intestinal stage of model digestion (**Figure 1F**). On the other hand, after model digestion of probiotics in the presence of water, approx. 12times increase of original number of cells was recorded. It is a model swallowing intake of a probiotic capsule together with 300 ml of water. Subsequently, the effect of protein (**Figure 1B**) on cell growth during digestion was tested. Two hours after addition of intestinal juices, a significant increase of cell concentration in sample with addition of 10 g/L of bovine albumin was observed. In this sample, about 3times more of the original number of cells was determined.

The effect of salt environment on cell growth during digestion is introduced in **Figure 1C**. After moderate increase of CFU in the environment of intestinal fluids, two hours after addition some decrease of cell concentration was observed (except the lowest concentration used). Moreover, with increasing salt concentration, the more intensive decrease of cell concentration was observed. For 0.4

A

B

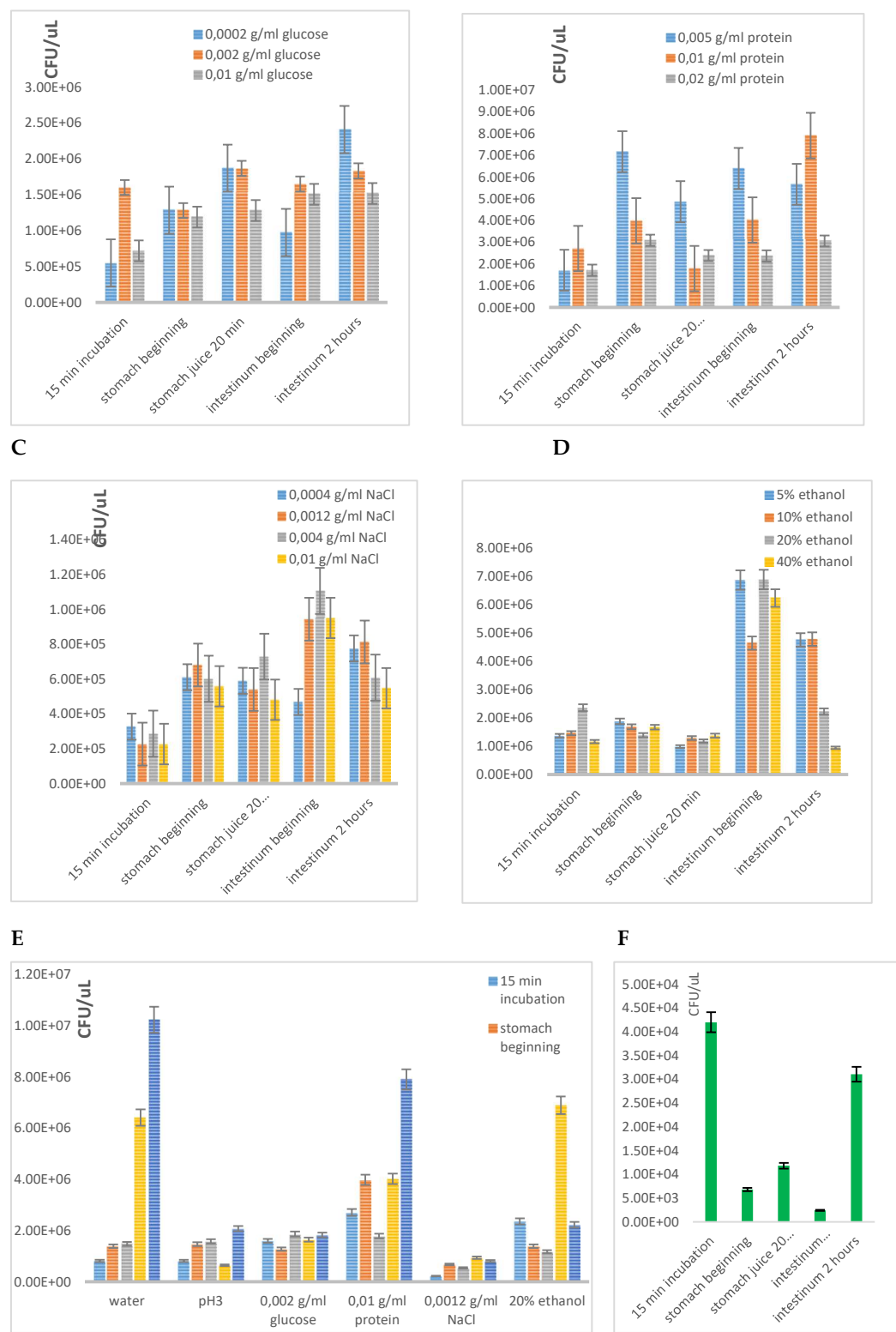


Figure 1. Viability of mixed culture of lactic acid bacteria during the process of digestion of model foods containing probiotics (commercial probiotic mixture) – A) sugar concentration, B) protein concentration; C) salt concentration; D) alcohol concentration; E) comparison of factors, pH 3 was set by acetic acid; F) incubation of cell suspension directly in digestive fluid.

g/L NaCl concentration, final CFU value after two hours of model digestion was about 2times higher than the original CFU value found at the beginning of exposition to artificial digestion.

Next, the effect of alcohol (**Figure 1E**) on cell growth during digestion was monitored. After the addition of intestinal juices, moderate increase of CFU was detected, but after a longer digestion a decrease of cell concentration was observed again. The decrease in cell number with increased alcohol concentration was observed as well. Nevertheless, low ethanol concentration (5-10%) exhibited relatively good effect on CFU (about 3-4x increase after complete model digestion).

Further, the effect of model food with glucose on cell growth during digestion was monitored (**Figure 1A**). The highest increase of cell number was recorded in the sample with 2 g/L of glucose. After two hours of model digestion, about 5times higher CFU in this sample was determined. Finally, the number of cells in acidic model food was tested (**Figure 1E**). At the beginning of the intestinal stage, a decrease of number of cells in acidic food was observed. However, after two hours of exposition to the intestinal juice, the CFU number for acidic food were already 2times higher.

Generally, according to total CFU numbers in model foods, as the most suitable environments for probiotic preparative intake were recognized: i) water, ii) protein at the concentration to 10 g/l, iii) glucose to 2 g/l and low ethanol concentration (5-10%). The most negative effect exhibited predominantly salt, which was caused probably by osmotic stress similarly to the effect of higher glucose concentration. Negative effect of ethanol as bacteriostatic agents was observed after long-term exposition to higher concentration (20 % and more); lower concentration of ethanol exhibited positive effect on probiotic viability. The reason could be cross-response to external stress effect of ethanol and probably also an ability of some probiotics to use ethanol as an additional carbon source.

3.1.2. Flow cytometry determination of viability of probiotics exposed to model digestion in liquid environments

In the following part of this work, the influence of model foods on the CFU number and viability of probiotic monocultures of *Lactobacillus acidophilus* and *Bifidobacterium breve* after model digestion was studied (**Figure 2**). For comparison, mixed probiotic culture was used. The scheme of experiment is described in Paragraph 2.5. After artificial digestion in stomach juice followed by incubation in intestinal juices, CFU number was directly determined by flow cytometry. At the end of incubation, the culture of *Lactobacillus acidophilus* exhibited high increase of CFU predominantly in model food containing protein, but slightly also in other model foods (**Figure 2A**). Conversely, the strain *Bifidobacterium breve* grew intensively predominantly in model foods containing saccharide (**Figure 2B**). In the commercial probiotic mixture, the highest increase of CFU number was found after the model digestion in the presence of water at pH=7 and in the presence of acidic solution (pH=3). Furthermore, the CFU increase was detected in foods containing protein and 10% alcohol as well (**Figure 2C**).

Flow cytometry was used also for determination of cell viability in the environment of clear (centrifugation 5 000 rpm, 10 min) meat and vegetable broth prepared according to Paragraph 2.1.2. Results are illustrated in **Figure 3**. When compared with model foods, monocultures as well as mixed probiotics culture exhibited similar trends corresponding ty some of typical model foods, such as protein (meat broth) or sugar (vegetable broth). The best environment for all tested samples was chicken broth, containing high concentration of proteins. Except this environment, monocultures grew quite slowly (approx.. 4-5x lower biomass formation) when compared with mixed culture. Growth of mixed culture in meat extract was lower than in the presence of 10 g/L of protein.

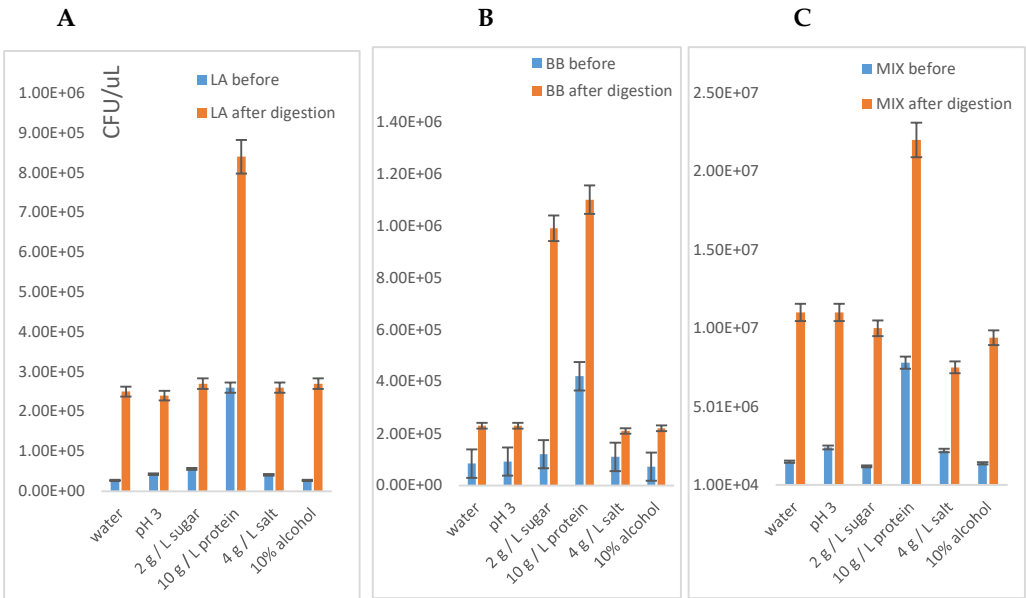


Figure 2. The influence of model foods and the type of probiotic on the number of viable cells after model digestion - A) *Lactobacillus acidophilus*; B) *Bifidobacterium breve*; C) Probiotic mixture

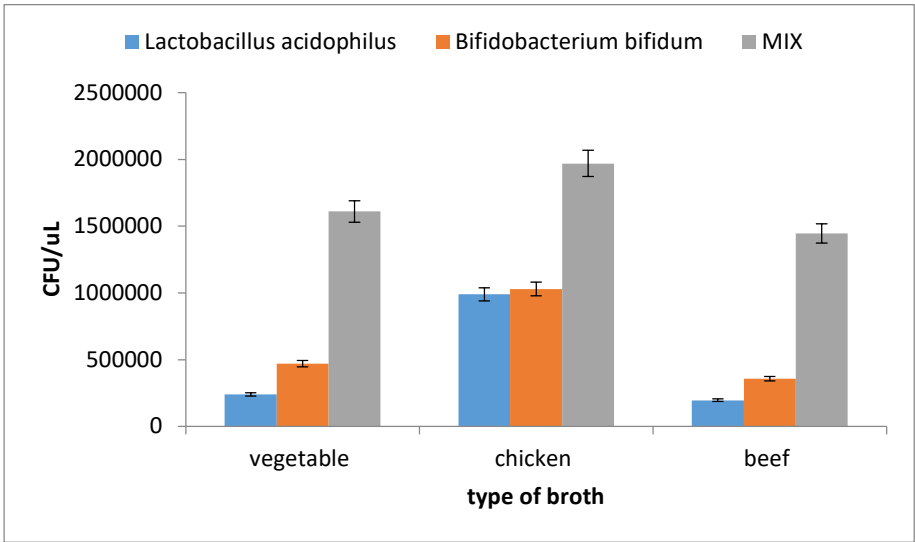


Figure 3. The influence of the type of food extract (broth) and the type of probiotic on the number of viable cells after model digestion

The resistance of probiotic mixture against acidic pH mentioned above is caused probably by addition of some protective agent to the preparative, which could prevent damage of cells by acidic stomach environment, as described by the producer. Similar study described as a suitable food environment for protection of lactic acid bacteria a neutral or slightly acidic pH (5–6) [19]. This pH was optimal for growth of probiotics during food manufacturing and cell survival during storage and digestion. Water activity can also improve cells protection during digestion, as well as solids and gels. Presence of highly fermentable sugars and fiber promoted survival cells during food storage and digestion, too. High fat content exhibited positive effects on cells mainly due to good buffering capacity [19].

Flow cytometry is highly sensitive method of cell viability determination, especially when compared to the cultivation techniques (Figure S1). About 1000 times lower amount of sample was sufficient for direct evaluation of cell viability (see Figure S2). We can suppose that clear beverages (water, tea; meat broth) and model solutions are suitable for flow cytometry measurements of cell viability, oppositely to colloid materials (e.g. milk), food homogenates and all environments containing some particles. Thus, the comparison of all tested real beverages was done by cultivation techniques to eliminate the interference of some impurities.

3.2 Influence of real foods and beverages on viability of probiotics

3.2.1. Growth and viability of probiotics in real beverages exposed to model digestion

In the next part of these work was tested the viability and growth of probiotic bacteria *Lactobacillus acidophilus*, *Bifidobacterium breve* and commercial mixture of probiotic strains after passing through the digestive tract with wide range of different type of food and beverages. Tested products can be divided into the following groups: drinks, soups, main courses and snacks (Table 1).

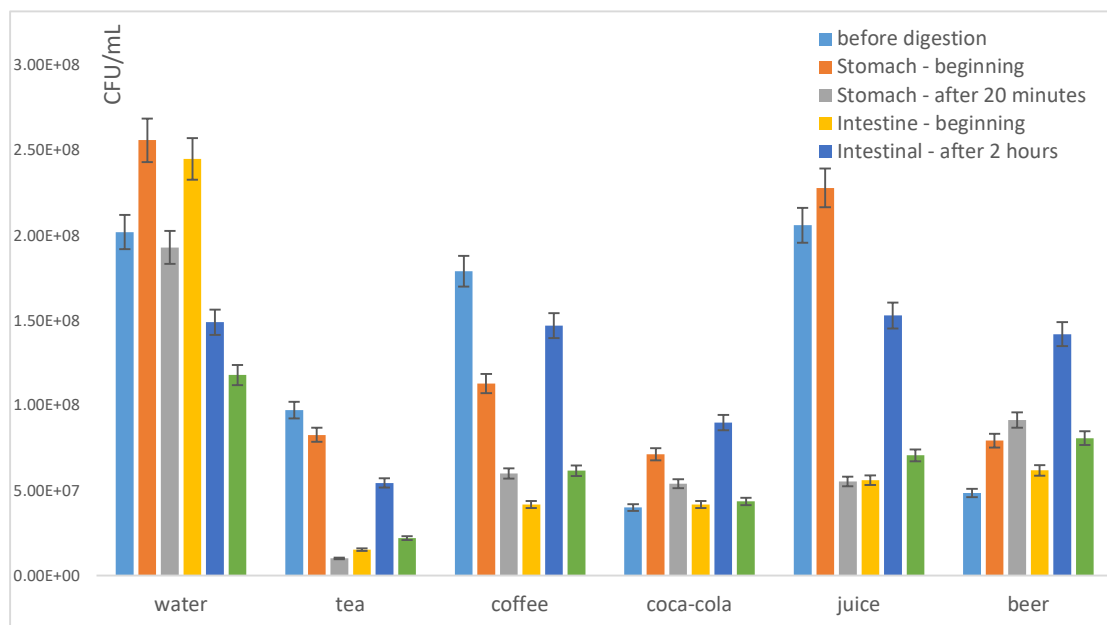
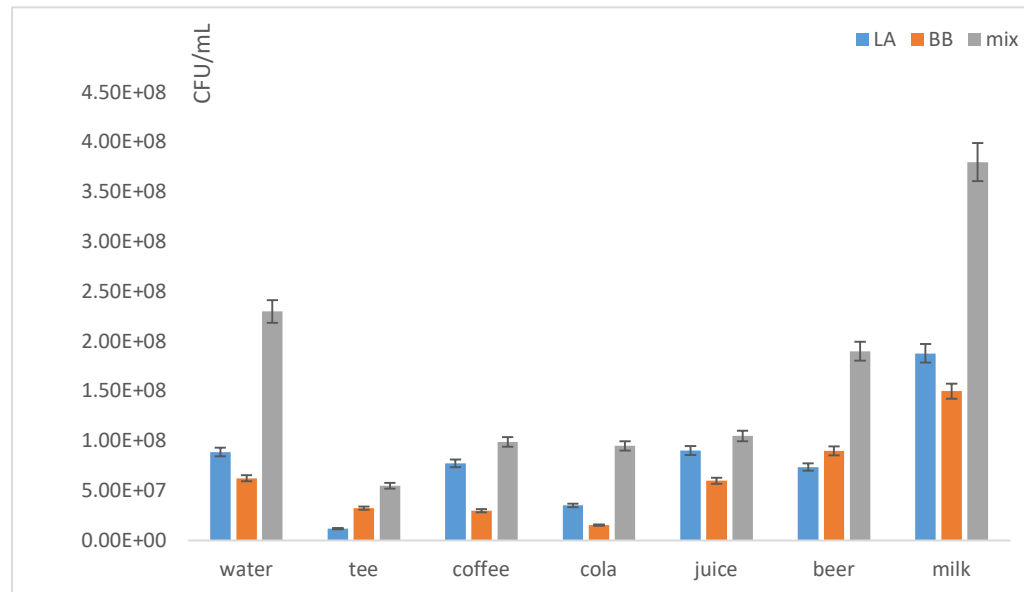


Figure 4. Viability of mixed probiotic culture in selected beverages during gradual process of model digestion

Traditionally, the use of probiotics in dairy beverages has been widely extended. However, since people who suffer from allergy to milk proteins or have severe lactose intolerance cannot consume dairy beverages, non-dairy beverages such as fruits, vegetables and cereals juices may also represent a suitable vehicle to deliver probiotics to consumers, with regard to the stability of the cells during storage [18]. In this study water, black tea, coffee, beer, juice, coca-cola and milk were tested. In all these beverages the highest amount of survived cells and increased growth of probiotic cells was observed in the intestinal environment (Figure 4). These results were also verified by flow cytometry.

The influence of real beverages on the CFU number and viability of probiotic monocultures of *Lactobacillus acidophilus* and *Bifidobacterium breve* after model digestion in comparison with probiotic mixture was studied (**Figure 5**) too. After artificial digestion in stomach juice followed by incubation in intestinal juices, CFU number was determined in monocultures and probiotic mixture.

A



B

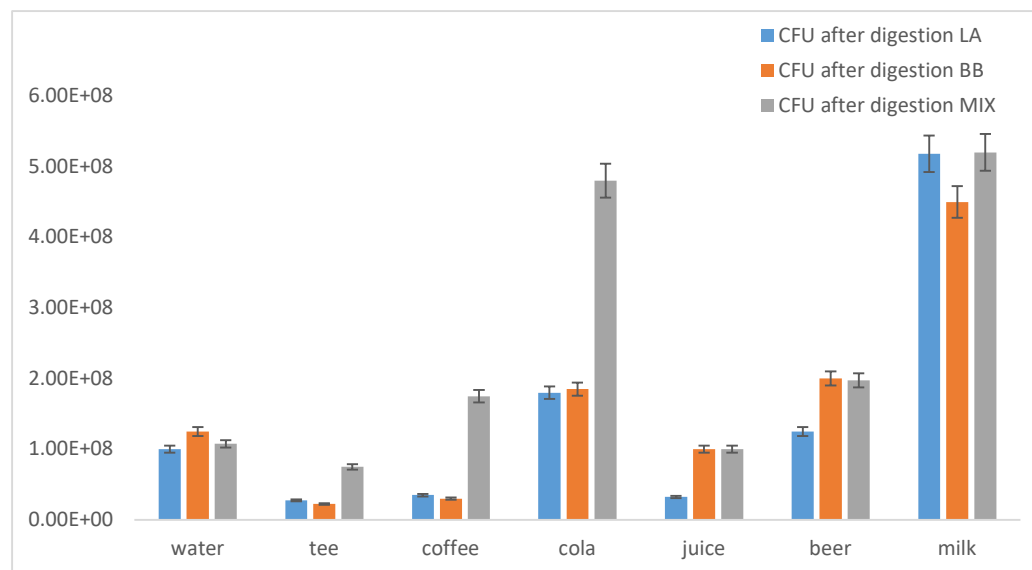


Figure 5. The influence of different types beverages and the type of probiotic on the number of viable cells after model digestion – comparison of monocultures *Lactobacillus acidophilus* (LA) and *Bifidobacterium breve* (BB) and mixed probiotic culture (MIX): **A**) viability after 15 min incubation; **B**) viability after complete model digestion (2 hours).

When the monocultures of *Lactobacillus acidophilus* or *Bifidobacterium breve* was used, significant increase (about 2.5 – 3x) of surviving cells was observed only in milk environment (**Figure 5A**). This finding confirms the fact, that milk is well-known suitable environment for probiotic bacteria. The probiotic cells from commercial preparation (**Figure 5B**) showed the highest increase of living cells

in a coca-cola and milk environment. Interesting was also the finding that increased CFU numbers of probiotic cells after passing through artificial digestion have also been observed in presence of coffee, beer and black tea. The mixture of probiotic strains is probably more stable in different environments when compared with monoculture.

3.2.2. Growth and viability of probiotics in some real liquid and solid meals during exposition to model digestion

Real meals were processed according to Paragraph 2.1.2 and divided into two groups: i) liquid meals (e.g. soups – instant and homemade) and ii) solid meals and snacks (see **Table 1**). Influence of all these environments on viability of monocultures of *L. acidophilus* and *B. breve* as well as on viability of probiotics in mixed culture was studied (**Figure 6**).

Regarding homogenates of tested soups (broth, pea soup, beef soup) (**Figure 6**), the number of viable probiotic cells of monocultures as well as commercial mixed culture after model digestion was similar in presence of all tested soups. The group of tested solid foods was formed by hamburger, instant pasta with cream sauce, fried pork meat with potato salad and chicken with rice as main courses (**Figure 6**). Here, the highest number of viable probiotic cells of monocultures as well as commercial mixed culture after model digestion was found in presence of potato salad with pork, porridge and chicken and rice. These meals are rich mainly in proteins, sugars and milk (in the case of porridge) (**Figure 6**). These meals are rich mainly in proteins, sugars and in the case of porridge also milk.

In the group of snacks (**Figure 6**), the absolutely highest CFU number of mixed probiotic culture after model digestion was observed in presence of chocolate spread. Increased CFU number in probiotic mixture was also observed in the presence of mixed fruits, chocolate pudding with whipped cream, salted chips, fruits and yoghurt (**Figure 6**). As in previous group, these foods exhibited similar character mainly regarding high sugar content. Important could be also the presence of fat, milk and, surprisingly, also salt stress in presence of salted chips was accepted by probiotics relatively well. Overall, similarly to beverages, the mixed probiotic culture was more stable in all tested food environments compared with monocultures (**Figure 6**).

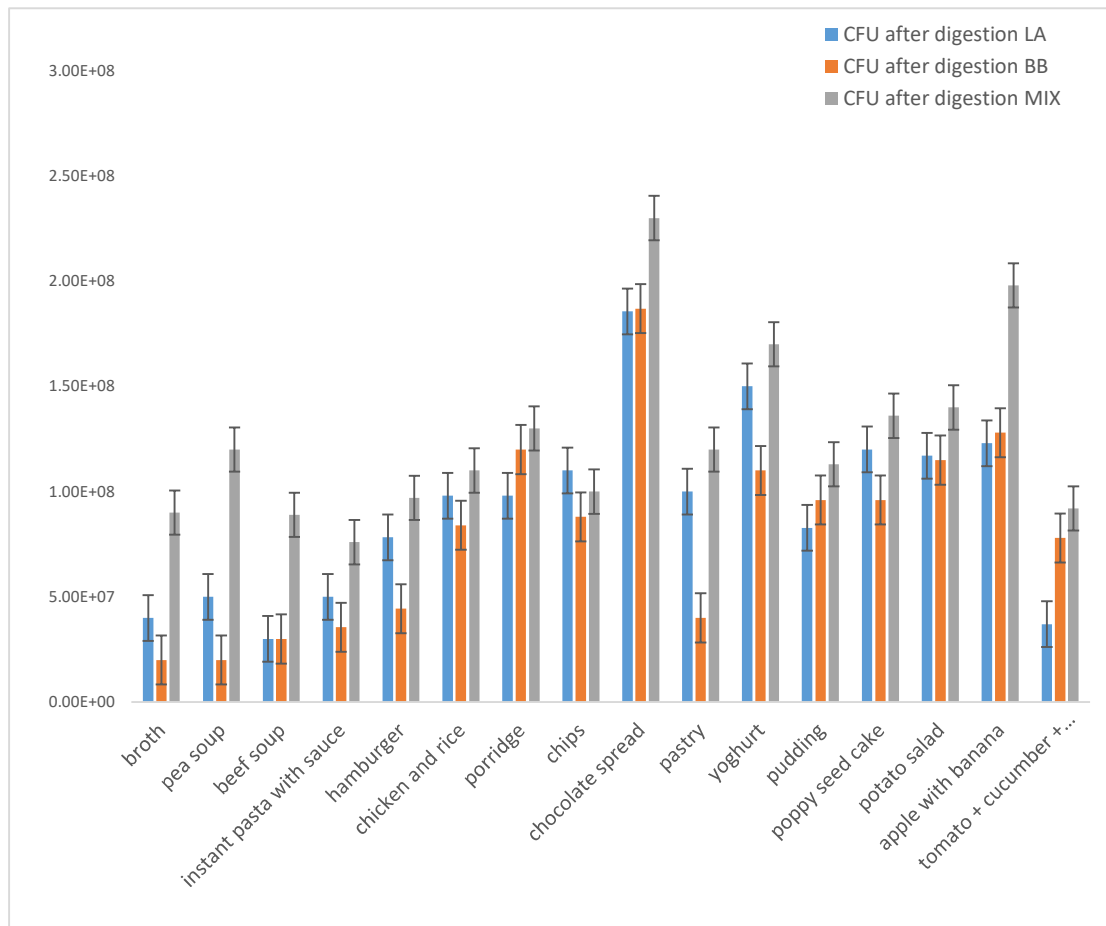


Figure 6. The influence of different types of food and the type of probiotic on the number of viable cells after model digestion (CFU/mL) - comparison of monocultures *Lactobacillus acidophilus* (LA) and *Bifidobacterium breve* (BB) and mixed probiotic culture (MIX)

3.2.3. Viability of mixed probiotic culture in the presence of probiotics and real foods and beverages

Finally, the addition of prebiotics and its influence of viability of mixture of probiotics in presence of different type of foods/beverages was observed (**Figure 7**). As a prebiotic in this study, inulin was used as one of the most studied and widely used prebiotics [10]. Mixed probiotic culture from capsule was incubated for 20 min in presence of individual foods with and without addition of inulin.

As supposed, the highest number of survived probiotic cells was recorded in presence of milk. Higher growth of probiotic cells compared to growth in distilled water was determined for fruits, yoghurt, fruit juice, black tea, cocs-cola and beef broth environment. When the probiotic mixture together with a prebiotic was used, similar effect was observed and the highest cell growth predominantly in presence of milk, beef broth and coke was determined (**Figure 7**). Addition of prebiotics/probiotics mixture led to an increase of viability of probiotics in presence of some “unhealthy” processed foods, such as hamburger and coke.

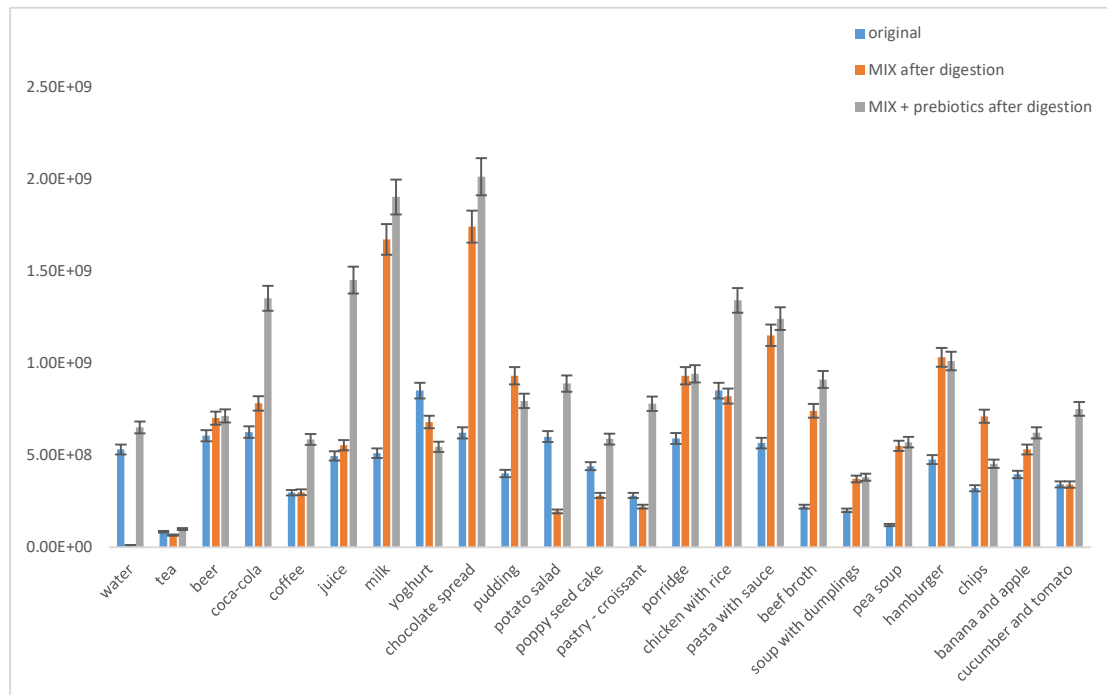


Figure 7. The effect of various foods and the addition of prebiotics (inulin) on the viability of probiotic cells in mixed probiotic preparative (MIX)

4. Discussion

Nowadays, probiotics belong to the most popular food supplements. They can be taken in different ways and forms, can be a part of different dietary regimes. A minimum dose of 10^6 colony forming units per mL or g (CFU/mL or CFU/g) must be reached if the food product will be labelled as probiotic [18]. Some researches even suggest increasing the dose up to 10^7 CFU/mL or CFU/g [19], because the viability of microorganisms is the key to achieve the health benefits. Viability depends on environmental condition in final product and its interaction with the probiotic strain. Thus, the food matrix's chemical composition and its physical state can substantially affect growth, stability and survival of probiotic microorganisms during digestion [19]. Microbial cultures should be capable of growing in substrate media, survive during technological processing and maintain their viability throughout the storage.

For stability and viability of probiotics not only the type of probiotic preparative and processing, but also the foods and beverages ingested simultaneously with probiotics can substantially influence the final effect of probiotics. Moreover, differences in the concentration and viability of probiotic cells when passing through the gastrointestinal tract may be observed. The present work deals mainly with the influence of different types of model and real foods/beverages on the viability of probiotic bacteria during digestion. The aim is to select foods that can increase cell viability during artificial digestion, which may further contribute to the positive effect of probiotic cells on human health.

Species of *Lactobacillus* and *Bifidobacterium* are the most commercial probiotics available in the foods market [18]. Therefore, in this work the influence of food and beverages was tested on

monocultures of *Lactobacillus acidophilus*, *Bifidobacterium breve* and for comparison also on commercial mixture of probiotics containing 9 different probiotic strains.

To reach probiotic status and ability to promote health benefits, it is necessary to evaluate cells resistance to the digestion process [10]. In this study, probiotic cultures were incubated shortly in selected foods and beverages and then the viability of probiotics was tested in model artificial digestive tract. The conception was proposed to find suitable and, conversely, completely inappropriate food taken into the body together with probiotics. First, incubation of probiotics in the environment of various model foods was performed, following by artificial digestion. As a model food, solutions with various concentrations of alcohol, sugar, salt, proteins and acid were prepared. Viability of probiotics in model solutions was measured directly by flow cytometry, which is a highly sensitive method sufficient for clear beverages and model solutions, oppositely to colloid materials, food homogenates and all environments containing some particles, which should be evaluated by cultivation techniques.

In further part, real foods and beverages were tested in similar way as model foods. Recently it was found that some of food matrices are during storage and simulated gastrointestinal conditions more protective than others [4,10,18]. In particular, special attention has been paid to dairy products such as cheese, yogurt, and fermented milk. Cheese has a potentially good matrix for delivery of probiotic due to several characteristics including its higher pH value, greater buffering capacity, greater fat content and nutrient availability, and lower oxygen content. Also fruits and vegetables have been found to be an ideal addition to probiotic foods, likely because they provide essential nutrients for bacterial growth [4]. As well as fiber-rich products such as fruit and grain can increase viability of probiotic bacteria during storage and simulated gastrointestinal conditions [18,19].

The tolerance of probiotic bacteria to gastric and small intestine conditions seems to be significantly influenced by the food carrier. Many studies showed that vegetable matrices could improve probiotics vitality during the gastric transit. The good protection of probiotics against simulated gastric juices was confirmed for carrot juice, similarly to dairy matrices [2]. In other studies, the food matrix impact on lactic acid bacteria viability during food digestion of dairy versus non-dairy products was compared [18]. Comparing milk and fruit juices, the high tolerance for bile acids and pepsin in milk environment was observed. Regarding different flavours of juice, banana and carrot juice exhibited highly positive effect when compared with orange juice [4]. Very good positive effects on probiotics similar to dairy product were reported for pasta [7]. When comparing different dairy products, the best results were obtained with cheese, milk and yoghurt, and finally ice cream [13]. However, for different strains, differences in the order of these matrices were recorded [19].

The purpose of this work was to test potential stress effect of food matrices on growth of probiotics and possible protection against the effects of the digestive system. This study contains a set of original data represented by results of incubation of probiotics in presence of many different types of complex food matrixes and exposed to model digestion. Meals containing combination of plant and animal foods such as meat, pasta, cream and some of the most popular beverages were tested. Some of our data agree with previously published studies focused on influence of fruit and dairy products [4,7,18-19]. Based on the results it can be concluded that the best way appears to be the combination of mixtures of probiotics with foods rich in proteins and sugars. As the best option among beverages seems to be milk. Nevertheless, other beverages such as tea, coffee, coca-cola and

beer can be recommended to consumption with probiotics. Further, we can conclude that probiotic microorganisms have survived better in presence of meals, when compared with consumption with the beverages only. Higher growth of probiotic cells was observed in foods containing high concentration of sugar, protein and fat or their optimal combination. These data are confirmed also by recent study, where carbohydrates are the most widely used protective compounds during dehydration, storage and exposure of probiotic to gastrointestinal tract [16]. Addition of prebiotics can positively influence the effect of processed and fast foods (hamburger, coca-cola) on probiotics viability in presence of these foods.

5. Conclusions

The present work dealt with the influence of different types of model and real foods on cell viability during digestion. The main goal of the work was to select a suitable food that demonstrates an increase in cell viability during digestion, which can further lead to an increase in the positive effect of probiotic cells on human health.

As model foods still water, acidified water (pH=3), and various concentrations of glucose, proteins, sodium chloride and ethanol were used. The viability of probiotic monocultures of *Lactobacillus acidophilus* and *Bifidobacterium breve* during model digestion were monitored and compared with commercial probiotic mixture. The highest growth of *Lactobacillus acidophilus* cells was detected in foods containing protein. On the contrary, *Bifidobacterium breve* exhibited the best growth in presence of foods containing saccharide. The best growth of mixed probiotic culture was determined in water at neutral pH and in acidic environment, as well as in the presence of foods containing protein and 10% alcohol.

Regarding real foods and beverages, the best environment food for increase of probiotic viability were complex meals with meat and fiber, milk products - porridge and yoghurt, chocolate spread and mixed fruits. Among beverages, the best option was milk, but acceptable for viability of probiotics were coca-cola, coffee, beer and black tea. We can conclude that probiotics are more viable when consumed with meals compared to the beverages only. Viability of mixed probiotic culture was higher in all environments when compared with both monocultures. Addition of prebiotics further positively increased viability of probiotics even in presence of processed foods. In general, the highest viability of probiotics during artificial digestion was observed in mixed probiotics culture in presence of protein, sugar and fat or their combination. The increase of cell viability observed in such foods during model digestion may further contribute to the positive effect of probiotics on human health.

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