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Effects of milk components and food additives on survival of three bifidobacteria strains in fermented milk under simulated gastrointestinal tract conditions

Małgorzata Ziarno* and Dorota Zaręba

Division of Milk Biotechnology, Department of Biotechnology, Microbiology and Food Evaluation, Warsaw University of Life Sciences, Szkola Główna Gospodarstwa Wiejskiego w Warszawie, Warsaw, Poland

Background: In the dairy industry, probiotic strains of *Bifidobacterium* are introduced into the composition of traditional starter cultures intended for the production of fermented foods, or sometimes are the sole microflora responsible for the fermentation process. In order to be able to reach the intestines alive and fulfil their beneficial role, probiotic strains must be able to withstand the acidity of the gastric juices and bile present in the duodenum.

Objective: The paper reports effects of selected fermented milk components on the viability of three strains of bifidobacteria in fermented milk during subsequent incubation under conditions representing model digestive juices.

Design: The viability of the bifidobacterial cells was examined after a 3-h incubation of fermented milk under simulated gastric juice conditions and then after 5-h incubation under simulated duodenum juice conditions. The *Bifidobacterium* strains tested differed in their sensitivity to the simulated conditions of the gastrointestinal juices.

Results: Bifidobacterial cell viability in simulated intestinal juices was dependent on the strain used in our experiments, and product components acted protectively towards bifidobacterial cells and its dose.

Conclusions: Bifidobacterial cells introduced into the human gastrointestinal tract as food ingredients have a good chance of survival during intestinal transit and to reach the large intestine thanks to the protective properties of the food components and depending on the strain and composition of the food.

Keywords: Bifidobacterium; *gastrointestinal conditions*; *milk protein*; *whey protein*; *fruit jam*; *prebiotic*

*Correspondence to: Małgorzata Ziarno, Faculty of Food Sciences, Warsaw University of Life Sciences – Szkoła Główna Gospodarstwa Wiejskiego (WULS-SGGW), Nowoursynowska Str. 159C, 02-776 Warsaw, Poland, Email: malgorzata_ziarno@sggw.pl

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Bifidobacteria have a positive effect on the health and functioning of the human gastrointestinal tract (GIT). Particularly important is their presence in the large intestine. *Bifidobacterium* can enter the human body with food or pharmaceutical formulas. The participation of these bacteria in the intestinal ecosystem is therefore very variable and often insufficient to ensure the proper functioning of the intestinal ecosystem. Studies have shown that the effectiveness of probiotics on the human digestive tract is largely dependent on the choice of an appropriate strain of the microorganism and the provision of a systemically appropriate number of live bacterial cells. It is recognised that the consumption of probiotics in the amount of 10^6–10^9 cells per day is the minimum effective therapeutic dose (1). It has been well known that the viability of bifidobacteria in gastric and intestinal juices is different at strain levels and is affected by a number of factors. These include the physiological state of the strain, the application of a probiotic carrier, and the addition of suitable prebiotics (2, 3). Also important are external factors to which the products are exposed after inoculation with bacterial starter culture, that is, technological conditions, fermentation, refrigeration, hydrogen ion concentration (pH value), water activity, and redox potential. The commercial Bb12 strain has already been extensively studied around many of these areas. Many studies are already available on the survival of bifidobacteria in simulated gastric and intestinal conditions (e.g. low pH, gastric enzymes, and bile salts) as well as on the effect of milk components, such as fat, and prebiotics, such as inulin.

In the dairy industry, probiotic strains of *Bifidobacterium* are used as a component of the starter culture intended for the production of fermented beverages, cottage cheeses,

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ice creams, and frozen desserts, or sometimes are the sole microflora responsible for the fermentation process (4–7). Most research and newly developed technologies with probiotic starter cultures are focused on dairy products. To reach the intestine alive and fulfil their beneficial role, probiotic strains must withstand the acidity of the gastric juices and bile present in the duodenum.

The aim of this study was to investigate the effects of selected preparations added to the fermented milks on the viability of three *Bifidobacterium* strains during subsequent incubation under conditions of *in vitro* model digestive juices. *Bifidobacterium bifidum* strain s1 was compared to two common probiotic strains of bifidobacteria, *B. animalis* ssp. *lactis* Bb-12 and *B. lactis* Bo.

Materials and methods

Raw material

The following *Bifidobacterium* strains were used in the experiments: *Bifidobacterium bifidum* s1 from the collection of the Division of Milk Biotechnology, Warsaw University of Life Sciences, *Bifidobacterium animalis* ssp. *lactis* Bb-12 (Chr. Hansen, Denmark), and *Bifidobacterium lactis* Bo (Danisco, Denmark). All bifidobacteria strains were stored deep-frozen in a mixture of MRS broth (Merck, Germany) and glycerol (Sigma-Aldrich, USA) mixed at a ratio of 8: 2. Before use in experiments, strains were defrosted and cultured anaerobically in 20 ml of BSM broth (Sigma-Aldrich, USA) for 12 h at 37°C. The average amount of bifidobacterial cells inoculated to the broth was 1.8×10^7 CFU per 20 ml of broth.

Simulated GIT conditions

A simulated gastric juice (SGJ) was prepared according to the recipe published by Clavel et al. (8), that is, 4.8 g NaCl (POCH, Poland), 1.56 g $NaHCO_3$ (POCH), 2.2 g KCl (POCH), and 0.22 g of $CaCl_2$ (POCH) were dissolved in 1 litre of distilled water, and then the pH was adjusted to 2.4 with 1 M HCl (POCH) or 1 M NaOH (POCH). The solution was drawn up in a 50 ml bottles, and batched and sterilized by autoclaving for 15 min at 121°C. Immediately prior to testing, to a portion of simulated gastric juice, 1.25 mg of crystalline pepsin (Sigma-Aldrich, USA) dissolved in 1 ml of sterile distilled water was added.

A simulated duodenum juice (SDJ) was prepared according to the recipe published by Marteau et al. (9) by dissolving 5 g of NaCl (POCH), 0.6 g KCl (POCH), 0.25 g $CaCl_2$ (POCH), and 8.5 g of bovine bile in 1 litre of 1 M solution of $NaHCO_3$ (POCH). This solution was adjusted to pH 7.0 with 1 M HCl (POCH) or 1 M NaOH (POCH), drawn up in a 50 ml bottles, batched and sterilised in an autoclave for 15 min at 121°C. Immediately prior to testing, to a portion of simulated intestinal juice, two capsules of Kreon 10,000 (Abbott Products, USA) were added. Each capsule contains 150 mg of active pancreatin equivalent to: 10,000 U. Ph. Eur. of lipase; 8,000 U. Ph. Eur. of amylase; and 600 U. Ph. Eur. of protease.

Experimental systems

Experiment No. 1: anaerobic incubation of broth cultures in simulated GIT conditions. Two millilitres of centrifuged live biomass of the strain to be tested were mixed with 50 ml of SGJ or SDJ. Then, 1 ml of the mixture was collected in order to determine the initial number of bacteria cells in the system. The remaining parts of SGJ or SDJ and bacterial biomass mixtures were placed in an incubator for 3 h at 37°C (in the case of SGJ) or for 5 h (in the case of SDJ). After this period, the sample was again mixed and 1 ml of the mixture was collected in order to determine the final number of live bacterial cells. The experiment was performed in five independent replications.

Experiment No. 2: anaerobic incubation of a sample of fermented milk with an increased content of non-fat solid mass (NFSM) in simulated GIT conditions. Milk with an increased content of NFSM was prepared by adding an appropriate amount of skimmed milk powder (containing 35.7 g of protein, 51.2 g of lactose, and 0.8 g fat per 100 g of product) in non-fat UHT milk. The amounts of skimmed milk powder were to reach the final content of NFSM as follows: 8.5%, 10.0%, 12.5%, and 15.0%. Prepared milk samples were heated in a water bath to 45°C, and after cooling in cold water, they were inoculated with 2 ml of a suspension of the living bifidobacteria monoculture and fermented anaerobically for 4 h at 37°C. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.8 ± 0.31 log CFU/ml, 7.6 ± 0.32 log CFU/ml, and 8.8 ± 0.22 log CFU/ml, respectively, and did not depend on NFSM content. The pH value of fermented milk was 4.47–4.67 and did not depend on the used strain or NFSM content. The resulting fermented milk (an amount of 50 ml) was precisely mixed with SGJ (50 ml), and then 1 ml of the mixture was collected in order to determine the initial number of bifidobacterial cells in the system. The remaining part of the mixture was placed in an incubator for 3 h at 37°C. After this period, the sample was again mixed and 1 ml of the mixture was collected in order to determine the final number of living bifidobacterial cells. The entire mixture was transferred to 50 ml of SDJ, mixed and again 1 ml of the mixture was collected in order to re-determine the number of bacterial cells. The mixture was again placed in the incubator for 5 h at 37°C, and after incubation again 1 ml of the mixture was collected in order to determine the number of bifidobacterial cells. The experiment was performed in five independent replications.

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Experiment No. 3: anaerobic incubation of a sample of fermented milk containing whey protein concentrate (WPC)-68 (Kowpol, Poland) under simulated GIT conditions. The preparation of milk for the fermentation, the fermentation of the milk, and incubation under simulated GIT conditions were preceded as in Experiment No. 2, where the addition of WPC-68 was 0%, 1.0%, 2.0%, 2.5%, and 5.0% by weight. The WPC-68 was added to the milk before the fermentation process. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.8 ± 0.22 log CFU/ml, 7.7 ± 0.23 log CFU/ml, and 8.9 ± 0.32 log CFU/ml, respectively, and did not depend on WPC-68 content. The pH value of fermented milk was 4.36–4.64 and did not depend on the used strain or WPC-68 content. The experiment was performed in five independent replications.

Experiment No. 4: anaerobic incubation of a sample of fermented milk containing an increased content of fat in simulated GIT conditions. The preparation of milk for the fermentation, the fermentation of the milk, and incubation under simulated GIT conditions were preceded as in Experiment No. 2, where in order to increase the fat content, the appropriate amount of homogenised UHT cream (of 30% fat content) was added. The amounts of UHT cream were to reach the final content of fat as follows: 0%, 0.5%, 2.0%, 4.0%, and 8.0%. The UHT cream was added to the milk before the fermentation process. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.9 ± 0.20 log CFU/ml, 7.6 ± 0.23 log CFU/ml, and 8.8 ± 0.33 log CFU/ml, respectively, and did not depend on fat content. The average pH value of fermented milk was 4.69 ± 0.24 and did not depend on the used strain or fat content. The experiment was performed in five independent replications.

Experiment No. 5: anaerobic incubation of a sample of fermented milk containing plum pulp (prepared in laboratory conditions, containing 54 g of sugar per 100 g, food grade, pH of 3.45) added under simulated GIT conditions. The preparation of milk for the fermentation, the fermentation of the milk, and incubation under simulated GIT conditions were preceded as in Experiment No. 2, where the addition of a plum pulp preparation (plum jam) after the fermentation was 0%, 5%, 10%, 15%, and 20% by weight. The plum pulp was added to the milk after the fermentation process. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.7 ± 0.22 log CFU/ml, 7.8 ± 0.21 log CFU/ml, and 8.7 ± 0.20 log CFU/ml, respectively, and did not depend on fruit pulp content. The addition of fruit pulp to the fermented milk samples resulted in a decrease of pH value of samples from initial 4.55 to final 4.47–4.23, respectively, in samples containing 5%, 10%, 15%, and 20% of fruit pulp. The experiment was performed in five independent replications.

Experiment No. 6: anaerobic incubation of a sample of fermented milk containing an inulin preparation (food grade, obtained from Raftiline HP, Orafti, Belgium) under simulated GIT conditions. The preparation of milk for the fermentation, the fermentation of the milk, and incubation under simulated GIT conditions were preceded as in Experiment No. 2, where the addition of an inulin preparation was 0%, 1.0%, 2.0%, 2.5%, and 5.0% by weight. The inulin preparation was added to the milk before the fermentation process. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.9 ± 0.23 log CFU/ml, 7.6 ± 0.27 log CFU/ml, and 8.9 ± 0.20 log CFU/ml, respectively, and did not depend on inulin level. The pH value of fermented milk ranged from 4.34 to 4.64 and did not depend on the used strain or inulin level. The experiment was performed in five independent replications.

Experiment No. 7: anaerobic incubation of a sample of fermented milk containing a Fibregum™ preparation (Colloids Naturels International, France, containing in excess of 90% soluble fibre, food grade) under simulated GIT conditions. The preparation of the milk for the fermentation, the fermentation of the milk, and incubation under simulated GIT conditions were preceded as in Experiment No. 2, where the addition of an inulin preparation was 0%, 1.0%, 2.0%, 2.5%, and 5.0% by weight. The Fibregum preparation was added to the milk before the fermentation process. The average cell number of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12 in fermented milks immediately after the fermentation process was 8.7 ± 0.23 log CFU/ml, 7.5 ± 0.25 log CFU/ml, and 8.7 ± 0.20 log CFU/ml, respectively, and did not depend on the Fibregum content. The pH value of fermented milk was 4.43–4.62 and did not depend on the used strain or the Fibregum content. The experiment was performed in five independent replications.

Microbiological analysis

The analysis included the determination of the bifidobacterial cell count in samples using the plate method, in two parallel replicates and five independent replicates for each analysed sample. BSM agar medium (Sigma-Aldrich, USA) was used for the determination of bifidobacterial cell number. Preparations of samples for microbiological analysis, as well as dilutions, were performed according to EN ISO 6887-5:2010 'Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 5: Specific rules for the preparation of milk and milk products'. Plates of BSM agar inoculated with sample dilutions were incu-

bated for 72 h at 37°C under anaerobic conditions (Anaerocult A, Merck, Germany). After anaerobic incubation, grown colonies of bifidobacteria were counted and the number of bacteria cells was converted into colony-forming units per 1 ml (CFU/ml), and then log CFU/ml has been calculated.

Statistical analysis

The mean values and standard deviations were calculated from the data obtained from the experiments. These data were compared using Tukey HSD's Multifactor ANOVA in Statgraphics Centurion XV (Statpoint Technologies, Inc., USA). Statistical significance was set at $P<0.05$. To assess the possibility of sample classification based on the survival of bacteria in fermented milk sample during subsequent incubation under conditions of *in vitro* model digestive juices, cluster analysis was performed. The statistical analyses were conducted with Statistica v.12 software (StatSoft Inc., USA).

Results

As our first experiment (Table 1) showed, a suspension of the same bifidobacterial cells under simulated GIT conditions caused the bacterial population to reduce drastically in the case of SDJ or to gradually in the case of SGJ. The biomass of live cells of *B. bifidum* s1 tolerated SGJ for 3 h worse than the living cells of *B. lactis Bo* and better than *B. animalis* ssp. *lactis* Bb12. *B. lactis* Bo and *B. animalis* ssp. *lactis* Bb-12 strains were tested as reference strains. The bifidobacteria strain selection criteria considered their probiotic status and numerous publications relating to research on the survival of these strains under different environmental conditions. Strains tested belonged to the three species of *Bifidobacterium*; this can be relevant for differentiating their behaviour in model simulated GIT conditions. The initial viable cell number of *B. bifidum* s1, *B. animalis* ssp. *lactis* Bb12, and *B. lactis* Bo was 7.9 ± 0.33 log CFU/ml, 7.8 ± 0.68 log CFU/ml, and 6.8 ± 0.51 log CFU/ml, respectively. After 3 h of incubation in simulated SGJ, the population of *B. bifidum* s1 and *B. animalis* ssp. *lactis* Bb12 decreased significantly to 4.7 ± 0.70 log CFU/ml and 3.1 ± 0.13 log CFU/ml, respectively. The final number of *B. lactis* Bo cells in SGJ was 6.0 ± 0.54 log CFU/ml and did not differ significantly from the initial value. However, regardless of the strain, all bifidobacterial population were reduced when placed in SDJ for 5 h. The pH values of SGJ and SDJ did not change after the inoculation of pure biomass of each *Bifidobacterium* strain. The average pH of SGJ was 2.56 ± 0.08 and SDJ was 7.06 ± 0.13.

The addition of milk components

Our further studies have shown that the behaviour of bifidobacterial cells under different simulated GIT conditions depends on the type and dosage of the additive used for milk. In our experiments, we have found that the addition of skimmed milk powder (Tables 2a, 3a, and 4a) or WPC (Tables 2b, 3b, and 4b) to milk fermented by bifidobacteria cultures has a statistically significant beneficial effect on bacterial cell survival under simulated GIT conditions, only at the highest level of applied dose. This phenomenon was found after increasing NFSM content up to 10.0% and 12.5% in the case of *B. bifidum* s1, and *B. lactis* Bo or *B. animalis* ssp. *lactis* Bb12, respectively (Tables 2a, 3a, and 4a). The final pH of samples ranged from 5.52 to 5.84 and did not depend on the used strain or NFSM content. The initial population of *B. bifidum* s1 ranged from 8.5 log CFU/ml to 8.0 log CFU/ml and after 3 h in SGJ and 5 h in SDJ it dropped to 7.5 ± 0.22 log CFU/ml in milk samples containing 8.5% of NFSM and to 8.1–8.2 log CFU/ml in milk samples containing 10% or more of NFSM (Table 2a). For comparison, the initial number of *B. lactis* Bo cells was 7.2–7.4 log CFU/ml and after 8-h incubation in SGJ and SDJ decreased to 6.5–6.7 log CFU/ml in milk samples containing 8.5% or 10% of NFSM and to 7.0–7.1 log CFU/ml in milk samples containing more than 10% of NFSM (Table 3a). Similar observations were made for *B. animalis* ssp. *lactis* Bb12 (Table 4a). At the beginning of incubation, their cell number was 8.5–8.6 log CFU/ml. After 3-h incubation in SGJ and 5-h incubation in SDJ, the population of *B. animalis* ssp. *lactis* Bb12 was 7.6–7.7 log CFU/ml in milk samples containing 8.5% or 10% of NFSM and to 7.9–8.1 log CFU/ml in milk samples

Table 1. Bifidobacterial cell number in growth bullion submitted to simulated gastric or duodenum juice

	Bifidobacterium bifidum s1	*Bifidobacterium animalis* ssp. *lactis* Bb12	*Bifidobacterium lactis* Bo
Simulated gastric juice			
0 h	7.9 ± 0.33a*	7.8 ± 0.68a	6.8 ± 0.51a
3 h	4.7 ± 0.70b	3.1 ± 0.13b	6.0 ± 0.54a
Simulated duodenum juice			
0 h	8.0 ± 0.23a	7.7 ± 0.65a	6.8 ± 0.38a
5 h	0.1 ± 0.07c	0.3 ± 0.18c	3.1 ± 0.46b

Means and standard deviations at the 95.0% confidence level, $n=5$, values are described as log CFU/ml. *Different letters in the same column indicate statistically significant differences at the 95.0% confidence level.

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Table 2. Average bacteria cell number of *Bifidobacterium bifidum* s1 in fermented milk submitted to simulated gastric or duodenum juices

	Simulated gastric juice		Simulated duodenum juice	
Dose	0 h	3 h	3 h	8 h
A) Fermented milk with different non-fat solid mass content				
8.5%	8.5 ± 0.22a*	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
10.0%	8.6 ± 0.54a	8.5 ± 0.54a	8.4 ± 0.54a	8.2 ± 0.54a
12.5%	8.6 ± 0.92a	8.5 ± 0.92a	8.4 ± 0.92a	8.2 ± 0.92a
15.0%	8.4 ± 0.22a	8.3 ± 0.22a	8.2 ± 0.22a	8.1 ± 0.22a
B) Fermented milk with addition of WPC preparation at different dose				
0%	8.5 ± 0.22a	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
1.0%	8.6 ± 0.22a	8.2 ± 0.80a	8.1 ± 0.80a	7.9 ± 0.80a
2.0%	8.4 ± 0.37a	8.3 ± 0.58a	8.2 ± 0.58a	8.0 ± 0.58a
2.5%	8.5 ± 0.53a	8.4 ± 0.61a	8.3 ± 0.61a	8.2 ± 0.61a
5.0%	8.6 ± 0.22a	8.2 ± 0.59a	8.1 ± 0.59a	8.0 ± 0.59a
C) Fermented milk with different fat content				
0%	8.5 ± 0.22a	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
0.5%	8.5 ± 0.22a	8.1 ± 0.22b	8.0 ± 0.22b	7.6 ± 0.39c
2.0%	8.6 ± 0.22a	8.3 ± 0.17b	8.2 ± 0.17b,c	8.0 ± 0.24c
4.0%	8.6 ± 0.22a	8.4 ± 0.27b	8.3 ± 0.27b,c	8.2 ± 0.25c
8.0%	8.4 ± 0.22a	8.4 ± 0.33b	8.3 ± 0.33b,c	8.0 ± 0.30c
D) Fermented milk with addition of fruit pulp preparation at different dose				
0%	8.5 ± 0.22a	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
5%	8.6 ± 0.22a	8.5 ± 0.22a	8.4 ± 0.22a	7.9 ± 0.22b
10%	8.4 ± 0.22a	8.2 ± 0.22a,b	8.1 ± 0.22a,b	8.0 ± 0.41b
15%	8.3 ± 0.22a	7.9 ± 0.22b	7.8 ± 0.22b,c	7.5 ± 0.22c
20%	8.4 ± 0.22a	7.9 ± 0.22b	7.8 ± 0.22b	7.2 ± 0.22c
E) Fermented milk with addition of inulin preparation at different dose				
0%	8.5 ± 0.22a	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
1.0%	8.6 ± 0.22a	8.2 ± 0.22a,b	8.1 ± 0.22b	7.8 ± 0.56b
2.0%	8.6 ± 0.22a	8.3 ± 0.22a,b	8.2 ± 0.22b	8.0 ± 0.35c
2.5%	8.6 ± 0.22a	8.3 ± 0.22a,b	8.2 ± 0.22b	8.1 ± 0.31b
5.0%	8.5 ± 0.22a	8.4 ± 0.22a	8.3 ± 0.22a,b	8.0 ± 0.38b
F) Fermented milk with addition of Fibregum™ preparation at different dose				
0%	8.5 ± 0.22a	8.0 ± 0.22b	7.9 ± 0.22b	7.5 ± 0.22c
1.0%	8.6 ± 0.22a	8.2 ± 0.22b	8.1 ± 0.22b	7.9 ± 0.22b
2.0%	8.6 ± 0.22a	8.4 ± 0.22a,b	8.3 ± 0.22a,b	8.1 ± 0.58b
2.5%	8.5 ± 0.22a	8.5 ± 0.22a	8.4 ± 0.22a	8.3 ± 0.22a
5.0%	8.6 ± 0.22a	8.5 ± 0.22a	8.4 ± 0.22a	8.1 ± 0.15b

Means and least significant difference at the 95.0% confidence level, $n = 5$, values are described as log CFU/ml. *Different letters in the same row indicate statistically significant differences at the 95.0% confidence level.

containing more than 10% of NFSM. Also, the increase of the WPC preparation to more than 1.0%, 2.0%, or 2.5% in the case of *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12, respectively, caused a similar effect (Tables 2b, 3b, and 4b). The final pH of samples with WPC addition ranged from 5.38 to 5.46 and did not depend on the used strain or WPC content.

Fat is another component of milk, which turned out to be important for bifidobacterial cell survival in GIT. In the case of *B. bifidum* s1, a beneficial effect was found with a fat content in milk of 2.0% or more (Table 2c). In the case of *B. lactis* Bo, the significant differences in survival were observed when the fat content in milk was 4.0 or more (Table 3c). From our experience on *B. animalis* ssp. *lactis* Bb12, fat content at a level of 0.5–4.0% was the most preferred level in fermented milk (Table 4c). The final pH of samples with different fat content ranged from 4.84 to 5.45 and did not depend on

Table 3. Average bacteria cell number of *Bifidobacterium lactis* Bo in fermented milk submitted to simulated gastric or duodenum juices

	Simulated gastric juice		Simulated duodenum juice	
Dose	0 h	3 h	3 h	8 h
A) Fermented milk with different non-fat solid mass content				
8.5%	7.4±0.10a*	7.0±0.11b	6.8±0.12b,c	6.5±0.42c
10.0%	7.3±0.07a	6.9±0.04b	6.9±0.14b	6.7±0.33b
12.5%	7.2±0.09a	7.1±0.07a	7.2±0.09b	7.0±0.04b
15.0%	7.2±0.19a	7.2±0.07a	7.2±0.12a,b	7.1±0.07b
B) Fermented milk with addition of WPC preparation at different dose				
0%	7.4±0.10a	7.0±0.11b	6.8±0.12b,c	6.5±0.42c
1.0%	7.4±0.25a	7.1±0.32a,b	7.0±0.34b	6.6±0.07c
2.0%	7.4±0.33a	7.3±0.26a	7.0±0.46a,b	6.7±0.23b
2.5%	7.3±0.26a	7.3±0.25a	7.1±0.30a	6.7±0.25b
5.0%	7.3±0.25a	7.3±0.26a	7.1±0.50a	6.8±0.45a
C) Fermented milk with different fat content				
0%	7.4±0.10a	7.0±0.10a,b	6.8±0.19b,c	6.6±0.48c
0.5%	7.2±0.21a	7.0±0.23a,b	6.8±0.27b	6.7±0.25b
2.0%	7.2±0.23a	7.0±0.11a,b	6.8±0.12b,c	6.5±0.42c
4.0%	7.3±0.19a	7.0±0.21a,b	6.9±0.22b	6.9±0.24b
8.0%	7.5±0.23a	7.0±0.23b	6.8±0.23b	6.8±0.25b
D) Fermented milk with addition of fruit pulp preparation at different dose				
0%	7.4±0.10a	7.0±0.11b	6.8±0.12b,c	6.5±0.42c
5%	7.5±0.44a	7.3±0.40a	6.9±0.50a	6.9±0.43a
10%	7.4±0.59a	7.2±0.55a	7.2±0.47a	7.1±0.50a
15%	7.4±0.58a	7.3±0.41a	7.2±0.51a	7.1±0.64a
20%	7.2±0.55a	7.1±0.50a	7.2±0.51a	7.1±0.55a
E) Fermented milk with addition of inulin preparation at different dose				
0%	7.4±0.10a	7.0±0.11b	6.8±0.12b,c	6.5±0.42c
1.0%	7.3±0.33a	7.1±0.40a,b	7.0±0.35a,b	6.8±0.28b
2.0%	7.4±0.35a	7.3±0.54a	7.2±0.36a	7.1±0.34a
2.5%	7.4±0.34a	7.4±0.33a	7.2±0.34a	7.1±0.33a
5.0%	7.4±0.33a	7.4±0.32a	7.2±0.33a	7.2±0.33a
F) Fermented milk with addition of Fibregum™ preparation at different dose				
0%	7.4±0.10a	7.0±0.11b	6.8±0.12b,c	6.5±0.42c
1.0%	7.3±0.09a	7.0±0.07b	6.9±0.01b	6.7±0.19c
2.0%	7.4±0.10a	7.0±0.43a,b	7.0±0.03b	6.8±0.31b
2.5%	7.2±0.12a	7.1±0.01a	7.1±0.07a	6.6±0.21b
5.0%	7.3±0.12a	7.3±0.23a	7.2±0.04a	6.8±0.05b

Means and least significant difference at the 95.0% confidence level, $n = 5$, values are described as log CFU/ml. *Different letters in the same row indicate statistically significant differences at the 95.0% confidence level.

the used strain or fat content. An increase in fat content in milk of more than 4% resulted in a reduction of *B. animalis* ssp. *lactis* Bb12 under SDJ conditions, but to a level better than that observed in the totally skimmed fermented milk.

The addition of fruit pulp

From our experience, high doses of the fruit pulp (at 15–20%) may adversely affect the survival of bifidobacteria under simulated GIT conditions (Tables 2d and 4d) perhaps because of the low pH value of the fermented milk with fruit pulp added. The final pH of samples containing plum pulp ranged from 5.28 to 5.77, depending on the added fruit pulp (the more fruit pulp, the lower pH value) and independently from the used strain. However, the addition of an amount of 5–10% by weight of the fruit pulp to fermented milk caused an improvement of the survival rate of *B. bifidum* s1 and *B. animalis* ssp. *lactis* Bb12. The initial population of *B. bifidum* s1 was 8.3–8.6 log CFU/ml and after 8-h incubation in

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Table 4. Average bacteria cell number of *Bifidobacterium animalis* ssp. *lactis* Bb12 in fermented milk submitted to simulated gastric or duodenum juices

	Simulated gastric juice		Simulated duodenum juice	
Dose	0 h	3 h	3 h	8 h
A) Fermented milk with different non-fat solid mass content				
8.5%	8.5±0.16a*	8.1±0.16b	8.0±0.16b	7.6±0.16c
10.0%	8.6±0.16a	8.0±0.16b	7.9±0.16b,c	7.7±0.16c
12.5%	8.6±0.37a	8.4±0.38a	8.3±0.38a	7.9±0.72a
15.0%	8.5±0.30a	8.3±0.41a	8.2±0.41a	8.1±0.25a
B) Fermented milk with addition of WPC preparation at different dose				
0%	8.5±0.16a	8.1±0.16b	8.0±0.16b	7.6±0.16c
1.0%	8.6±0.16a	8.1±0.19b	8.0±0.19b	7.8±0.47b
2.0%	8.6±0.16a	8.3±0.18b	8.2±0.18b	7.8±0.32c
2.5%	8.8±0.16a	8.5±0.25a	8.4±0.25a,b	8.0±0.65b
5.0%	8.7±0.16a	8.4±0.22a	8.3±0.22a	7.9±0.37b
C) Fermented milk with different fat content				
0%	8.5±0.16a	8.1±0.16b	8.0±0.16b	7.6±0.16c
0.5%	8.6±0.39a	8.6±0.27a	8.5±0.27a	8.3±0.32a
2.0%	8.7±0.27a	8.6±0.25a	8.5±0.25a	8.4±0.21a
4.0%	8.5±0.35a	8.4±0.28a	8.3±0.28a	8.3±0.24a
8.0%	8.5±0.16a	8.2±0.16b	8.1±0.16b,c	7.9±0.16c
D) Fermented milk with addition of fruit pulp preparation at different dose				
0%	8.5±0.16a	8.1±0.16b	8.0±0.16b	7.6±0.16c
5%	8.5±0.16a	8.2±0.16a,b	8.1±0.16b	8.1±0.39b
10%	8.3±0.07a	8.2±0.07a	8.1±0.07a,b	7.9±0.40b
15%	8.2±0.16a	8.1±0.16a,b	8.0±0.16a,b	7.8±0.33b
20%	8.1±0.16a	7.9±0.16a	7.8±0.16a,b	7.5±0.34b
E) Fermented milk with addition of inulin preparation at different dose				
0%	8.5±0.16a	8.1±0.16b	8.0±0.16b	7.6±0.16c
1.0%	8.7±0.46a	8.5±0.26a	8.4±0.26a	8.4±0.26a
2.0%	8.6±0.16a	8.7±0.17a	8.6±0.17a	8.6±0.28a
2.5%	8.8±0.46a	8.7±0.18a	8.6±0.18a	8.5±0.25a
5.0%	8.7±0.42a	8.6±0.20a	8.5±0.20a	8.5±0.24a
F) Fermented milk with addition of Fibregum™ preparation at different dose				
0%	8.5±0.16a	8.1±0.16b	8.0±0.16b	7.6±0.16c
1.0%	8.6±0.16a	8.4±0.16a,b	8.3±0.16b	7.8±0.16c
2.0%	8.4±0.07a	8.3±0.07a,b	8.2±0.07b	8.0±0.16c
2.5%	8.3±0.16a	7.9±0.16b	7.8±0.16b	7.4±0.16c
5.0%	8.2±0.16a	7.9±0.16b	7.8±0.16b	7.3±0.16c

Means and least significant difference at the 95.0% confidence level, $n = 5$, values are described as log CFU/ml. *Different letters in the same row indicate statistically significant differences at the 95.0% confidence level.

simulated GIT conditions the population dropped to 7.9–8.0 log CFU/ml in milk samples containing 5% or 10% of fruit pulp and to 7.2–7.5 log CFU/ml in milk samples containing 0% or 15–20% of fruit pulp (Table 2d). For comparison, the starting number of *B. animalis* ssp. *lactis* Bb12 was 8.1–8.5 log CFU/ml and after 8-h incubation in SGJ and SDJ decreased to 7.9–8.1 log CFU/ml in milk samples containing 5 or 10% of fruit pulp and to 7.5–7.8 log CFU/ml in milk samples containing 0% or 15–20% of fruit pulp (Table 4d). In the case of *B. lactis* Bo, a significant difference was observed between milk samples containing fruit pulp as an additive and milk samples without fruit pulp (Table 3d). The initial population of *B. lactis* Bo ranged from 7.2 log CFU/ml to 7.5 log CFU/ml and after 3 h in SGJ and 5 h in SDJ it dropped to 6.5±0.42 log CFU/ml in milk samples without fruit pulp and to 6.9–7.1 log CFU/ml in milk samples containing fruit pulp. The increase in the viability of bifidobacteria

with the medium addition of fruit pulp was interesting. The reason for this may be the composition of the pulp (high sugar content may act protectively). The highest dose used did not cause the improved survival of almost all tested strains of bifidobacteria, which can also be explained by the acidity of the pulp (3.45).

The addition of inulin or Fibregum

It is known that certain prebiotics have a protective effect on bacterial cells, such as inulin. Prebiotics may have a great influence on the survival of probiotic bacteria in large intestine of the human GIT tract, especially bifidobacteria. Our experiments have shown that the inulin almost used in each tested dose stimulated the cells of bifidobacteria to develop better tolerance to environmental conditions (Tables 2e, 3e, and 4e). At the beginning of incubation of milk samples with *B. bifidum* s1, the number of bacterial cells was 8.5–8.6 log CFU/ml. After 3-h incubation in SGJ and 5-h incubation in SDJ, the population of bifidobacteria decreased to 7.5 ± 0.22 log CFU/ml in milk samples without inulin and to 7.8–8.1 log CFU/ml in milk samples with inulin (Table 2e). Also, the increase of inulin to 1% or more in the case of *B. lactis* Bo caused a similar effect (Table 3e). For comparison, under the same conditions and time of incubation, the population of *B. animalis* ssp. *lactis* Bb12 decreased from initial 8.5–8.8 log CFU/ml to $7.6 \log \pm 0.16$ CFU/ml in milk samples without inulin and to 8.4–8.6 log CFU/ml in other milk samples (Table 4e). The final pH of samples with inulin ranged from 5.09 to 5.19 and did not depend on the used strain or inulin level. An additional prebiotic preparation used in our experiments was the Fibregum. We found that the addition of this preparation to fermented milk stimulated *B. bifidum* s1 and *B. lactis* Bo cells to better survival under simulated GIT conditions (Tables 2f and 3f). However, an excessive dose of this preparation (2.5–5.0%) acted negatively, leading to a worse survival for *B. animalis* ssp. *lactis* Bb12 than that in the pure milk (Table 4f). The initial *B. animalis* ssp. *lactis* Bb12 cell number ranged from 8.2 log CFU/ml to 8.6 log CFU/ml and after 8-h incubation in simulated GIT conditions decreased to 7.6–8.0 log CFU/ml in milk samples containing 2.0% or less of Fibregum. In the case of higher dose of Fibregum, the final population of *B. animalis* ssp. *lactis* Bb12 was at 7.3–7.4 log CFU/ml. Interestingly, the final pH of samples with Fibregum did not depend on additive level or the used strain and was 5.33 ± 0.11.

Comparison of the model samples based on the survival of bifidobacteria in fermented milk samples during subsequent incubation under conditions of *in vitro* model digestive juices using cluster analysis helped establish close similarities between the survival of bifidobacteria depending on the incubation time (Fig. 1). A comparison

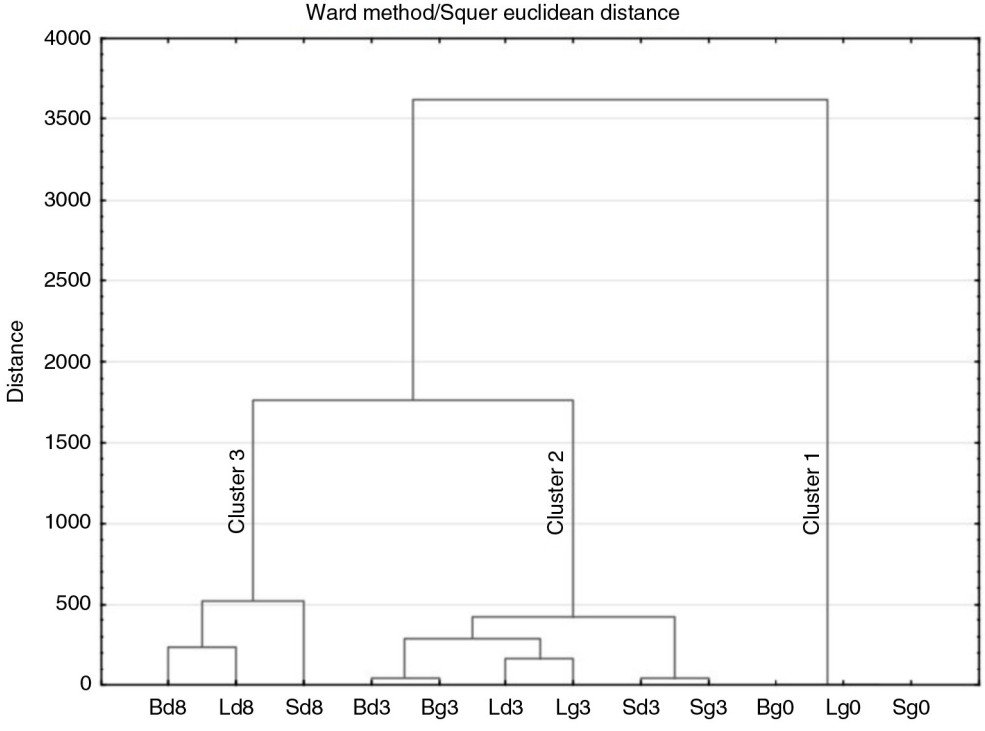

Fig. 1. Dendrogram representing the similarity relationships among the bifidobacteria survival rate in fermented milk sample during subsequent incubation under conditions of *in vitro* model digestive juices. B, L, S – the samples fermented by *B. bifidum* s1, *B. lactis* Bo, and *B. animalis* ssp. *lactis* Bb12, respectively; d, g – simulated duodenum and gastric juices, respectively; 8, 3, 0 – incubation time (hours) in model digestive juices.

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of the survival of bifidobacteria at the different stages of the digestive system allowed showing a significant effect of the model gastric juice on the number of viable cells of bifidobacteria, what has been marked as a separate cluster (cluster 1 in Fig. 1) of the cases of zero time (Bg0, Lg0, and Sg0). The analysis showed similar survival rate after 3 h incubation in gastric juice and after the transfer of the fermented milk samples to intestinal juice (Bg3, Lg3, Sg3, and Bd3, Ld3, Sd3, respectively). Cluster 2 (in Fig. 1) was isolated as a result of this analysis. A separate cluster (cluster 3 in Fig. 1) was created by the sample after 8 h incubation in intestinal juice (Bd8, Ld8, and Sd8). It confirms that the incubation in *in vitro* model digestive juices had the greatest influence on the bifidobacterial population. The length of lines connecting successive clusters (expressed in Fig. 1 as the distance on the Y-axis) shows that the classification of species was important, but not as important as incubation in *in vitro* model digestive juices.

Discussion

Our studies have shown the influence of selected additives to fermented milk on survival of bifidobacteria in the static conditions of the GIT tract model. We proved that food components and prebiotics may be important protective factors for bacterial cells, since in each case the introduction of bifidobacteria with fermented milk resulted in better survival rates for bacterial cells under simulated GIT conditions as compared to a suspension of bacterial cells mixed with the culture broth. Moreover, in most cases the dosage of the additive to milk prior to fermentation significantly determined the final number of bacterial cells surviving incubation under simulated GIT conditions. We demonstrated that the sequential passaging of bacterial cells suspended in the fermented milk in the simulated GIT juices meant that the bacterial cells are less resistant to the second step (i.e. SDJ) than to the first step (i.e. SGJ). It is known that the same probiotic strain exposed to different components or storage conditions may present different responses regarding their susceptibility to the adverse conditions or viability in the GIT tract (10). A great advantage of our study is that the impact of selected additives and their doses has been demonstrated on one biological material prepared in the same laboratory conditions. Thanks to this; we observed a great diversity of results obtained as a consequence of not only the dose of additives used but also bacterial strains.

The results of our first experiment confirm the literature data that different strains of bifidobacteria behave very differently when exposed to an *in vitro* simulated intestinal environment. Some bifidobacteria strains survive very well, while others do not (11). As expected, strains of *Bifidobacterium* tested by us behave differently in model simulated GIT conditions. They belong to the three species of *Bifidobacterium*, and it is known that species isolated from animals (*B. animalis*, *B. lactis*) are more acid resistant in comparison to human GIT tracts (*B. bifidum*). Strains isolated from human or animal intestinal tract (*B. animalis*, *B. bifidum*) should exhibit better adaptation to the conditions of the GIT tract compared to strains isolated from milk and fermented milks (*B. lactis*). Although Lankaputhra and Shah (12) and Martin (13) indicated that many strains of bifidobacteria are able to survive at a high population levels under conditions simulating the gastric pH, it should be noted that the models simulating GIT digestive juices used in the cited studies differ from one another. Many authors use solutions of sodium chloride at reduced pH as gastric juice models, while other researchers study systems at reduced pH, while other researchers study systems enriched in digestive enzymes (14–17). A similar situation can be observed in the case of the intestinal juice model, where the simulation often consists of solutions of sodium chloride and bile salt, with the optional addition of enzymes (18, 19). So far, many *in vitro* and *in vivo* studies have been conducted on the influence of various factors on the survival of probiotics in GIT. These factors include the degree of acidity in the stomach, the duration of exposure to acids in the gastric juice, the concentration and duration of exposure to bile salts, the level of bile salt hydrolase (BSH) activity, the enzymes present in GIT, intestinal motility, as well as the bacterial phase growth and how to deliver the bacteria to the digestive tract (9, 10, 20–24). It has been proved that the survival of the bacteria also depends on the initial number of bacterial cells (24, 25). Generally, the greater the number of introduced microorganisms, the more bacterial cells can survive in the intestinal juice. Other studies have demonstrated that one of the factors affecting the survival of bacteria in GIT is the choice of an appropriate strain (15, 16, 26–29). For example, Vernazza et al. (30) showed that *B. animalis* ssp. *lactis* Bb-12 was the most resistant to low pH values and bile salts of the five test strains of *Bifidobacterium*.

The addition of milk components

In our experiments the addition of selected milk components (NFSM or WPC-68) or prebiotics (inulin or Fibregum) to processed milk had a significant effect on the pH of test samples. It is clear that the buffering capacity of fermented milk samples is an important physico-chemical characteristic that corresponds to the survival of bifidobacteria. Fermented milk samples contain significant levels of organic acids, proteins, and phosphates, but the addition of selected components to processed milk increases buffering capacity. As has been demonstrated by many researchers (14, 25, 31–33), food components are an important protective factor for bacterial cells, as components of the food buffer environment

in which bacteria are suspended. This has been proved in our studies. Modifications applied to the milk used in our experiments for fermentation show, however, that it is possible to improve bifidobacterial cell survival in sequential passaging by simulated digestive juices. The addition of skimmed milk powder to yoghurt is a commonly encountered treatment intended to increase the dry matter content in the product, resulting in, among others, an improvement in the rheological characteristics of the product. Similarly, products obtained from whey, for example WPC, can be used. Al-Saleh et al. (34) showed that the addition of 1% of skimmed milk powder to MRS broth improved the resistance of all the tested strains, in particular bifidobacteria, at low pH values. As we know, tolerance of the acidic conditions in the stomach and bile salts and enzymes in the duodenum differ between strains (35, 36). The tolerance of bacterial cells to acid is attributed to their ability to maintain a constant pH gradient between the environment and the pH of the cytoplasm (20, 37). This may explain the variability of the results published in the literature, depending on the dose of the additive and the test strain. In the case of intestinal juices, bile salts have a huge impact on the survival of bacteria. Bile affects phospholipids and bacterial cell membrane proteins and disrupts the stability of macromolecules and cellular homeostasis (38). In the case of *Bifidobacterium*, an increased resistance to bile salts is associated with the presence of the *K* gene in the DNA which encodes the protection function against many adverse conditions, including heat shock and osmotic stress. It has been found that the expression of this gene in bifidobacteria is increased after exposure to bile salts (39).

The enzymes are present in both GIT juices, they affect the survival of bacteria in the digestive tract, and they contribute to the enzymatic digestion of nutrients which protect bacterial cells (40, 41). This is confirmed by our experience. The use of additives such as milk components (proteins or fat) to the processed milk reduces the effect of gastric enzymes (pepsin, for example) or bile salts and intestinal enzymes (lipases, amylases, proteases) on the bacterial cells. There has been published work on the influence of milk protein on the survival of probiotics in the human digestive tract (42). In contrast, Charteris et al. (14) observed that milk protein improves the tolerance of most test strains under conditions of simulated gastric juice and simulated intestinal juice. Moreover, the products of enzymatic digestion of milk components may enhance the bifidobacteria growth, what is documented in the literature (31, 43, 44). These data indicate that some strains of *Bifidobacterium* may survive passage through the human stomach, particularly when they are consumed in the form of fermented milks and food products produced with milk proteins. The addition of milk protein may stimulate bifidobacteria survival in another way; for example, it has been proved that sialic acid, a component of casein macropeptide, stimulates the growth of *Bifidobacterium*. The addition of 2% of casein macropeptide to milk also caused a marked increase in the population of *B. animalis* ssp. *lactis*. Therefore, casein macropeptide is considered a potential prebiotic substance (44).

The results of our experiments on the effect of fat content in fermented milk on the survival of bifidobacteria and lactic acid bacteria in GIT are not supported by the data of the literature, because there are no publications in this field. It should be noted that under *in vivo* conditions the influence of fat content in fermented milk containing *Bifidobacterium* may be different from those observed under *in vitro* conditions. It is known that lipids present in food increase the level of bile salts secreted in GIT, and thus they can increase the inhibitory effect of bile on the bifidobacterial cells (45, 46). It is believed that the survival of bifidobacteria and some lactic acid bacteria in GIT is correlated with their ability to produce the enzyme, bile salt hydrolase, BSH (35, 47, 48). BSH activity is a component regulating the composition and quantity of gut microflora in the human host. The release of deconjugated forms of bile salts protects bacterial cells against the growth of a competitive microflora (48). The literature indicates that the activity of BSH, observed in bifidobacteria and selected strains of *Lactobacillus*, is not observed in typical dairy starter cultures (47). Clark and Martin (49) demonstrated very good survival of *B. longum* cells in the presence of 2% and 4% bile salt concentration. Perhaps this is related to the fact that *Bifidobacterium* has the ability to produce the enzyme BSH, while some researchers argue that there is no correlation between the ability of bacteria to produce BSH and their resistance to the toxic effect of bile salts (47, 48).

The addition of fruit pulp

From our experience, 15–20% doses of the fruit pulp may adversely affect the survival of bifidobacteria under simulated GIT conditions. The explanation for this phenomenon may be the acidity of the fermented milk, as well as the acidity of the added fruit pulp. As is known, fruit pulp may have a pH substantially lower than that of fermented milk, and the addition of fruit pulp to milk may lead to an excessive increase in the acidity of the mixture. According to Martin and Chou (50), to reduce the pH to 3.6 may be a reason for inhibiting the growth of *Bifidobacterium*, a minimum pH allowing the survival of these microorganisms is in the range of 5.5–5.6. There are no studies on the effects of fruit pulp added to the fermented milk on the survival of bifidobacteria in GIT. There are some publications concerning the impact of components of fruit preparations, such as pectin and sugars (36), on the survival of lactic acid bacteria.

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The addition of inulin or Fibregum

We demonstrated that inulin used in a tested dose stimulated the cells of bifidobacteria to develop better tolerance to environmental conditions; this is confirmed by other authors. According to Gibson and Roberfroid (51), prebiotics are non-digestible food ingredients, preferably affecting the host organism and having the ability to selectively stimulate the growth and activity of selected bacterial species in the colon. Alkalin et al. (52) observed better cell survival of *Bifidobacterium* upon the addition of fructooligosaccharides. The prebiotic effect of inulin and oligofructose under *in vivo* conditions was confirmed by Buddington et al. (53) and Kleesen et al. (54). An additional preparation used in our experiments was the Fibregum. We found that the addition of Fibregum preparation to fermented milk stimulated bifidobacterial cells to better survival under simulated GIT conditions. Discussion of the results is difficult due to the lack of publications on the study of the survival of bifidobacteria and lactic acid bacteria in simulated digestive juices in the presence of a preparation of Fibregum. It is known that Fibregum, also known as 'gum arabic' or 'gum Acacia', is very slowly fermented by intestinal microflora and is not hydrolysed by digestive enzymes. As a prebiotic, it selectively stimulates the growth of beneficial bacterial flora in the human gut (known as a bifidogenic factor). Acacia gum is used for the encapsulation of probiotic cultures in order to protect them during spray-drying, storage, and passage through the human GIT (55). Additionally, it has also been proved that gum arabic in humans made it possible to increase the *Bifidobacterium* population in stools, and Fibregum is known as a very well-tolerated dietary fibre (56).

The cluster analysis demonstrated that, irrespective of the samples composition, incubation in *in vitro* model digestive juices is a more important factor than the choice of bifidobacteria strain.

Conclusions

Based on the results of our study, it can be concluded that the components of the products have a protective effect on bifidobacteria and contribute to increasing the degree of survival under simulated GIT conditions, when cells are sequentially transferred through the section of the GIT tract. Our research has shown that the most serious obstacle to bifidobacterial cells in the GIT tract is the intestinal conditions (duodenum juice), especially when the cells have been the moment before in contact with gastric juice. Thus, bifidobacterial cells introduced into the human GIT with food ingredients have a good chance of survival during intestinal transit and to reach the large intestine, thanks to the protective properties of the food components and depending on the strain and composition of the food. The species of bifidobacteria most widely used in the food industry are *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. longum*, *B. animalis*, and *B. infantis*. We investigated the effects of selected food components on the viability of *B. bifidum* s1 compared to two common strains of bifidobacteria, *B. lactis Bo* and *B. animalis* ssp. *lactis* Bb12, during subsequent incubation under conditions of *in vitro* model digestive juices. Our results showed that incubation in successive digestive juices is a more important factor for bacteria cell survival than the kind of bifidobacteria strain. A great advantage of our study is that the impact of selected food components or other additives and their doses has been demonstrated on one biological material prepared in one laboratory conditions and at the same time. It is worth noting that the survival of the cells of three *Bifidobacterium* strains studied depended on the strain used and the specific response to a given preparation and its dose.

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