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INVESTIGATION OF SURVIVAL AND VIABILITY OF PROBIOTIC STRAINS DURING STORAGE

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Abstract: Correct taxonomy identification, viability and transit tolerance of the probiotic strains are crucial for achievement of the expected effects of probiotic supplements. Probiotics strains may be incorporated in dietary supplements and other food matrices which are expected to have up to 24 months of stability. However, the viability of probiotic cultures depends on production technology, type of the product and storage conditions during the entire shelf-life. Survival of probiotic bacteria are also affected by the high concentration of digestive enzymes and the low value of pH, during their passing through the gastrointestinal system. To increase the transit tolerance of probiotic strains, capsule dosage form is often used. In the present study the survival of probiotic strains was monitored at different periods of the product storage. Also, the effect of pepsin and pancreatin on probiotic strains in various commercial formulations was tested. The impact of the capsule on the viability of probiotic strains in terms of storage has been studied. *In vivo* transit tolerance of the probiotic strains under simulated conditions was visualized by the fluorescence microscopy method. Our results showed that the strains tested were more sensitive to the simulated gastric juice conditions, and many of them did not survive after 90 minutes and were more resistant to small intestine conditions. Viability of the probiotic strains at pH 3 is higher than at pH 2. At pH 3 all strains in products showed similar stability, and their number only slightly decreased. It was observed that after 90 days of storage, the number of bacteria significantly decreased but was still in the order of 106 CFU/ml.

INTRODUCTION

An increasing number of commercial probiotic products are available on the Bulgarian market. This makes the decision about the choice of the product

difficult both for the consumer and the specialist (Fredua-Agyeman and Gaisford, 2019) It is generally recommended for a given probiotic microorganism to satisfy a number of requirements in order to achieve "probiotic" status (Holzapfel and Schillinger, 2002, Balamurugan et al., 2014). There is no global agreement on the minimum number of bacteria per gram or ml of product necessary for functionality. It is generally accepted that at the point of consumption, probiotic products should have a minimum concentration of >1×106 cfu/ml or gram and probiotic microorganisms should be present in sufficient number by the end of the shelf life (Galat et al., 2016). However, commercial probiotic products do not always contain the number of bacterial cells indicated on the label (Astashkina *et al.,* 2014). In this regard, the quantitative content of probiotic bacteria was examined in the present study.

The presence of viable probiotic bacteria in dietary supplements does not guarantee their efficiency. Probiotic bacteria selected for commercial use in foods and dietary supplements must maintain their viability during processing and storage and, after consumption, survive transit through the gastrointestinal (GI) tract and are fully able to grow and colonize in the colon on arrival (Toumola *et al.,*2001, Ventura and Perozzi, 2011). Approximately 2.5 liters of gastric juice and 1 liter of bile juice are secreted into the human digestive tract every day (Begley *et al.,*2005). This determines the tolerance of LAB to gastric juice and small intestine conditions as one of the most prominent selection characteristics of probiotic strains (Talwalkar and Kailaspathy, 2004, Ventura and Perozzi, 2011, Jensen *et al.,*2012, Ashraf and Smith, 2016).

There are two main challenges in formulating probiotics — the stability over shelf life and efficient delivery of the probiotic to the appropriate site in the gastrointestinal tract (Feldman *et al.,* 2017). The life stability of probiotic products is influenced by storage humidity, dosage form, moisture content, temperature, pressure and temperature during the tableting process, solvent use and drying conditions during coating process, dissolved oxygen content due to process conditions, pH of the final product and individual strain characteristics (Shah and Lankaputhra, 1997; Vinderola *et al.,*2002, Feldman *et al.,*2017). There are also evidences in the literature for the importance of an adequate selection of a proper food matrix for probiotic strains, considering functional variabilities and levels of viable cells (Vinderola et al., 2011a, Galinoiu et al., 2016). Vinderola et al., (2011b) showed that different L. casei strains in fermented milks, which maintained adequate levels of viable cells during refrigerated storage, experienced changes in gastric resistance depending on the temperature of storage. In addition, variations in manufacturing process, quality between batches, and packaging material may have an impact on the final products (Szajewska *et al.,*2016). To improve the viability of probiotics in different food products during their production and storage until the time of consumption, many manufacturers use different methods. The probiotics in dietary supplements are primarily utilize in

the freeze-dried powder form. Capsules, tablets, and powder in stick packaging or sachets are the most commonly found formats and are usually stored at ambient conditions. The capsules are the most applicable approach for probiotic delivery (Fenster *et al.,*2019, Feldman *et al.,* 2017). They create a physical barrier to environmental conditions. In the capsules, the bacteria are protected during transit through the gastrointestinal tract and are released into the desired target intestine. Specific capsules were developed to ensure passage of probiotic through stomach. There are examples of using different type of capsule outer shell as gelatin and hypromellose (HPMC). For more than 100 years, capsules were only made of gelatin. Gelatin capsules typically contain 13 to 16 percent moisture for shell pliability of the capsule. This moisture can transfer inside to the product and may cause premature activation of the probiotic cells. This can lead to significant decrease of the viable probiotic cells (Feldman e*et al.,*2017). Standard single capsule had stability of 9 months. Consumer demand for alternatives to animal products and the need for more technically advanced products led to the development HPMC capsules. They have a lower water content - between 3 to 9% which maintains stability of the capsule. The product remained stable throughout a 24-month period. Hence, the aim of this study was to investigate the survival of probiotic species in various commercial formulations, available on Bulgarian market and to explore the impact of the capsules on the viability of probiotic strains during the long term storage and protection from stomach acidity.

MATERIALS AND METHODS

Probiotic products

A total of sixteen commercially available probiotic formulations were selected in the present study, as thirteen probiotic products were purchased from local pharmacies and three powdered substances were obtained from a local manufacturer (**Table 1**). Seven of the products (PI, PII, PV, PVII, PVIII, PIX and PX) were capsule dosage form, five were in a powder-form packed in sachets (PIII, PIV, PXI, PXII and PXIII) and one was liquid (PVI) form. The products PI, PII, PIII, PIV, PV and PVI were imported, the others PVII, PVIII, PIX, PX, PXI, PXII, PXIII, PXIV, PXV, and PXVI were produced by local manufacturers. All products were stored according to manufacturer's requirement and analyzed at least 6 months in duplicates before their expiration date.

Isolation and enumeration of viable strains in selected probiotic products

Isolation and enumeration of probiotic strains was performed according to the standardized procedure described in ISO 15214 (Horizontal method for the enumeration of mesophilic lactic acid bacteria). The content of a capsule or sachet of the freeze-dried products was rehydrated in 10 mL peptone water (pH 7.4) for 2 h at 37°C. The suspensions were diluted tenfold and aliquots of 100μL were spread-plated on MRS agar (De Man Rogosa Sharpe agar), M17 agar and NA

(Nutrient agar), in triplicates of each dilution. Liquid product was serially diluted and spread-plated on indicated culture media (Merck Millipore, Germany). The number of viable cells were counted after 48 hours of incubation at 37°C in anaerobic conditions (Anaerocult A, Merck Millipore, Germany).

Depending on the number of morphological types of colonies on each petri dish, 2-3 colonies of each type were randomly selected and transferred to a new plate. Pure cultures were subcultured in MRS broth (HiMedia Laboratories, India) and were phenotypically identified. The species designation of the isolates was investigated by Gram-stain, colonial appearance, cell morphology, spore forming, catalase activity, oxidase activity and substrate fermentation (API 50 CHL System; BioMerieux SA, France).

Transit tolerance

The gastrointestinal tolerance of isolated *Lactobacillus* was investigated according to the method described by Charteris *et al.,*(1998). Selected probiotic strains were subjected to gastric and intestinal stress and were tested for survival in simulated gastric juice at pH 2 (3 g/l pepsin, Merck Millipore, dissolved in 0.5% NaCl, pH was adjusted with 37% HCl) and pancreatin solution at pH 8.0 (1g/l pancreatin, Merck Millipore, dissolved in 0.5% NaCl pH was adjusted with 5M NaOH). The solutions were prepared fresh daily and sterilized using bacteriological filter - 0.22 μm pore size (Merck Millipore, Germany).

Preparation of washed cell suspension

The LAB strains were cultivated in 10 ml MRS broth at 37ºC for 24 hours. Cultures were centrifuged at 5000 g for 5 min at 4ºC. Cells were harvested and washed three times in phosphate-buffered saline (PBS, pH 7.4) and finally resuspended in PBS. The total viable count of the washed bacterial suspension was adjusted to 0.5 McFarland ($1.5x10^8$ cfu/ml) and was determined prior to assay of transit tolerance.

***In vitro* resistance of LAB in simulated gastrointestinal conditions**

The washed cell suspensions (1.0 ml) were added to prepared 9.0 ml solutions of pepsin (pH 2.0) and/or pancreatin (pH 8.0). The materials were vortexed for 5-10 s for complete dispersion of the cells and incubated at 37°C. Aliquots of 0.1 ml were taken at 1, 90, 180 min for pepsin assay. Aliquots were removed at 1 and 240 min for pancreatin assay. Total viable counts of survival LAB were determined on MRS agar using a pour-plate method after serial 10-fold dilution. Plates were incubated at 37ºC for 48 hours. The strains in PBS without stress were used as control. The viability was calculated from colony-forming units (CFU) of appropriate dilutions from the control and stress-treated bacterial cells.

Visualization of gastrointestinal transit tolerance by fluorescent microscopy

The impact of the gelatin capsule on the viability of probiotic strains under gastrointestinal stress were investigated by fluorescent microscopic method. The survival of probiotic strains at the end of pepsin stress (at pH 2 and pH 3) was

monitored using the LIVE/DEAD bacterial viability method. The live and dead bacteria were visualized as green/or blue and red fluorescent cells, respectively under a fluorescence microscope (Leica M5500, at magnifications 40 and 62 objectives) and photomicrographs were captured using a digital camera (Olympus DP70, Tokyo, Japan).

Two probiotic products, one formatted as capsule dosage (PVIII) form and one as powder form (PXVI) were subjected to gastric stress according to the method described by Charteris et al. (1998). Each sample contained 9 ml pepsin solution at pH 2.0/ or pH 3.0, two intact capsules of probiotic product or equal content of powder probiotic product. The mixtures were vortexed for 5-10 s and incubated at 37ºC. Aliquots for analysis were taken at 1, 90, 180 min. The samples were stained with DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) (Sigma, Germany) as follows: 10 µl of each sample was mixed with 0.2 µl DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride, 10 mg/ml, Sigma, Germany) and 0.2 µl propidium iodide solution (10 mg/ml, Sigma, Germany,).

Viability during long-term storage

The viability of probiotic products PVII, PVIII, PIX, PXIII, PXIV during long-term storage was determined by the pour-plate method using MRS and M17 agar media. Each dosage form was rehydrated in peptone water for 2 h at 37°C. The viability of each product suspension was examined by the procedure described for enumeration of the probiotic strains in the current study. The analysis was carried out immediately after production processing, after one and three months of storage in conditions according to the manufacturer's recommendations. In each experiment we used a new pack from the same batch.

RESULTS AND DISCUSSION

Enumeration and isolation of probiotic LAB

The total number of probiotic lactic acid bacteria (LAB) was enumerated on MRS and M17 media. The viable probiotic bacteria in the products must be at least 106-107 CFU/g (Galat *et al.,* 2016). The present investigation determined that the content of viable bacteria from genus *Lactobacillus* in none of the products (from PI to PVI), bought from the market, fully meet their quantity, indicated on the label. In PII and PIV, the number was below the declared content. Two products PI and PIII from different companies showed no growth on MRS and M17 media (**Table 1**). The number of bacteria in all products (PVII - PXVI) provided from a local manufacturer was in accordance with label information or even higher. This may be due to the fact that these products were freshly produced (Zawistowska-Rojek *et al,* 2016).

Of about 885 colonies that were formed on the two media, 322 small, round and opaque, and white colonies showed characteristics of LAB: Gram-positive, non-spore forming rods or cocci, catalase and oxidase negative, and aero tolerant.

Fifty-five presumptive LAB strains were purely isolated and were phenotypically characterized. Ninety percent of the isolates were rod-shaped and 10% were spherical in short and medium long chains.

The microbiological quality of the tested products was determined on nutrient agar for evaluation of the unacceptable microbial contamination. The colonies, isolated on nutrient agar did not have the characteristics of lactic acid bacteria or yeast. The analysis of the microbiological quality showed that more than 37% of the tested probiotic supplements contained unacceptable microbial contamination. Undesirable microbial growth was detected from products PIII, PXIV, PXV and PXVI (Table 1). This could be due to inadequate quality control in manufacturing or contamination during transport or storage of the products.

The tested probiotic products should contain different species as *Lactobacillus bulgaricus, L. acidophilus, Streptococcus thermophilus, L. lactis, L. casei, L. plantarum, L. helveticus, L. reuteri, L. rhamnosus, Bifidobacterium breve, B. longum, B. bifidum* (**Table 1**). In this study, we did not isolate all morphological types of colonies of the lactic acid bacteria corresponding to the mentioned product specification. Only in products PIV, PV and PVI one morphological type of colonies was recognized, corresponded to the specific morphology of the expected species *L. bulgaricus, L. acidophilus,* and *L. rhamnosus* respectively. In product PIX only 3 types of colonies were found out, but according to the information given on the label, the product should be containing five *Lactobacillus* species (*L. plantarum, L. rhamnosus, L. bulgaricus, L. lactis, L. casei, L. helveticus*). The situation was similar with product PII. One morphological type of colonies on MRS agar was isolated, instead of two *Lactobacillus* species corresponding to the product specification (*L. rhamnosus* and *L. acidophilus*). These results showed that the differentiation and subsequent enumeration of strains in mixed culture products, using only MRS medium (according ISO 15214) does not allow a correct assessment. Some of the reasons may be due to the similarity in growth requirements of the closely related LAB. That's way a wide range of culture media have been developed for selective and differential enumeration of probiotic bacteria mainly in mixed populations (Sule, 2014, Tabasco, 2007). Nevertheless, food manufacturers still tend to rely on conventional plating techniques on MRS agar for *Lactobacillus* species for enumeration purposes (Elahi, 2008, Miranda *et al.,* 2011, Davis, 2014).

Some probiotic products showed a greater morphological diversity than expected. Products XIII and XIV should contain four LAB species (*L. rhamnosus, L. acidophilus, L. lactis,* and *S. thermophilus*), but eight morphological types of colonies were isolated. This variety could be due to the use of incorrectly taxonomically defined species (in product XIII) or to the massive contamination (in product XIV). Products (PX, PXI, PXII, PXIII, PXV and PXVI) possessed smaller deviations concerning the correlation between the variety of species included and the number of colony types.

Table 1. Enumeration of probiotic bacteria in selected probiotic products.

Product dosage form	Strains identified	Information given on label		Enumeration of bacteria (CFU/g)		
		Probiotic microorganisms	Total count (CFU/g)	MRS agar	M 17 agar	NA agar
PI capsule		*L. acidophilus, L. rhamnosus*	NG*			
		L. bulgaricus, S. thermophilus		NG	NG	NG
PII capsule		*L. acidophilus* Rosell-11	$2{,}4x12^{12}$	$1x10^{5}$	NG	NG
		L. rhamnosus Rosell-52				
		B. longum Rosell-175				
		S. boulardii	$1{,}5\ x10^{10}$	ND**	ND	
PIII sachet		*L. acidophilus, L. casei, L. lactis*	$1x10^{9}$	NG	NG	$4x10^{5}$
		Bifidobacterium sp.	$5x10^{9}$	ND	ND	
PIV sachet	*L. bulgaricus* 4B	*L. bulgaricus* GLB44	$2x10^{9}$	$4x10^{5}$	NG	NG
PV capsule	*L. acidophilus* M8	*L. acidophilus*	$9{,}5x10^{9}$	$6{,}5x10^{6}$	NG	NG
PVI drops	*L. rhamnosus* M6	*L. rhamnosus* GG	$1{,}4x10^{9}$	$3x10^{5}$	NG	NG
PVII capsule	*L. acidophilus* N1	*L. acidophilus, L. casei*	$1x10^{9}$	$4{,}9x10^{9}$	$3{,}5x10^{5}$	NG
		Bifidobacterium sp.				
		S. boulardii	$5x10^{9}$	ND	ND	
PVIII capsule	*L. acidophilus* N2	*L. acidophilus, L. casei*	$1{,}7x10^{10}$	$1{,}8x10^{8}$	$1{,}5x10^{9}$	NG
		Bifidobacterium sp.				
		S. boulardii	$2{,}6x10^{9}$	ND	ND	
PIX capsule	*L. plantarum* 84	*L. plantarum, L. rhamnosus*	$1x10^{10}$	$1x10^{10}$	$1{,}9x10^{9}$	
		L. bulgaricus, L. lactis, L. helveticus,				
		Bifidobacterium				NG
PX capsule		*L. rhamnosus L.acidophilus, L. plantarum, S. thermophilus,*	$1{,}94\ x10^{9}$	$1{,}6x10^{9}$	$1{,}1x10^{9}$	
		Bifidobacterium				NG
		S. boulardii	ND	ND	ND	
PXI sachet	*L. bulgaricus* 52	*L. helveticus, L. acidophilus*	$1x10^{9}$	$4{,}3x10^{9}$	$1{,}1x10^{9}$	$1{,}3x10^{9}$
		L. bulgaricus, S. thermophilus,				
		Bifidobacterium sp.				
PXII sachet		*L. acidophilus, L. casei*	$\geq 1\ x\ 10^{9}$	$2{,}6\ x10^{9}$	$4{,}9x10^{9}$	$1{,}2x10^{10}$
		Bifidobacterium sp.				
		S. boulardii	$\geq 5x10^{9}$	ND	ND	
PXIII sachet	*L. rhamnosus* 57	*L. rhamnosus, L. lactis*	$\geq 2{,}5x10^{9}$	$7x10^{9}$	$2x10^{10}$	NG
		L. acidophilus				
		Str. thermophilus				
PXIV raw mat	*L. acidophilus* 64	*L. rhamnosus, L. lactis*	$\geq 6{,}2x10^{9}$	$4{,}7x10^{10}$	$2{,}6x10^{9}$	$8{,}5x10^{8}$
		L. acidophilus				
		Str. thermophilus				
PXV raw mat	*L. bulgaricus* 70	*L. bulgaricus L. acidophilus*	$2{,}5x10^{10}$	$4{,}8x10^{10}$	$6{,}7x10^{9}$	$7{,}9x10^{9}$
		Str. thermophilus				
		Bifidobacterium sp.				
PXVI raw mat	.	*L. acidophilus, L. casei*	$1{,}5x10^{10}$	$9{,}2\ x10^{10}$	$1{,}1x10^{10}$	$2{,}4x10^{10}$
		Bifidobacterium sp.				
		S. boulardii	$\geq 5x10^{9}$	ND	ND	

*** NG – no growth, ** ND – not determined**

According to the biochemical profiles of isolates only 10 strains were identical to the expected species: *L. bulgaricus* (3 strains), *L. acidophilus* (4 strains), *L. rhamnosus* (2 strains) and *L. plantarum* (1 strain). According to the specifications, most probiotic products were supposed to contain the species *Lactobacillus delbrueckii* ssp. *bulgaricus*. Our studies confirmed its presence in only three probiotic products. Similar results were obtained for *L. acidophilus.*

The results of our study revealed that almost none of the tested probiotic products was satisfactory either qualitatively or quantitatively. Similarly, other authors, investigating the quality of probiotic supplements have reported that none of the products tested in their studies contained all of the types described on the label and most of the products contained species, other than those listed (Kolacek et al., 2017, Wannaprasat *et al.,* 2009). Recent studies also have shown that in many products strains were not identified or many were misidentified (Farahmand, 2015). Another problem is that commercial products did not contain the stated cell numbers (Lin *et al.,* 2006), but had significantly lower levels than reported (Coeuret et al. 2004, Al-Otaibi, 2009). Probiotic preparations must meet strict criteria related to quality, safety and functionality (Vankerckhoven *et al.,* 2008). Hence, the lower content of viable probiotic bacteria in some of the tested product has a negative impact on their potential health benefits, because the numbers were below the minimal beneficial effective dose for probiotic bacteria mentioned above (FDA, 2006).

***In vitro* transit tolerance of probiotics**

The effects of stimulated gastrointestinal conditions at different pH level on viability of the ten probiotic strains were examined. The isolated strains 4B, 52, N1, N2, M6, M8, 70, 64, 84, 57 were chosen because their identification at genus and species level was confirmed by biochemical method as *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus, L. rhamnosus* and *L. plantarum. In vitro,* gastric juice was simulated by incubating the strains in pepsin-containing medium, at pH 2.0. The viable cell population was determined at 1, 90 and 180 min of incubation on MRS agar plates by the plate count method. The data obtained were shown on (**Table 2.**). The tolerance level to the simulated acidic conditions of the species tested was variable. The number of the strains was lowered with 1 to 2 log orders immediately after the cells were introduced into the pepsin solution. Further incubation at pH 2 caused cell death in the most of the strains examined. Only the strains *L. delbrueckii* subsp. *bulgaricus* 52, *L. acidophilus* N2, *L. acidophilus* 64 and *L. plantarum* 84 were able to survive for 90 min in pepsin solution (pH 2). They showed a slight resistance at 90 minutes and counts decreased 5-6 log orders than the control. It was recorded, that none of the strains has survived at 180 minute.

Table 2. Transit tolerance of probiotic strains to the gastric juice pH 2.0

Strain	Inoculum of initial bacterial concentration	Total viable of LAB on MRS agar (CFU/ml) in vitro incubation at:		
		1min	90min	180min
4B *(L. bulgaricus)*	1.5×10^8	4.2×10^6	NG	NG
52 *(L. bulgaricus)*	1.5×10^8	1.66×10^5	2.1×10^2	NG
N1 *(L. acidophilus)*	1.5×10^8	2.6×10^7	NG	NG
N2 *(L. acidophilus)*	1.5×10^8	4.6×10^6	3.5×10^2	NG
M6 *L. acidophilus)*	1.5×10^8	5.1×10^6	NG	NG
M8 *(L. rhamnosus)*	1.5×10^8	2.6×10^6	NG	NG
70 *(L. bulgaricus)*	1.5×10^8	9.1×10^6	NG	NG
64 *(L. acidophilus)*	1.5×10^8	7.2×10^6	1.6×10^3	NG
84 *(L. plantarum)*	1.5×10^8	1.2×10^7	1.1×10^3	NG
57 *(L. rhamnosus)*	1.5×10^8	9.3×10^6	NG	NG

NG – no growth

Charteris et al. (1998) indicated that the resistance of *L. delbrueckii* subsp. *bulgaricus* to pepsin is very weak (2 to 5% of cells remain viable). The strains of *L. delbrueckii* subsp. *bulgaricus* tested in this study had significantly higher pepsin resistance compared to the above-mentioned authors (25% and 17% viable cells). According to Jensen *et al.* (2012), some of the *Lactobacillus* species studied by them, retain vitality up to 180 minutes, reducing their viability by about 1 to 4 times, but others were totally destroyed. Similar results for the species *L. acidophilus, L. rhamnosus* and *L. plantarum* have been described by Ashraf and Smith, 2016, who found out that the survival rate of the mentioned strains is about 50% after 120 minutes of treatment with simulated gastric juice at pH 2. The same authors reported that at pH 3, there was a significant increase in survival rate even after 120 minutes. Although probiotics, included in the probiotic products, are specifically tested for resistance to gastric acid and bile, only 5-10% of living bacteria reach the large intestine (Shenderov, 1997).

Simulation of conditions corresponding to the small intestine was done by incubating the test strains in pancreatin solution, pH 8.0. The number of surviving cells was determined on the first minute and 240 min. The results of the analysis are presented on Table 3. All examined strains were less sensitive to pancreatin conditions compared to the gastric juice. At the first minutes of contact with pancreatin, the bacterial cells showed better resistance and had a stable count or decrease by 1 or 2 log orders of their initial count. At the end of the intestinal stress, the number of surviving cells decreased by 3-4 log orders. Jensen *et al.* (2012) conducted a similar experiment, also demonstrating that the strains used

by them are significantly more resistant to pancreatin than pepsin. According to them, some of the strains of *L. plantarum, L. sakei,* and *L. reuteri* studied even remained unaffected by pancreatin throughout the 240-minute period of treatment with the simulated intestinal juice. For other strains there was a decrease in the number in the 240th minute by 10-20%.

Table 3. Transit tolerance of probiotic strains to the pancreatin solution

Strain	Inoculum of initial bacterial concentration	Total viable of LAB on MRS agar (CFU/ml) in vitro incubation at:	
		1 min	240 min
4B (*L. bulgaricus*)	1.5×10^8	6.3×10^6	4.6×10^6
52 (*L. bulgaricus*)	1.5×10^8	1.3×10^{10}	1.86×10^5
N1 (*L. acidophilus*)	1.5×10^8	3.6×10^8	5.0×10^6
N2 (*L. acidophilus*)	1.5×10^8	1.1×10^6	1.6×10^6
M6 *L. acidophilus*)	1.5×10^8	6.6×10^5	3.6×10^5
M8 (*L. rhamnosus*)	1.5×10^8	4.6×10^6	3.0×10^5
70 (*L. bulgaricus*)	1.5×10^8	1.6×10^7	2.6×10^5
64 (*L. acidophilus*)	1.5×10^8	2.3×10^6	1.4×10^6
84 (*L. plantarum*)	1.5×10^8	4.1×10^7	4.3×10^6
57 (*L. rhamnosus*)	1.5×10^8	5.2×10^3	9.6×10^4

Our results showed that the strains tested were more sensitive to the simulated gastric juice conditions, and many of them did not survive after 90 minutes and more resistant to small intestine conditions. Depending on the composition of the food intake, the pH in the stomach varies usually between 2.5 and 3.5 (Holzapfel *et al.,* 1998). Therefore, according to our data, it may be assumed that some of the strains tested belong to *L. bulgaricus, L. acidophilus,* and *L. plantarum* have the potential to survive in real conditions.

Influence of the capsule dosage form on the viability of probiotic strains

To investigate the effects of the gelatine capsule on the survival of probiotic strains, the action of pepsin at different pH (pH 2 and pH 3) on two probiotic products (PVIII and PXVI) was examined. Products PVIII and PXVI had identical strains composition and just differed in their dosage form (capsule and powder dosage form). Probiotics should contain the following species: *L. acidophilus, L. casei, Bifidobacterium* sp. and *Saccharomyces boulardii.* The method of fluorescence microscopy was used to visualize the ratio between live and dead cells in the simulated gastric conditions. The ratio was determined as a qualitative

reaction on the base of dominant color corresponding to the live or dead cells. The living cells were stained by membrane-binding DAPI dye, which gave blue or green color. Bacterial cells are colored in blue, and yeast cells in green. The dead cells were differentiated by propidium iodide, which has the ability to incorporate among the nucleotide bases of DNA. Dead cells are stained in orange to red. The control of the product PVIII (capsule dosage form) did not show dead cells, while in the control of the product PXVI (powder dosage form) was observed some dead cells coloured in red. (**Fig. 1)**. Viability in all prepared samples was detected at the 1 min, 90 min and 180 min of pepsin exposure. Survival during the incubation was lower in powder dosage form probiotic. The capsule dosage form showed a smaller number of dead cells (24 red cells) compared to the powder (36 red cells) one on the base of the color changes of the microscopic pictures. This is probably due to the protective function of the gelatin capsule, which limits the access of the aggressive acid medium to the cells.

Viability of the selected probiotics at pH 3 is higher than at pH 2. At pH 3 all strains in products showed similar stability, and their number slightly decreased (the blue/green color remained dominant) during incubation (**Fig. 3**). Our data shows that capsule dosage form enhance the survival of the target microorganisms in the gastrointestinal environment than the powder one and protecting the cells from lower pH and digestive enzymes.

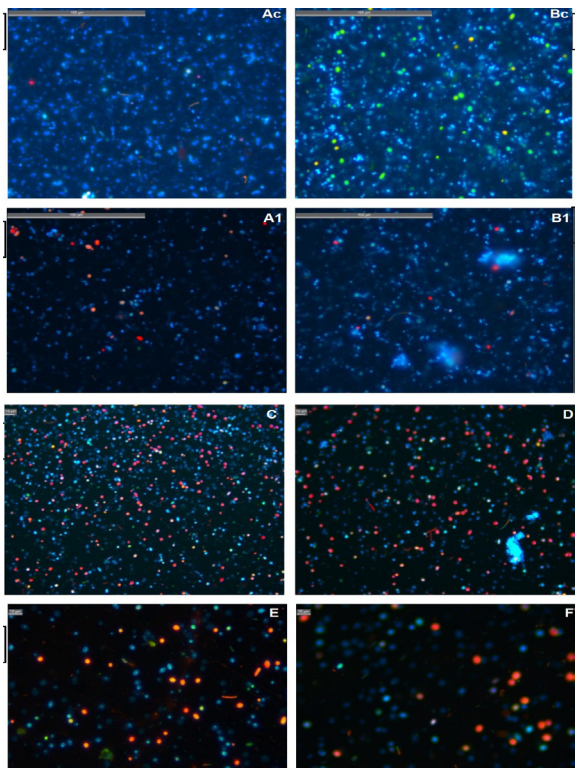

Fig. 1. Visualization of gastrointestinal transit tolerance at pH 2 by fluorescent microscopy.

Non treated probiotic products: **Ac** –PXVI, powder dosage form; **Bc** - PVII, capsule dosage form; *Pepsin treated probiotic products:* 1 min treatment: **A1** – PXVI powder dosage form; **B1** – PVIII capsule format of product; after 90 min treatment: **C** –PXVI powder dosage form; **D** - PVIII capsule format; after 180 min treatment: **E** - PXVI powder dosage form; **F** - PVIII capsule dosage form. Magnification x 40 for Ac, A1, Bc and B1, magnification x 63 – C, D, E and F.

Impact of storage on the viability of probiotic strains

The quantity of viable bacteria during storage in six probiotic products (PVII, PVIII, PIX, PIX, PXIII, PXIV) was investigated. The products were produced by local manufacturer and differed in dosage form. The experiment was carried out in three intervals after production: 1 day, 30 and 90 days of the storage according to the manufacturer recommendations. Table 4 shows the changes in the number of probiotic microflora of the selected products during 90 days of storage.

Table 4. The viability of probiotic strains during storage

Product	Storage, days, CFU/g,		
	1 day	30 days	90 days
PVII/capsules	4.9×10^9	2.6×10^9	3.23×10^6
PVIII/capsules	1.8×10^{10}	1.69×10^9	4×10^6
PIX/capsules	1×10^{10}	1.88×10^9	6.16×10^6
PXIII/sachets	7×10^9	2.7×10^9	4×10^6
PXIV/ powdered	4.7×10^{10}	1.6×10^{10}	4.83×10^6

The results clearly showed the progressive reduction in the number of viable cells during the storage period. It is observed that after 90 days of storage, the number of bacteria significantly decreased. In the end of our last reporting period (90 days of manufacture), the total number of all products tested was in the order of 106 CFU/ml, which still corresponds to the WHO eligibility limit according to the FDA, 2006. Feldman et al., (2017) tested two storage environments, one at 4 to 8°C and one at 25oC with a relative humidity of 60%. The test was conducted over 36 months and the capsules were packaged in a polypropylene bottle, without a moisture-protection barrier. Also they tested different types of capsules. They used a special designed capsule DUOCAPR and standard single capsule. Their results revealed that DUOCAPR had 36-months stability under both conditions, while the standard single capsule only had stability of 9 months. Most probiotic products have a shelf-life between 1 and 2 years from the date of manufacture. Our investigations also showed that the viability decreased after 3 months even their properly stored. We could have suggested that after 6 or 9 months of storage more the stability of products would be significantly reduced.

CONCLUSIONS

Microbial populations in selected probiotic supplements were investigated and obtained in present study results indicated the following problems: the total viable cells in the most probiotic products did not correspond to the cell number given in the label. There were deviations from the information provided concerning strain composition of the product and some of the products were

contaminated. Our results revealed problems with the quality of the commercial probiotic products and issues related to the accurate strain identification and labeling of the already established products in the market. Hence, it was deemed necessary to review the status of the available probiotic products on Bulgarian market and screen their quality.

DECLARATION OF INTEREST STATEMENT

All authors declare no conflict of interest.

AUTHORS CONTRIBUTION STATEMENT

YD and VM have made substantial contribution to acquisition of data. IR and YK have made the analysis and interpretation of the data and drafting the article. PH have made substantial contribution to conception and critically revising the article for the content and final approval of the version to be published.

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