

Investigation of the Stability of Freeze-Dried Probiotic Products in Capsules

Yordanka Dermendzhieva, Maria Pandova, Yoana Kizheva, Petya K. Hristova*

Department of General and Applied Microbiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria

Abstract

The probiotic supplement market is extremely large, highly dynamic, and offers an abundance of probiotic products from both established leaders and emerging manufacturers. Each manufacturer is expected to offer a probiotic product whose qualities comply with the regulations and what is stated on the label, both at the time of manufacturing and throughout its shelf life. Given the unstable nature of lyophilized probiotics, the production of a quality and stable probiotic product is a difficult task, requiring a complex and scientifically sound approach to the selection, evaluation, and control of a number of factors that affect the quality of the product. The present study is focused on the investigation of the stability of probiotic products in a capsule, by assessing various factors influencing the quality of the product over time: type of primary packaging, condition of desiccant, gas composition in the bottle, quantity of capsules in the bottle, and composition of the excipients. Thirty-three R&D samples of probiotic products prepared for the purposes of the present study, stored under long-term stable conditions corresponding to climate zone II (25°C and 60% Relative humidity (RH)) and analyzed four times during their storage period within twelve months, were tested. The results clearly showed that the stability of the probiotic product is influenced by all monitored factors.

Keywords: manufacturing of probiotics, stability of probiotic products, viability, probiotic microorganisms, lactic acid bacteria

Резюме

Пазарът на пробиотични добавки е изключително голям, силно динамичен и предлага изобилие от пробиотични продукти, както от утвърдени лидери, така и от нововъзникващи производители. От всеки производител се очаква да предлага пробиотичен продукт, чиито качества отговарят на разпоредбите и на посоченото на етикета, както по време на производство, така и през целия му срок на годност. Предвид нестабилния характер на лиофилизираните пробиотици, производството на качествен и стабилен пробиотичен продукт е трудна задача, изискваща сложен и научно обоснован подход към избора, оценката и контрола на редица фактори, които влияят върху качеството на продукта. Настоящото изследване е фокусирано върху изследването на стабилността на пробиотичните продукти в капсула, чрез оценка на различни фактори, влияещи върху качеството на продукта във времето – вид първична опаковка, състояние на десиканта, състав на газа в бутилката, количество капсули в бутилката и състав на помощните вещества. Тествани са тридесет и три научноизследователски и развойни проби от пробиотични продукти, приготвени за целите на настоящото изследване, съхранявани при дългосрочни стабилни условия, съответстващи на климатична зона II (25°C и 60% относителна влажност (RH)) и анализирани четири пъти по време на периода им на съхранение в рамките на дванадесет месеца. Резултатите ясно показаха, че стабилността на пробиотичния продукт се влияе от всички наблюдавани фактори.

Introduction

The growing consumer demand for a healthier lifestyle has evolved into the concept of functional foods, which should have enhanced nutritional and therapeutic values. Thus, the production of probi-

otic products has gradually become the most developed sector, which now accounts for more than 60% of the global functional food (Misra *et al.*, 2021). Moreover, the global probiotics market has a compound annual growth rate (CAGR) of about

*Corresponding author: pkabad@biofac.uni-sofia.bg
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7% and is expected to reach USD 77.09 billion by 2025. The first probiotic drink, marketed in 1935 in Japan (Yakult Honsha Co. Ltd., Tokyo, Japan), was based on the properties of *Lactocaseibacillus casei* Shirota (Fukushima and Hurt, 2011). Later, another group of bacteria, such as *Bifidobacterium*, was included in probiotic products after their benefits in maintaining a healthy gut microbiome were established. Currently, members of the family *Lactobacillaceae* and the genus *Bifidobacterium* are the most commonly used probiotic microorganisms in dietary supplements.

Probiotics are defined as living organisms that, in adequate amounts (10^6 – 10^7 CFU/g), produce a positive effect on host health (FAO/WHO, 2006). Moreover, the World Health Organization's assessment documents for probiotic products now require declaration of the number of viable bacteria both at the beginning and at the end of the shelf life of these supplements (FAO/WHO, 2002; Pineiro and Stanton, 2007). This determines the development of viable probiotics with a significant non-refrigerated shelf life (Chavez and Ledebor, 2007). The development of probiotic products is associated with a number of challenges, such as the selection of active strains with good technological qualities that are able to survive during the processing and storage of the products (Celik and O'Sullivan, 2013). At the same time, a growing number of scientific publications report discrepancies in the data indicated in the labels of probiotic products. For example, three of four probiotic products containing *Lactobacillus* sp. sold on the market do not contain the number of bacteria that is in accordance with the packaging label (Begum *et al.*, 2001). Our previous study supports the aforementioned statement (Marinova *et al.*, 2019). Of the 26 probiotic products examined, none of the commercial products contained all of the labeled LAB, and some of them contained unacceptable microorganisms.

Manufacturers of probiotic products are expected to create a product whose small dosage form contains a large number of probiotic microorganisms and to provide their stability throughout their shelf life, often under storage conditions far from optimal for them (Jofre *et al.*, 2015). Long-term storage and stabilization of probiotic bacteria requires high-yield fermentation, a gentle drying method, and protective matrix composition (Poddar *et al.*, 2012). Several technologies can be applied to dry mixtures of probiotic bacteria and matrix, including freeze drying, spray drying, and fluidized bed drying. Each of these processes re-

sults in the inevitable gradual loss of viability of microorganisms. In freeze-drying, the loss of viable bacteria is due to ice crystal formation within the cell and rupture of the cell membrane (Fonseca *et al.*, 2004). Spray drying uses a high temperature at which thermal inactivation of the cells occurs (Ananta *et al.*, 2005). Fluidized bed drying applies mild temperatures that minimize bacterial inactivation, but this method has rarely been used to produce probiotic products. (Bayrock and Ingledew, 1997). The freeze-drying method more effectively preserves the viability of probiotic cultures (Celik and O'Sullivan, 2013).

To minimize stress and increase the stability of probiotics during freeze-drying and subsequent storage, the composition and structure of the lyoprotective matrix are essential. The inclusion of carbohydrates, proteins, and fats has a protective role for the survival of bacteria and has been successfully used as components of the desiccant matrix. The influence of trehalose and lactose as lyoprotectants has been most widely studied (Cui *et al.*, 2023). For example, lactose substitutes the hydrogen-bonded water in cell membrane phospholipid bilayers (Santivarangkna *et al.*, 2008), while other carbohydrates create a high viscosity that acts as a protective capsule for bacteria. (Morgan *et al.*, 2006). Proteins slow the crystallization of sugars and maintain the protective amorphous state (Shrestha *et al.*, 2007). Similarly, fat can delay lactose crystallization (Knudsen *et al.*, 2002).

There are a number of factors that alone or in combination can influence the quality of probiotic products during storage – moisture content, dosage form of finished product, conditions of manufacturing of finished product, composition of excipients and type of primary packaging (Poddar *et al.*, 2014; Savedboworn *et al.*, 2019) (Fig. 1). These factors cannot be ranked in order of importance and neglecting even one of them can have a negative impact on product quality, despite the investment in controlling the others. The manufacture of a quality probiotic product is a difficult and complex task and requires a clear strategy for assessing all these factors. The aim of this study was assessing some key factors influencing the stability of probiotic products (primary packaging, excipients, marked in black in Fig. 1, while fixing the other factors (selection of suitable strains, manufacturing of probiotic raw material, dosage form, manufacturing process of finished product, marked in gray in Fig. 1 to improve the stability of freeze-dried probiotic products in capsules.

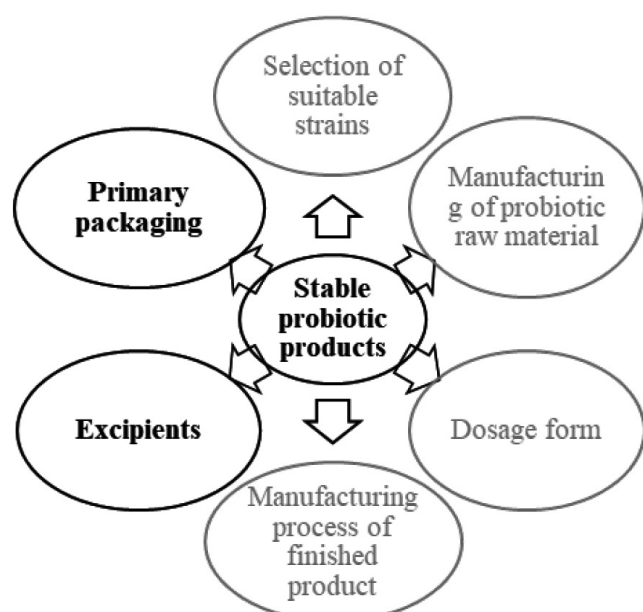


Fig. 1. Factors influencing the stability of a probiotic product

Materials and Methods

Samples of probiotic products

Thirty-three R&D samples of probiotic products in capsules were prepared under equal manufacturing conditions for the purposes of the current study. All samples are produced with the cooperation of Kendy Ltd., Bulgaria. All probiotic raw materials included in the products tested in this test

were purchased from the same manufacturer. Five different types of probiotic blends in 14 different packages were tested. A description of the main compositions (excluding excipient variations) is presented in Table 1, while the variations of their primary packaging are described in Table 2.

The composition of the five lyophilized probiotic raw materials produced under the same conditions is listed in Table 3. For the preparation of probiotic raw materials, fifteen strains indicated in Table 4 were used in different combinations. The initial quantity of microorganisms in each probiotic blend is also presented in Table 4.

The R&D samples of probiotic products were stored in a stability chamber under conditions corresponding to climate zone II (25°C/60% RH) in four replicates to be tested at: 1/ the time of manufacturing; 2/ three months; 3/ six months, and 4/ twelve months after their manufacture.

Evaluation of appearance, taste, and odor

The appearance, taste, and odor of the probiotic products and their compliance with the product specification (“light yellow to light brown powder” and “characteristic lactic acid taste and odor of the capsule contents” respectively), were determined visually and organoleptically according to the procedure described in BDS 15612:1983.

Table 1. General composition of R&D samples of probiotic products

R&D Product	Composition	(mg/capsule)
R&D product I	Probiotic blend I:	175
	Lynside Pro SCB:	65
	Maltodextrin IT 19:	155
	Magnesium stearate:	5
	V Caps:	90
R&D product II	Probiotic blend II:	250
	Orafti GR (Inulin powder):	150
	Magnesium stearate:	5
	V Caps	90
R&D product III	Probiotic blend III:	380
	Probiotic blend III:	20
	Magnesium stearate	4
	V Caps, DR:	90
R&D product IV	Probiotic blend IV:	200
	Orafti GR (Inulin powder):	200
	Magnesium stearate:	5
	HGC Caps:	90
R&D product V	Probiotic blend V:	385
	Magnesium stearate:	5
	HGC Caps:	90

Table 2. Description of primary packaging

Used designation of primary packaging	Description	
Blister I	PVC foil	PVC 60 µm
	Aluminum foil	Alu 20 µm
Blister II	PVC foil	PVC 60 µm + PE 60 µm + PVDC 33 µm
	Aluminum foil	Alu 20 µm
PVC bottle I	PVC bottle	180 mL
	PVC cap with foil	
	Silica gel	sachet with 1 g
PVC bottle II	100 capsules	
	PVC bottle (Kendy)	150 mL
	PVC cap with foil	
PVC bottle III	Silica gel	sachet with 2 g
	60 capsules	
	PVC bottle (Korea)	150 mL
PVC bottle IV	PVC cap with foil	
	Silica gel	sachet with 1.8 g
	60 capsules	
PVC bottle V	PVC bottle	50 mL
	PVC cap with foil	
	Silica gel	sachet with 1 g
PVC bottle VI	20 capsules	
	PVC bottle	150 mL
	PVC cap with foil	
PVC bottle VII	Silica gel	sachet with 1 g
	15 capsules	
	PVC bottle	180 mL
PVC bottle VIII	PVC cap with foil	
	Silica gel	sachet with 1 g
	18 capsules	
PVC bottle IX	PVC bottle	180 mL
	PVC cap with foil	
	Silica gel	3 sachets with 1 g
PVC bottle X	100 capsules	
	PVC bottle	180 mL
	PVC cap with foil	
	Silica gel	5 sachets with 1 g
	18 capsules	

Used designation of primary packaging	Description	
PVC bottle XI	PVC bottle	75 mL
	PVC cap with foil	
	Silica gel	sachet with 1 g
	15 capsules	
PVC bottle XII	PVC bottle	180 mL
	PVC cap with foil	
	Silica gel	sachet with 1 g
	15 capsules	
Alu bottle I	Aluminum bottle:	125 mL
	Closure with 2.3 g silica gel	
	60 capsules	
PVC tube I	PVC tube	d = 33 mm
	PVC closure, 3.5 g silica gel	
	66 capsules	

Table 3. Composition of probiotic raw materials

Probiotic blend	Composition of strains		Total count microorganisms (≥ CFU/g)
Probiotic blend I	<i>Bifidobacterium bifidum</i>	NBIMCC 8328	5.0 x 10 ¹⁰
	<i>Bifidobacterium infantis</i>	NBIMCC 8909	
	<i>Bifidobacterium longum</i>	NBIMCC 8329	
	<i>Lactobacillus acidophilus</i>	NBIMCC 8759	
	<i>Lactocaseibacillus rhamnosus</i>	NBIMCC 8769	
Probiotic blend II	<i>B. bifidum</i>	NBIMCC 8328	2.0 x 10 ¹¹
	<i>B. infantis</i>	NBIMCC 8909	
	<i>B. longum</i>	NBIMCC 8329	
	<i>L. acidophilus</i>	NBIMCC 8242	
	<i>L. bulgaricus</i>	NBIMCC 8917	
	<i>Streptococcus thermophilus</i>	NBIMCC 8714	
Probiotic blend III	<i>B. bifidum</i>	NBIMCC 8328	2 x 10 ¹¹
	<i>Bifidobacterium lactis</i>	NBIMCC 8908	
	<i>B. longum</i>	NBIMCC 8329	
	<i>L. bulgaricus</i>	NBIMCC 8917	
	<i>Lactocaseibacillus paracasei</i>	NBIMCC 8948	
	<i>L. rhamnosus</i>	NBIMCC 8769	
	<i>S. thermophilus</i>	NBIMCC 8714	
Probiotic blend IV	<i>B. bifidum</i>	NBIMCC 8328	2.5 x 10 ¹⁰
	<i>B. infantis</i>	NBIMCC 8909	
	<i>B. longum</i>	NBIMCC 8329	
	<i>L. acidophilus</i>	NBIMCC 8242	
	<i>L. bulgaricus</i>	NBIMCC 8917	
	<i>S. thermophilus</i>	NBIMCC 8714	

Table 3. Composition of probiotic raw materials

Probiotic blend	Composition of strains		Total count microorganisms (≥ CFU/g)
Probiotic blend V	<i>B. bifidum</i>	NBIMCC 8328	3.0 x 10 ¹⁰
	<i>B. infantis</i>	NBIMCC 8909	
	<i>B. longum</i>	NBIMCC 8329	
	<i>L. bulgaricus</i>	NBIMCC 8915	
	<i>Lactobacillus helveticus</i>	NBIMCC 8268	
	<i>Lactobacillus lactis</i>	NBIMCC 8764	
	<i>Lactiplantibacillus plantarum</i>	NBIMCC 8765	
	<i>L. plantarum</i>	NBIMCC 8766	
	<i>Lacticaseibacillus rhamnosus</i>	NBIMCC 8769	

Table 4. Probiotic strains

Probiotic strains	
<i>B. bifidum</i>	NBIMCC 8328
<i>B. infantis</i>	NBIMCC 8909
<i>B. lactis</i>	NBIMCC 8908
<i>B. longum</i>	NBIMCC 8329
<i>L. acidophilus</i>	NBIMCC 8759
<i>L. acidophilus</i>	NBIMCC 8242
<i>L. bulgaricus</i>	NBIMCC 8917
<i>L. bulgaricus</i>	NBIMCC 8915
<i>L. helveticus</i>	NBIMCC 8268
<i>L. lactis</i>	NBIMCC 8764
<i>L. paracasei</i>	NBIMCC 8948
<i>L. plantarum</i>	NBIMCC 8765
<i>L. plantarum</i>	NBIMCC 8766
<i>L. rhamnosus</i>	NBIMCC 8769
<i>S. thermophilus</i>	NBIMCC 8714

Moisture

Two grams of the sample were dried on an automatic moisture analyzer at 80°C for fifteen minutes till reaching the absolute dry weight according to ISO 5537:2004. The percentage of moisture in the sample was determined according to the difference in the weight of the sample before and after drying. The measurements were performed in two replicates.

Moisture (%) = 100-(x/y.100); x- the weight before drying (g); y - weight after drying (g)

In cases where the difference between the values is less than 0.5%, this parameter is defined as insignificant.

Total count of viable probiotic microorganisms

The total count of probiotic microorganisms was determined according to the methods specified in the harmonized ISO 7889: 2005 and ISO 29981:

2010. The samples were tenfold diluted in saline separately, followed by poor-plate method in three replicates of the last three significant dilutions in a suitable selective culture medium, according to the composition of the product. MRS agar was used for *Lactobacillus* sp., TOS agar + Mupirocin for *Bifidobacterium* sp., and M17 agar for *S. thermophilus*. The samples were cultured anaerobically for lactobacilli and bifidobacteria on agar and aerobically for streptococci at 37°C for 72 hours. At the end of the incubation period, the number of the colony forming units on each medium was counted

$$N = \frac{\sum C}{(n_1 + 0.1 n_2) d V}, \text{ where}$$

and calculated according to the formula:

N – the numerical value of the number of microorganisms (CFU/g) per unit of test sample;

∑C – the numerical value of the sum of all colonies formed in the petri dishes from two consecutive dilutions;

n₁ – the numerical value of the listed plates;

n₂ – the numerical value of the listed plates in the second dilution;

d – the dilution factor.

V – amount of inoculum (mL).

The total count of probiotic microorganisms was presented as the sum of the obtained results of the respective culture medium, calculated from the weight of the capsule content of the product (CFU / caps.).

When interpreting the results for this parameter, the difference between the values obtained in the different test periods, less than 1 x 10^x CFU / capsule, was determined to be insignificant, which roughly corresponds to the confidence interval at 95 % confidence level.

Rate of reduction of probiotic microorganisms

A criterion for uniform assessment of the rate of reduction in probiotic microorganisms in all samples (R_x) was used, which was calculated by the following formula:

$$R_x = \log_{10} N_x - \log_{10} N_y, \text{ where:}$$

R_x – rate of reduction;

N_x – total count probiotic microorganisms (CFU/caps.) at the time of manufacturing;

N_y – total count probiotic microorganisms (CFU/caps.) at the particular test period.

When interpreting the results for this parameter, the difference between the values obtained in the different test periods of less than 0.1 was determined to be insignificant.

Results and Discussion

The present study aimed to investigate the stability of probiotic products in capsules. Two main factors affecting product quality were monitored: type and conditions of primary packaging and composition of excipients. The other factors (selection of suitable strains, manufacturing of probiotic raw material, dosage form, manufacturing process of finished product) were not changed.

The strategy in our study was as follows:

- *Selection of suitable strains* – fixed parameter. The strains involved in the probiotic products subject to this study are listed in Table 4.

- *Manufacturing of probiotic raw material* – fixed parameter. All probiotic raw materials included in the products tested in this test were purchased from the same manufacturer. They are made on equal terms.

- *Dosage form* – fixed parameter. The stability of probiotic products in the most common dosage form – capsules – was tested.

- *Manufacturing process of finished product* – fixed parameter. All R&D probiotic products for this study were manufactured under equal conditions – in a modern production base, in clean class D rooms (according to GMP), at a temperature below 20°C, and relative humidity below 35% RH.

- *Excipients* – the stability of R&D probiotic products was tested, which included various excipients and combinations between them.

- *Primary packaging* – the stability of different R&D probiotic products was tested, which had different types of primary packaging (blisters and bottles), desiccants, and gas composition in the bottle and quantity of product in the packaging.

The current extensive study included an evaluation of thirty-three R&D probiotic products at four time-control points within one year of manufacture (0, 3, 6, and 12 months), by comparing results on the four control parameters (appearance, moisture, total number of probiotic microorganisms, and rate of reduction) – a total of 528 tests.

As expected, there was a tendency between the results of the four monitored indicators in all samples, expressed in the following: the increase in moisture in the capsule content leads to deterioration of its appearance, reduction of the total number of viable probiotic microorganisms in a capsule, and an increase in their rate of reduction.

Our study went sequentially through the stages shown in Figure 2, and the results obtained at each stage will be discussed separately below.

Assessment of the influence of the type of primary packaging

The effect of seven types of primary packaging on the stability of three types of R&D probiotic products in capsules was studied (Table 5). The description of the composition of the products and of the selected primary packaging is presented in Table 1 and Table 2, respectively.

R&D product I was packaged in three types of primary packaging – Blister I, Blister II and PVC bottle I. The obtained results clearly showed that the composition of Blister I cannot provide reliable protection of the capsules, and only after three months of storage, the appearance of the capsule contents is changed to a degree not corresponding to the specification, the condition of the product did not allow for correct measurement of moisture, and the reduction rate of viable microorganisms was

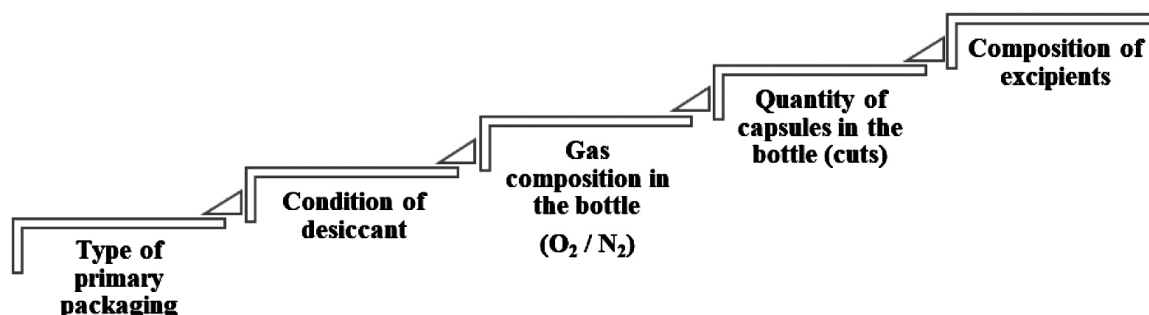


Fig. 2. Stages of study

Table 5. Type of primary packaging

Test period	<u>Appearance</u>								
	R&D product I			R&D product II			R&D product III		
	Blister I	Blister II	PVC bottle I	Blister II	PVC bottle I	PVC tube I	PVC bottle II	PVC bottle III	Alu bottle I
0 month	✓	✓	✓	✓	✓	✓	✓	✓	✓
3 rd month	X	✓	✓	✓	✓	✓	✓	✓	✓
6 th month	X	✓	✓	✓	✓	✓	✓	✓	✓
12 th month	X	✓	✓	✓	✓	✓	✓	X	✓
Test period	<u>Moisture (%)</u>								
	R&D product I			R&D product II			R&D product III		
	Blister I	Blister II	PVC bottle I	Blister II	PVC bottle I	PVC tube I	PVC bottle II	PVC bottle III	Alu bottle I
0 month									
3 rd month	N/A	3.23	3.96	3.66	3.46	3.28	3.52	4.44	3.04
6 th month	N/A	4.17	4.27	4.38	3.46	3.57			
12 th month	N/A	4.36	4.34	4.53	4.26	4.39			
Test period	<u>Total count probiotic microorganisms (CFU/caps.)</u>								
	R&D product I			R&D product II			R&D product III		
	Blister I	Blister II	PVC bottle I	Blister II	PVC bottle I	PVC tube I	PVC bottle II	PVC bottle III	Alu bottle I
0 month	1.8×10^{10}	1.8×10^{10}	1.8×10^{10}	5.0×10^{10}	5.0×10^{10}	5.0×10^{10}	7.5×10^{10}	7.5×10^{10}	7.5×10^{10}
3 rd month	3.7×10^8	9.2×10^8	1.6×10^9	1.7×10^9	1.5×10^9	2.3×10^9	4.2×10^{10}	4.5×10^{10}	6.5×10^{10}
6 th month	$< 10^2$	3.5×10^8	4.6×10^7	1.6×10^8	2.5×10^8	1.7×10^8	8.5×10^9	2.5×10^9	9.4×10^9
12 th month	$< 10^2$	N/A	N/A	7.7×10^7	1.0×10^8	1.1×10^8	4.2×10^8	3.4×10^7	3.1×10^9
Test period	<u>Rate of reduction (Rx)</u>								
	R&D product I			R&D product II			R&D product III		
	Blister I	Blister II	PVC bottle	Blister II	PVC bottle I	PVC tube I	PVC bottle II	PVC bottle III	Alu bottle I
3 rd month	1.69	1.30	1.06	1.47	1.52	1.34	0.26	0.23	0.07
6 th month	> 8.26	1.72	2.60	2.50	2.30	2.47	0.95	1.48	0.91
12 th month	N/A	N/A	N/A	2.81	2.70	2.66	2.24	3.33	1.39

✓ conform to specification (light yellow to light brown powder);

X non-conforming to specification (light yellow to light brown powder);

N/A not applicable.

drastic. Blister II and PVC bottle I retain the appearance of the capsule contents corresponding to the end of the study, and by the third month, the results of the remaining parameters are in favor of PVC container I, but after the sixth registered results are in favor of Blister II. The probable reason for this is that the container leaks a larger amount of moisture, and by the third month, the desiccant in it manages to compensate for this and protect the product, but after the sixth its efficiency has decreased, and the quality of the product in it begins to deteriorate faster than the product in Blister II. Unfortunately, a correct analysis of viable lactoba-

cilli and bifidobacteria in R&D product I after the twelfth month was not possible due to the presence of *S. boulardii* in its composition. Yeast is far more stable than lactic acid bacteria, and after the twelfth month had a significant numerical advantage over them, which prevented the correct enumeration of lactobacilli at this stage, because when inoculated at a lower dilution in MRS agar, *S. boulardii* forms a lot of small colonies. This did not allow a clear differentiation between the protective properties of Blister II and PVC bottle I and led to the re-inclusion of these two packages in subsequent studies.

R&D product II was packaged in three types

Table 6. Condition of desiccant

Test period	<u>Appearance</u>	
	R&D product IV	
	PVC bottle IV 1 g silica gel	PVC bottle IV 1 g dried silica gel
0 month	✓	✓
3 rd month	✓	✓
6 th month	✓	✓
12 th month	✓	✓
Test period	<u>Moisture (%)</u>	
	R&D product IV	
	PVC bottle IV 1 g silica gel	PVC bottle IV 1 g dried silica gel
0 month	3.30	3.30
3 rd month	4.53	2.64
6 th month	4.61	4.19
12 th month	4.87	4.21
<u>Total count probiotic microorganisms (CFU/caps.)</u>		
Test period	R&D product IV	
	PVC bottle IV 1 g silica gel	PVC bottle IV 1 g dried silica gel
0 month	1.3 x 10 ¹⁰	1.3 x 10 ¹⁰
3 rd month	3.4 x 10 ⁸	4.6 x 10 ⁸
6 th month	8.5 x 10 ⁷	2.0 x 10 ⁸
12 th month	1.6 x 10 ⁷	3.8 x 10 ⁷
Test period	<u>Rate of reduction (Rx)</u>	
	R&D product IV	
	PVC bottle IV 1 g silica gel	PVC bottle IV 1 g dried silica gel
3 rd month	1.58	1.45
6 th month	2.18	1.81
12 th month	2.91	2.51

✓ conform to specification (light yellow to light brown powder).

of primary packaging – Blister II, PVC bottle I, and PVC tube I. The storage conditions that provide PVC bottle I and PVC tube I were chosen as close as possible to assess the impact only on the package. All three packages kept the appearance of the samples unchanged. When monitoring the moisture in them, it became clear that the difference between the values obtained is not significant, and on this indicator, we cannot take into account the advantage of one package over another. The registered results of the rate of reduction of microorganisms showed that Blister II has the worst protective properties, and between the properties of PVC bottle I and PVC tube I, there is no clear difference.

R&D product III was packaged in three types

of primary packaging – PVC bottle II, PVC bottle III, and Aluminum bottle I. The results clearly and unambiguously showed that in the aluminum container, the product is maximally protected, while the results of the product in PVC bottle III are the worst.

Assessment of the influence of the condition of the desiccant

In most cases, manufacturers of desiccants offer their products in various forms and cuts, ensuring their effectiveness, regardless of the product and the conditions in which they will be used. Practice shows that this is not true and the correct choice of the type of desiccant (one or a combination), the form in which it will be applied, the packaging and

cuts in which it will be delivered and the production conditions under which it will be applied is difficult and in each one case must be approached individually.

At this stage of our study, we evaluated the influence on the stability of the same R&D probiotic product (R&D product IV), packaged in the presence of sachets of 1 g of silica gel, applied as supplied by the manufacturer and in parallel, in the presence of sachets of silica gel from the same batch, which before application were pre-dried in a dry oven at 100° C for 1h (Table 6). The results obtained clearly show that the appearance of the product remains unchanged in both cases, but the other results are significantly better for packages containing pre-dried silica gel.

Assessment of the influence of the gas composition in the bottle

The stability of the same R&D probiotic product (R&D product V), packed in a PVC bottle V in the presence of atmospheric oxygen, as well as in the injection of compressed nitrogen into the package, was monitored (Table 7).

The observed results for all parameters showed that the packages in which the atmospheric oxygen is replaced by nitrogen protect the product better and ensure its better stability during its shelf life.

Assessment of the influence of the quantity of capsules in the bottle (cuts)

R&D product II was packaged in six types of primary packaging: R&D – PVC bottle I, VI, VII, VIII, IX, and X (Table 8). A PVC bottle with a volume of 180 mL was used for all packages, but the ratio between the number of capsules and the amount of silica gel was different.

The obtained results showed that at the same ratio between the number of capsules and the volume of the container (18 caps./180 mL and 100 caps./180 mL), the higher the amount of silica gel, leads to better results, as the fewer capsules the package contains (respectively the greater the free volume in the container), the better results are recorded. The best results were reported for the sample in PVC bottle X (18 caps./180 mL/5 g silica gel).

A more detailed comparison of the results between the three pairs of packages with the same ratio between the volume of the container and the amount of silica gel (180 mL/1 g silica gel, 180 mL/3 g and 180 mL/5 g), but with a different number of capsules (18 capsules or 100 capsules), it

became clear that in the first months of testing the fewer capsules in the package, the better results are recorded.

However, this changes over time, as the more silica gel the package contains, the better the results are in packages with fewer capsules. For example, for packages with 1 g of silica gel, the results are in favor of the 18 capsules by the 3rd month, for packages with 3 g of silica gel – by the 6th month, and for packages with 5 g of silica gel – by the 12th month. The probable reason for this is that the fewer capsules and respectively moisture is introduced into the bottle at the time of manufacturing, the longer the silica gel manages to compensate for the incoming moisture through the packaging during the storage of the product.

Assessment of the influence of the composition of excipients

In the course of our study, the effect of a total of seven excipients added alone or in combination to the composition of three probiotic products was evaluated (Table 9).

The original compositions of R&D products V and III do not contain excipients, and the weight of their capsule content is less than the weight of their derivatives with added excipients, the amount of product imported into the container at the time of manufacturing is less. This reflects on the moisture in the packaging at the time of manufacturing and makes the comparison of their results with those of their derived products with added excipients incorrect. Therefore, the results obtained for R&D products V and III with their original compositions are presented in Table 9, but will not be taken into account in the interpretation of the data.

The original composition of R&D product V included 5 mg of three excipients (Prosolv, N-Zorbit, and Sipernat) alone, representing 1.23% by weight of the capsule content. The results clearly showed that the R&D product containing Sipernat was the most unstable in each test period. In the 12th month, the best results were registered for the R&D product containing Prosoolv. However, it is difficult to compare them with the results of the R&D product containing N-Zorbit, as the trends emerging in the different test periods are unstable and the differences between the two excipients can be defined as vague and insignificant. Therefore, these two excipients were re-included in the next stage of our study.

The original composition of R&D product I contains 155 mg of Maltodextrin IT 19, representing 38.75% by weight of the capsule content. All

Table 7. Gas composition in the bottle

		<u>Appearance</u>	
		R&D product V	
Test period		PVC bottle V 1 g silica gel	PVC bottle V 1 g dried silica gel + N ₂
0 month		✓	✓
3 rd month		✓	✓
6 th month		✓	✓
12 th month		X	X
		<u>Moisture (%)</u>	
		R&D product V	
Test period		PVC bottle V 1 g silica gel	PVC bottle V 1 g dried silica gel + N ₂
0 month			
3 rd month	4.85	4.64	
6 th month	6.09	5.62	
12 th month	7.78	7.28	
		<u>Total count probiotic microorganisms (CFU/caps.)</u>	
		R&D product V	
Test period		PVC bottle V 1 g silica gel	PVC bottle V 1 g dried silica gel + N ₂
0 month	2.3 x 10 ¹⁰	2.3 x 10 ¹⁰	
3 rd month	4.4 x 10 ⁸	7.3 x 10 ⁸	
6 th month	4.7 x 10 ⁷	5.5 x 10 ⁷	
12 th month	2.5 x 10 ⁴	3.5 x 10 ⁴	
		<u>Rate of reduction (Rx)</u>	
		R&D product V	
Test period		PVC bottle V 1 g silica gel	PVC bottle V 1 g dried silica gel + N ₂
3 rd month	1.72	1.50	
6 th month	2.69	2.62	
12 th month	5.96	5.82	
✓ conform to specification		(light yellow to light brown powder).	

or part of Maltodextrin was replaced by four excipients alone or in combination (Table 8) – N Zorbit (33.78% of the capsule content), Prosolv (33.78%), Maltodextrin + Sipernat (34.66% in total), and Dextrose + Sipernat (33.78% in total). The results showed that the most unstable throughout the test period was the R&D product, which included Dextrose (125 mg) and Sipernat (5 mg). The best results were registered for R&D products containing Maltodextrin, followed by the R&D product containing Prosolv and the one containing N-Zorbit.

80 mg of probiotic blend was removed from the original composition of R&D Product III, and 100 mg of two excipients (Maltodextrin IT 6 and

Vivapur 102), representing 23.58% by weight of the capsule content, were included alone. The results clearly showed that the R&D product containing Maltodextrin IT 6 was more stable throughout the storage period than that containing Vivapur 102.

Conclusion

The nature of probiotic lyophilized products determines their strong hygroscopicity and requires ensuring their maximum protection during their entire shelf life.

The choice of the type and composition of the primary packaging is essential for preserving the qualities of the product, as the aluminum bottle showed the best protective qualities, followed

Table 8. Quantity of the capsules in the bottle (cuts)

<u>Appearance</u>								
Test period	R&D product II				Product V			
	PVC	PVC	PVC	PVC	PVC	PVC	PVC	PVC
	bottle I	bottle VI	bottle VII	bottle VIII	bottle IX	bottle X	bottle XI	bottle XII
	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(75 mL	(180 mL
	100 caps.	18 caps.	100 caps.	18 caps.	100 caps.	18 caps.	15 caps.	15 caps.
	1 g sil. gel)	1 g sil. gel)	3 g sil. gel)	3 g sil. gel)	5 g sil. gel)	5 g sil. gel)	1 g sil. gel)	1 g sil. gel)
0 month	✓	✓	✓	✓	✓	✓	✓	✓
3 rd month	✓	✓	✓	✓	✓	✓	✓	✓
6 th month	✓	✓	✓	✓	✓	✓	✓	✓
12 th month	✓	✓	✓	✓	✓	✓	✓	X
<u>Moisture (%)</u>								
Test period	R&D product II				Product V			
	PVC	PVC	PVC	PVC	PVC	PVC	PVC	PVC
	bottle I	bottle VI	bottle VII	bottle VIII	bottle IX	bottle X	bottle XI	bottle XII
	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(75 mL	(180 mL
	100 caps.	18 caps.	100 caps.	18 caps.	100 caps.	18 caps.	15 caps.	15 caps.
	1 g sil. gel)	1 g sil. gel)	3 g sil. gel)	3 g sil. gel)	5 g sil. gel)	5 g sil. gel)	1 g sil. gel)	1 g sil. gel)
0 month								
3 rd month	3.46	3.18	3.19	2.96	2.86	2.41	4.41	4.21
6 th month	3.46	3.44	4.12	3.90	4.19	2.73	4.31	4.47
12 th month	4.26	4.56	4.31	4.01	3.65	3.34	6.01	5.62
<u>Total count probiotic microorganisms (CFU/caps.)</u>								
Test period	R&D product II				Product V			
	PVC	PVC	PVC	PVC	PVC	PVC	PVC	PVC
	bottle I	bottle VI	bottle VII	bottle VIII	bottle IX	bottle X	bottle XI	bottle XII
	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(75 mL	(180 mL
	100 caps.	18 caps.	100 caps.	18 caps.	100 caps.	18 caps.	15 caps.	15 caps.
	1 g sil. gel)	1 g sil. gel)	3 g sil. gel)	3 g sil. gel)	5 g sil. gel)	5 g sil. gel)	1 g sil. gel)	1 g sil. gel)
0 month	5.0 x 10 ¹⁰	5.0 x 10 ¹⁰	5.0 x 10 ¹⁰	5.0 x 10 ¹⁰	5.0 x 10 ¹⁰	5.0 x 10 ¹⁰	2.6 x 10 ¹⁰	2.6 x 10 ¹⁰
3 rd month	1.5 x 10 ⁹	2.6 x 10 ⁹	2.5 x 10 ⁹	6.9 x 10 ⁹	2.3 x 10 ⁹	1.2 x 10 ¹⁰	9.8 x 10 ⁸	5.1 x 10 ⁸
6 th month	2.5 x 10 ⁸	2.0 x 10 ⁸	2.4 x 10 ⁸	4.4 x 10 ⁸	2.4 x 10 ⁸	1.8 x 10 ⁹	8.0 x 10 ⁷	6.6 x 10 ⁷
12 th month	1.0 x 10 ⁸	7.6 x 10 ⁷	1.4 x 10 ⁸	1.2 x 10 ⁸	1.4 x 10 ⁸	1.9 x 10 ⁸	6.3 x 10 ⁶	3.7 x 10 ⁶
<u>Rate of reduction (Rx)</u>								
Test period	R&D product II				Product V			
	PVC	PVC	PVC	PVC	PVC	PVC	PVC	PVC
	bottle I	bottle VI	bottle VII	bottle VIII	bottle IX	bottle X	bottle XI	bottle XII
	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(180 mL	(75 mL	(180 mL
	100 caps.	18 caps.	100 caps.	18 caps.	100 caps.	18 caps.	15 caps.	15 caps.
	1 g sil. gel)	1 g sil. gel)	3 g sil. gel)	3 g sil. gel)	5 g sil. gel)	5 g sil. gel)	1 g sil. gel)	1 g sil. gel)
3 rd month	1.52	1.29	1.30	0.86	1.34	0.62	1.42	1.70
6 th month	2.30	2.40	2.32	2.06	2.32	1.44	2.52	2.60
12 th month	2.70	2.82	2.55	2.62	2.55	2.42	3.61	3.84
✓	conform to specification (light yellow to light brown powder);							
X	non conform to specification (light yellow to light brown powder).							

Table 9. Composition of excipients

<u>Appearance</u>												
Test period	Original composition	Product V PVC bottle XI			Original composition	Product I Blister II			Original composition	Product III PVC bottle II		
		+ 5 mg Prosolv	+ 5 mg Sipernat	+ 5 mg N Zorbit		- 155 mg Maltodextrin + 125 mg N Zorbit	- 155 mg Maltodextrin + 125 mg Prosolv	- 30 mg Maltodextrin + 5 mg Sipernat		- 155 mg Maltodextrin + 125 mg Dextrose + 5 mg Sipernat	,	- 80 mg probiotic blend + 100 mg Maltodextrin
0 month	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3 rd month	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
6 th month	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓
12 th month	✓	✓	✓	✓	✓	✓	✓	✓	X			
<u>Moisture (%)</u>												
Test period	Original composition	Product V PVC bottle XI			Original composition	Product I Blister II			Original composition	Product III PVC bottle II		
		+ 5 mg Prosolv	+ 5 mg Sipernat	+ 5 mg N Zorbit		- 155 mg Maltodextrin + 125 mg N Zorbit	- 155 mg Maltodextrin + 125 mg Prosolv	- 30 mg Maltodextrin + 5 mg Sipernat		- 155 mg Maltodextrin + 125 mg Dextrose + 5 mg Sipernat	- 80 mg probiotic blend + 100 mg Vivapur 102	- 80 mg probiotic blend + 100 mg Maltodextrin
0 month												
3 rd month	4.41	4.25	4.80	4.14	3.23	5.88	3.50	3.47	4.03	3.52	4.10	4.18
6 th month	4.31	4.67	5.41	4.27	4.17	5.86	5.42	5.07	N/A			
12 th month	6.01	5.25	6.43	6.26	4.36	5.83	5.17	5.22	N/A			
<u>Total count probiotic microorganisms (CFU/caps.)</u>												
Test period	Original composition	Product V PVC bottle XI			Original composition	Product I Blister II			Original composition	Product III PVC bottle II		
		+ 5 mg Prosolv	+ 5 mg Sipernat	+ 5 mg N Zorbit		- 155 mg Maltodextrin + 125 mg N Zorbit	- 155 mg Maltodextrin + 125 mg Prosolv	- 30 mg Maltodextrin + 5 mg Sipernat		- 155 mg Maltodextrin + 125 mg Dextrose + 5 mg Sipernat	- 80 mg probiotic blend + 100 mg Vivapur 102	- 80 mg probiotic blend + 100 mg Maltodextrin
0 month	2.6 x 10 ¹⁰	1.9 x 10 ¹⁰	1.8 x 10 ¹⁰	2.2 x 10 ¹⁰	1.8 x 10 ¹⁰	1.8 x 10 ¹⁰	1.8 x 10 ¹⁰	1.8 x 10 ¹⁰	1.8 x 10 ¹⁰	1.8 x 10 ¹⁰	5.6 x 10 ¹⁰	5.7 x 10 ¹⁰
3 rd month	9.8 x 10 ⁸	1.4 x 10 ⁹	1.4 x 10 ⁸	1.4 x 10 ⁹	9.2 x 10 ⁸	1.3 x 10 ⁸	1.7 x 10 ⁹	1.2 x 10 ⁹	1.3 x 10 ⁸	4.2 x 10 ¹⁰	2.1 x 10 ¹⁰	2.8 x 10 ¹⁰
✓ conform to specification (light yellow to light brown powder); X non conform to specification (light yellow to light brown powder); N/A not applicable.												

Table 9. Composition of excipients

Total count probiotic microorganisms (CFU/caps.)												
Test period	Product V PVC bottle XI				Product I Blister II				Product III PVC bottle II			
	Original composition	+ 5 mg Prosolv	+ 5 mg Sipernat	+ 5 mg N Zorbit	Original composition	- 155 mg Maltodextrin + 125 mg NZorbit	- 155 mg Maltodextrin + 125 mg Prosolv	- 30 mg Maltodextrin + 5 mg Sipernat	- 155 mg Maltodextrin + 125 mg Dextrose + 5 mg Sipernat	Original composition	- 80 mg probiotic blend + 100 mg Vivapur 102	- 80 mg probiotic blend + 100 mg Maltodextrin
6 th month	8.0 x 10 ⁷	5.9 x 10 ⁷	8.3 x 10 ⁶	1.2 x 10 ⁸	3.5 x 10 ⁸	7.5 x 10 ⁶	8.6 x 10 ⁶	3.2 x 10 ⁷	3.5 x 10 ⁶	8.5 x 10 ⁹	1.1 x 10 ⁹	2.5 x 10 ⁹
12 th month	6.3 x 10 ⁶	5.8 x 10 ⁶	4.8 x 10 ⁵	4.6 x 10 ⁶						4.2 x 10 ⁸	2.9 x 10 ⁷	1.3 x 10 ⁸
Rate of reduction (Rx)												
Test period	Product V PVC bottle XI				Product I Blister II				Product III PVC bottle II			
	Original composition	+ 5 mg Prosolv	+ 5 mg Sipernat	+ 5 mg N Zorbit	Original composition	- 155 mg Maltodextrin + 125 mg NZorbit	- 155 mg Maltodextrin + 125 mg Prosolv	- 30 mg Maltodextrin + 5 mg Sipernat	- 155 mg Maltodextrin + 125 mg Dextrose + 5 mg Sipernat	Original composition	- 80 mg probiotic blend + 100 mg Vivapur 102	- 80 mg probiotic blend + 100 mg Maltodextrin
3 rd month	1.42	1.13	2.11	1.19	1.30	2.15	1.03	1.18	2.15	0.26	0.43	0.31
6 th month	2.52	2.51	3.34	2.26	1.72	3.38	3.33	2.75	3.72	0.95	1.71	1.36
12 th month	3.61	3.52	4.58	3.68						2.24	3.29	2.65
✓ conform to specification (light yellow to light brown powder); X non conform to specification (light yellow to light brown powder); N/A not applicable.												

by the high-density plastic bottle and an Alu/PVC blister with PVC foil composition PVC 60 µm / PE 60 µm / PVDC 33 µm. The Alu/PVC blister with single-layer PVC foil (60 µm) showed the worst protective properties.

The condition of the desiccant is essential for its efficiency. When silica gel is chosen, it must be dried and protected from environmental moisture until it is applied in the packaging of the probiotic product.

The gas composition in the container affects the stability of the probiotic product. Our research has shown that when atmospheric oxygen is displaced by nitrogen in the container, the properties of the product are better preserved.

The ratio between the number of capsules to be introduced into the bottle, the volume of the bottle, and the amount of silica gel determines the amount of moisture introduced into the package at time of manufacture, the free volume in the container, the contact area of the bottle through which environmental moisture will enter and the efficiency of the desiccant during storage. This ratio is highly dynamic and significantly affects the quality of the product. The results of our study defined the package with the best protective qualities as the one with the fewest capsules, respectively, more free volume, and the largest amount of desiccant, which can compensate for a longer time the moisture introduced in the bottle with the product at the time of

manufacture and the moisture constantly entering from the environment.

The presence of excipients in the capsule influences the stability of the probiotic product. The results of our study determined that the R&D product containing 1.27% Sipernat was more unstable compared to similar products containing the same amount of Prosolv and N Zorbit. The most unstable among another group of products was the R&D product containing 33.33% Dextrose in combination with 1.33% Sipernat, compared to a similar product containing the same amounts of Maltodextrin IT 19 and Sipernat, as well as N Zorbit (33.78%), Prosolv (33.78%), and Maltodextrin IT 19 (38.75%). The product containing 23.58% Vivapur 102 was found to be more unstable compared to a similar product containing the same amount of Maltodextrin IT 6. The results of the studied product groups highlighted Maltodextrin IT 6 and Maltodextrin IT 19 as excipients with the best protective properties. The review of the results of similar samples containing equal amounts of Prosolv and N Zorbit did not outline sustainable trends and did not allow us to identify the differences between them as clear and significant.

The results of the present study clearly showed that all monitored factors influence the stability of probiotic capsules.

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