

# Improving the stability of a probiotic product with *Lactiplantibacillus plantarum* 299v by introducing flow pack bags

Gabor I. Katona<sup>1</sup>, Davor J. Korčok<sup>1,2</sup>, Nada A. Tršić-Milanović<sup>2</sup> and Nataša M. Jovanović-Lješević<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy Novi Sad, Trg mladenaca 5, Novi Sad, Serbia

<sup>2</sup>Abela Pharm d.o.o., Viline vode bb, Belgrade, Serbia

## Abstract

Probiotic products are becoming more common in everyday use around the world, while at the same time, the interest of scientists in researching probiotic production and use is increasing. Stability of a probiotic product in pharmaceutical production is affected by the choice of probiotic strain, formulation, and packaging. Packaging is the final stage of production and presents a crucial factor for the stability of probiotic products to maintain declared probiotic viability during the products' shelf life. The present research describes the influence of additional packaging material on the encapsulated probiotic product, which contains *Lactiplantibacillus plantarum* 299v. In specific, the effect of additional blister protection within flow pack bags was investigated. Blisters were made of a chloride/polyvinylidene chloride/polyethylene-triplex foil (PVC/PVdC/PE foil) and aluminum foil. Viability of probiotic lactobacilli cells protected in blisters only was compared to those packed in flow pack bags filled with nitrogen as an inert gas. Better protection of probiotic cells from oxygen, light, and moisture was determined in the capsules in the latter case. In specific, introduction of additional blister protection in flow pack bags resulted in ~11 % higher probiotic viability when compared to the other blister samples without such protection after 24 months, and therefore it enabled more efficient storage of the product during use.

**Keywords:** quality of packaging; blisters; preservation of viability; packaging materials.

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## 1. INTRODUCTION

The increasingly widespread use of probiotic products worldwide is undoubtedly a result of proven, preventive and beneficial effects of probiotics on the improvement of numerous disorders, primarily in the gastrointestinal system [1]. Therefore, probiotic products are becoming more common in everyday use worldwide.

In recent decades, the most common definition of probiotics has been the one provided by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), according to which probiotics are: "Live microorganisms which, when administered in adequate amounts confer a health benefit on the host" [2].

Probiotics are microorganisms, bacteria and fungi, which contribute to the host's health by stimulating the growth of beneficial bacteria, suppressing pathogens by inhibiting their mucosal adhesion, and by stimulating production of antimicrobial agents [3]. Probiotics are used in food, as dietary supplements, and as medicines.

One of the most frequent probiotic strains in commercial use is *Lactiplantibacillus plantarum* 299v (*L. plantarum* 299v) [4], which is present in over 60 clinical studies on humans, proving its positive effects on relieving symptoms of irritable bowel syndrome (IBS) and contributing to iron absorption [5]. This strain is safe for human use (QPS list- Qualified Presumption of Safety) and it can survive in conditions of the human gastrointestinal tract by binding to mannose on epithelial cells [6-8].

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Corresponding authors: Davor J. Korčok, Abela Pharm d.o.o., Viline vode bb, Belgrade, Serbia

E-mail: [davor.korcok@abelapharm.rs](mailto:davor.korcok@abelapharm.rs)

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The use of probiotic microorganisms within pharmaceutical dosage forms is certainly more effective than the use in food because pharmaceutical products usually contain selected probiotic strains with proven positive effects in an optimal formulation and involve monitoring the viability of probiotic cells in clinically confirmed product doses. The therapeutic effect of probiotics is confirmed in a daily dose of  $10^8$  to  $10^{10}$  probiotic cells [7-9].

The final production phase of a probiotic product with the required number of microorganism cells is essential for preserving the selected strain's activity and, therefore, the product. This research describes the selection of the additional packaging material for encapsulated commercial probiotic products with the strain *Lactiplantibacillus plantarum* 299v. The effect of additional blister protection in flow pack bags was investigated. Blisters were made of a polyvinyl chloride/polyvinylidene chloride/polyethylene-triplex foil (PVC/PVdC/PE foil) and an aluminum foil and then such blisters were packed in flow pack bags filled with inert gas [10].

Based on the previous research on saccharomycetes, this paper aims to select the optimal packaging material for probiotics with lactobacilli, since these microorganisms are more affected by temperature. The number of lactobacilli probiotic cells has to be larger than that of saccharomycetes cells in the final product in order to produce a beneficial effect [10].

The stability of a probiotic product is primarily affected by light, moisture, and oxygen. Other factors that affect the survival of probiotics are temperature, pH value, storage time, water activity, other ingredients, and packaging material [11,12].

Oxygen is one of the key factors influencing the viability (activity of live strains) and stability of a probiotic strain, whether it is a bacterial strain or a yeast strain. Its effect is directly related to the technological process of production, which has to ensure the probiotic strain viability during all production phases as well as during the product shelf life [10,13]. Optimization of the technological production process aims to maintain the probiotic strain characteristics while adhering to all criteria that ensure its quality, bioavailability, and optimal therapeutic effects [6,14]. Prevention of the oxygen effects on the encapsulated probiotic strain provides a more stable probiotic product during its predicted shelf life and primary and secondary packaging materials have the crucial role in this attempt. Primary packaging consists of a material that is in direct contact with the product, while the secondary packaging material is an additional, protective packaging that preserves products packed in the primary packaging. Under the influence of oxygen, probiotic strains have reduced viability [15,16]. The information on the probiotic product declaration has to correspond to the product characteristics, which is the manufacturer's responsibility [17]. To maintain the probiotic viability, it is necessary to use a packaging material that acts as an absolute barrier to oxygen. This precondition is necessary, and this research aims to confirm that the choice of primary packaging material (primary product protection), which was used in this study, should result in the maximum protection for probiotic strains, in this case, lactobacilli. Thus, it was necessary to confirm that the selected packaging materials for blisters and flow pack bags of the encapsulated probiotic product Flobian® capsules will provide the appropriate protection so to meet the requirements for probiotic viability. The studies included investigations of viability and stability. To ensure the stability of the product and the perseverance of crucial characteristics of the probiotic strain, it is, firstly, necessary to limit the influence of oxygen. Therefore, this paper presents a hypothesis that an additional packaging material for blisters would provide greater stability to the encapsulated probiotic strain and, thus, greater efficiency during the shelf life of the product. With this aim, the influence of inert gas (nitrogen) as an additional factor that reduces the effect of oxygen on the probiotic product was also considered in this research.

## 2. EXPERIMENTAL

### 2. 1. Active ingredients, excipients, capsule

*Lactiplantibacillus plantarum* 299v (DSM 9483) was procured as a lyophilized powder from Probi AB (Probi AB, Lund, Sweden).

The following excipients were used: maize starch with 2 % moisture (UniPure FL, Germany), magnesium stearate (Magnesia, Germany), and colloidal silicon dioxide (Evonik, Belgium). For encapsulation, vegetable-origin capsules were used from the manufacturer ACG Lukaps, India.

Commercial dietary product Flobian® capsules were produced by using the listed active substances and excipients by filling capsules made of hydroxypropyl methylcellulose (HPMC) [20].

## 2. 2. Packaging material

A combination of PVC/PVdC/PE and aluminum foil was used as the primary packaging material, and the following samples were prepared:

1. Samples 1,2 (Flobian 1,2): blister combination of PVC/PVdC/PE foil and aluminum foil. Samples 1 and 2 represent technical replicates.
2. Sample 3 (Flobian 3): blister combination of PVC/PVdC/PE foil and aluminum foil enclosed in a flow pack bag filled with inert gas – nitrogen.

PVC/PVdC/PE foil had the following characteristics: PVC/PVdC/PE foil (polyvinyl chloride/polyvinylidene chloride/polyethylene-triplex foil) with the thickness of 383 µm.

For Sample 3, a secondary packaging, flow-pack foil, was used as an additional packaging material (also a triplex foil). This foil for flow pack bags in which the blisters were packed was made of PET/Al/TPE (polyethylene terephthalate/aluminum/transparent polyethylene).

Before sealing the flow pack bags, nitrogen as an inert gas, was injected to the flow pack bags.

## 2. 3. Production of capsules, blisters, and flow pack bags

A flow chart of the technological process for production of capsules and flow pack bags is presented in Figure 1.

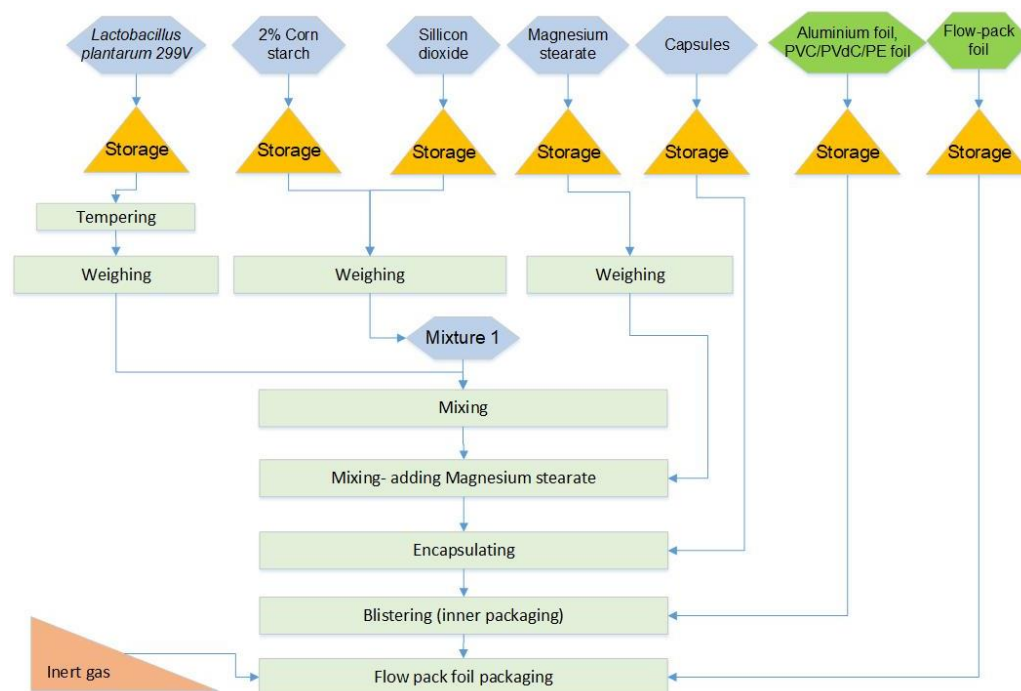


Figure 1. Flowchart of the production process of capsules, blisters, and flow pack bags of Flobian® samples

As presented in Figure 1, the production of capsules begins with weighing the active component and excipients. The active component was measured and dosed inside the capsule in such a way as to provide a minimum of  $30 \times 10^9$  lactobacilli cells in each capsule immediately after manufacturing. The excipients were weighed and mixed with the active substance, and the encapsulation mass was formed inside a cubical-type device (Omniprojekt, Serbia). The mass was encapsulated, *i.e.* divided into individual doses (capsules) on an automatic encapsulation machine (Macofar, Italy).

During the following production phase, the capsules were blistered in all tested samples by using a blistering machine (Uhlmann, Germany), which combines two foil types: a PVC/PVdC/PE foil and an aluminum foil. These foils were fused to form blister packs containing ten capsules each. The PVC/PVdC/PE and Al blister packs for the sample 3 were then packed within flow pack bags (Sigmaproces, Serbia) made of a PET/Al/TPE triplex foil and filled with nitrogen. Each flow pack bag contained 1 blister pack.

All capsule and blister production phases were performed in strictly controlled ambient conditions at temperatures of  $26 \pm 1$  °C and humidity levels of  $32 \pm 2$  %.

The Flobian® blister is shown in Figure 2, while the appearance of a pack bag is shown in Figure 3.



Figure 2. Front and back side of a standard Flobian® blister pack used in all samples



Figure 3. Flow pack bag with 1 Flobian® blister pack inside (Sample 3)

#### 2. 4. Analytical methods

The number of viable cells of the lactobacilli probiotic strain was determined in capsules within three investigated sample groups. In each sample group, lactobacilli were counted in 20 representative samples of blisters/flow pack bags.

Following the recommendations from the manufacturer, the probiotic strain *Lactiplantibacillus plantarum* 299v was cultivated in a MRS broth (Biokar Diagnostics, France) [18]. The cell count was determined by using the agar plate method (NMKL method, 2007, Probi). Probiotic strain samples in the form of powder (capsule content) were rehydrated in a sterilized purified water solution containing sodium-chloride 0.85 % w/v (Sigma Aldrich, USA), peptone 0.1 % w/v (Sigma Aldrich, USA), and then diluted in serial solutions. Aliquots ( $0.1 \text{ cm}^3$ ) of the last two diluted solutions were transferred to the agar plates (Sigma Aldrich, USA), which were then incubated under anaerobic conditions at 37 °C for

two days. After the incubation period, the colonies formed on the plates were counted. The results are presented as the number of colonies or CFU (colony forming units) per powder mass (*i.e.* the capsule content). The described procedure was performed three times, and each result presents the mean value of these three counts as recommended in literature [9,19].

This method of determining the number of lactobacilli was applied immediately after producing the commercial batches of the product Flobian® batch number 190793 for all the investigated samples (July 2019). Then the analysis was repeated at the end of the product shelf life, *i.e.* after 24 months (July 2021). Testing lasted longer than 24 months to confirm the product expiry date and determine whether it could be prolonged over 24 months. The total duration was 30 months, while the testing was performed at 24, 27, and 30 months. All the samples were stored at  $25\pm 2$  °C and at air humidity of  $55\pm 5$  % RH, which follows the prescribed storage conditions.

## 2. 5. Statistical analysis

Twenty blister samples in each group were analyzed (Samples 1 - 3). The Shapiro-Wilks test was used for the analysis of distribution normality. One-way ANOVA test and the post-hoc Tukey test was used for the inter-group comparison. The results for the three samples are expressed as the mean values  $\pm$  standard deviations of all the obtained analyses.

## 3. RESULTS AND DISCUSSION

To preserve activity of a commercial probiotic product, one of the most important strategies is to increase its low resistance to environmental and technological factors. In this sense, capsules can provide primary protection against environmental factors and represent a dosage form that is easy to swallow [15,16,20].

Packaging of commercial probiotic products with lactobacilli should preserve the cell viability by protecting the cells from external influences. Hydroxypropyl methylcellulose capsules were used in this research as the primary protective element for lactobacilli during product storage at room temperature as shown in literature to preserve viability of this microorganism [20].

Materials containing polyethylene are recommended for protection against moisture and oxygen [16].

This paper is a continuation of previous research, which confirmed that packaging of saccharomycetes probiotics in capsules packed in PVC/PVdC/PE blister packs inserted in flow pack bags is the optimal choice of packaging for that microorganism [10]. The introduction of additional blister protection in flow pack bags greatly contributed to preserving the probiotic viability. Therefore, it enabled better quality of the product before its opening during the product shelf life. As a confirmation of the hypothesis in the present work, samples of Flobian® capsules of the same production batch were divided into three groups and tested simultaneously. Two samples were 20 blisters each without flow pack bags (Samples 1 and 2), and one sample presented 20 blisters each packed in a flow pack bag (Sample 3).

For this commercial dietary product, the manufacturer has declared on the product packaging the required number of living lactobacilli cells, which is at least  $20\times 10^9$  per capsule for two years (shelf life).

The obtained viability results together with statistical analysis results are shown in Table 1, together with the changes in average numbers of viable cells over time for each sample group.

Table 1. Cell numbers per capsule in the three tested sample groups at four measurement times with the statistical analysis results

Sample	Mean cell number $\pm$ standard deviation, CFU $\times 10^9$ / caps				<i>P</i> <sup>@</sup>
	Immediately after production- Start	24 months after production	27 months after production	30 months after production	
Sample 1	31.4 $\pm$ 1.9	23.0 $\pm$ 1.7 <sup>aaa</sup>	18.5 $\pm$ 1.2 <sup>aaa,bbb</sup>	/	<0.001
Sample 2	33.5 $\pm$ 2.0	24.5 $\pm$ 1.8 <sup>aaa</sup>	19.2 $\pm$ 1.5 <sup>aaa,bbb</sup>	/	<0.001
Sample 3	32.6 $\pm$ 1.9	27.5 $\pm$ 1.6 <sup>aaa</sup>	25.5 $\pm$ 2.2 <sup>aaa,b</sup>	21.2 $\pm$ 2.9 <sup>aaa,bbb,ccc</sup>	<0.001
<i>P</i> <sup>&amp;</sup>	ns	< 0.001 1 vs. 3: <0.001 2 vs. 3: <0.001	<0.001 1 vs.3: <0.001 2 vs. 3: <0.001	/	/

Data are presented as means  $\pm$  standard deviations; *P* from one-way ANOVA test with posthoc Tukey test; ns - nonsignificant;

<sup>aaa</sup> *P* <0.001 vs. start; <sup>b</sup> *P* <0.05 vs. 24 months; <sup>bbb</sup> *P* <0.001 vs. 24 months; <sup>ccc</sup> *P* <0.001 vs. 27 months.

<sup>&</sup> difference between different samples in every study point; <sup>@</sup> difference between different times (study points) for any distinct sample





The lactobacilli numbers in the three samples immediately after production were similar amounting to about  $32 \times 10^9$  lactobacilli per capsule (in the range  $31.4\text{--}33.5 \times 10^9$  CFU/caps). The differences were not statistically significant. These results indicate that the production process of the encapsulated form of probiotic microorganisms corresponds to the defined technological procedure and that the targeted probiotic activity at the beginning of the shelf life in all three groups of samples is achieved (Table 1).

The stability study of Samples 1 and 2 showed that the number of lactobacilli decreased during the shelf life of 24 months yielding the survival rate of about 73 % without significant difference between the samples. Still, the final cell numbers of 23.0 to  $24.5 \times 10^9$  CFU/caps met the required, declared value of  $20 \times 10^9$  CFU/caps. Similarly, the number of lactobacilli in Sample 3 decreased during the shelf life but to a lower extent as compared to the other samples. The number of probiotic cells at the end of the shelf life (*i.e.*  $27.5 \times 10^9$  CFU/caps) was also above the declared value, and the survival rate was 84.4 %, which is for ~11 % higher when compared to the other samples after 24 months. Furthermore, this difference was found to be statistically significant,  $P < 0.001$ .

Thus, these results proved that the blisters packed in flow pack bags were protected significantly better from external ambient conditions as compared to blisters without this additional protective material resulting in significantly greater viability of the probiotic cells. Namely, the number of cells was significantly higher in Sample 3 as compared to Samples 1 and 2 at both time points: the 24<sup>th</sup> month and 27<sup>th</sup> month of the study (Table 1). Furthermore, after 27 months, the number of lactobacilli was below the declared number in Samples 1 and 2, whereas the number of lactobacilli cells in Sample 3 was above the declared value even after 30 months amounting to  $21.2 \times 10^9$  CFU/caps. Thus, the results presented in Table 1 confirmed the influence of the novel package on the lactobacilli number decrease retardation in comparison to samples in standard packages. Sample's 3  $P$  was  $< 0.05$  for the difference in the 27<sup>th</sup> month cells number, compared to both  $P$  (for Samples 1 and 2) which was  $< 0.001$  for the same study points comparison. This shows the beneficial effects of flow pack bags in lactobacilli cell protection, while the further research will be focused on evaluating these effects under conditions of potential exposure of the product to higher temperatures than the declared room temperature (for example under conditions of traveling).

Additional protection of the flow pack bags against light, moisture, and oxygen can be explained by the impermeable laminate foil that protects blisters and capsules from the exposure to light and moisture, while the inert gas within the bag further prevents penetration of oxygen to the product. Further research will focus on the individual impacts that light, moisture, and oxygen can have on the bacterial viability in the product.

#### 4. CONCLUSION

In the age of increasing use of probiotics, a more significant commitment of manufacturers is needed to preserve the cell viability in commercially available probiotic products. Preservation of viability is a prerequisite for achieving the probiotic action of the selected strain during the shelf life and product use. In addition to optimizing the production processes, one of the crucial factors in preserving viability is the choice of packaging material for the probiotic product.

In this paper, the initial hypothesis was that for the commercial probiotic product Flobian® containing *Lactiplantibacillus plantarum* 299v probiotic strain, it is beneficial to pack the blisters containing capsules within flow pack bags to better preserve the viability of the probiotic strain during the product use.

First it was proved that the probiotic capsules packed within a PVC/PVdC/PE foil blister pack and stored at room temperature during the shelf life, at the declared storage conditions, retained the declared number of viable probiotic cells per capsule. However, the viability of lactobacilli cells was slightly but significantly higher (~11 %) after 24 months when the blisters were packed within additional flow pack bags filled with the inert gas (*i.e.* nitrogen) compared to the samples without this type of packaging material. Moreover, due to the probiotic higher viability, prolongation of the product shelf life to 30 months when stored at or below 25 °C can be considered.

Optimization of the production process and packaging conditions of probiotics is a continuous and constant challenge for the pharmaceutical industry because it involves continuous monitoring and control of process parameters and the possible introduction of modern and improved packaging materials. This research study is a contribution in this direction.

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## Poboljšanje stabilnosti probiotskog proizvoda sa *Lactiplantibacillus plantarum* 299v uvođenjem laminatnih („flow pack“) kesica

Gabor Katona<sup>1</sup>, Davor J. Korčok<sup>1,2</sup>, Nada A. Tršić-Milanović<sup>2</sup> i Nataša M. Jovanović-Lješević<sup>1</sup>

<sup>1</sup>Farmaceutski fakultet Novi Sad, Trg mladenaca 5, Novi Sad, Srbija

<sup>2</sup>Abela Pharm d.o.o., Viline vode bb, Beograd, Srbija

(Stručni rad)

Izvod

U svakodnevnoj upotrebi širom sveta sve su zastupljeniji probiotski proizvodi. Paralelo s tim, i u stručnoj javnosti raste interesovanje za istraživanja njihove proizvodnje i primene. Stabilnost probiotskog proizvoda se u farmaceutskoj proizvodnji obezbeđuje izborom probiotskog soja, formulacije i izborom pakovanja. Pakovanje je završna faza proizvodnje i presudni je faktor za stabilnost probiotika radi održanja vijabilnosti u toku roka upotrebe. U ovom radu opisan je izbor dodatnog pakovanja kapsuliranog probiotika *Lactiplantibacillus plantarum* 299v. Ispitivan je efekat dodatne zaštite blistera u laminatnim kesicama (engl. *flow pack bag*). Odabrani su blisteri napravljeni od PVC/PVdC/PE (polivinil hlorid/poliviniliden hlorid/polietilen) folije i aluminijumske folije, koji su zatim paralelo ispitani u poređenju sa blisterima pakovanim u dodatne kesice punjene inertnim gasom. Poređenjem vijabilnosti probiotskih ćelija laktobacila u uzorcima blistera u kesicama u odnosu na dva uzorka blistera bez tih kesica uočena je bolja zaštita probiotskih ćelija od kiseonika, svetlosti i vlage u dodatnom pakovanju. Uvođenjem dodatne zaštite blistera u kesicama dobijena je za 11 % veća vijabilnost probiotika u poređenju sa uzorcima bez kesica posle 24 meseci što omogućava komformnije čuvanje proizvoda tokom upotrebe.

*Ključne reči:* Kvalitet pakovanja, blisteri, očuvanje održivosti, materijali za pakovanje