Improving the viability and stability of a probiotic product with *Saccharomyces boulardii* DBVPG

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**Abstract**

In recent decades, probiotic products have been increasingly used to prevent certain gastrointestinal and urogenital disorders, to improve the general condition of the body and as a supplement to pharmacological therapy. They are most often registered as dietary supplements, and less often as drugs in the form of capsules, powders, and solutions. Optimization of technological processes of production and packaging of these products aims to maintain probiotic characteristics while adhering to all criteria during production that ensure quality, bioavailability and optimal therapeutic effects. In this paper, the importance of choosing primary and secondary packaging materials was explored with the aim of preserving the viability of probiotic cells in capsules for two years, i.e. during the shelf life. By comparing the applications of polyvinyl chloride (PVC) and PVC/polyvinylidene chloride/polyethylene blister foils, better protection of probiotic cells was observed by applying the multilayer foil. In addition, in this research, further improvements of probiotic cell protection were achieved by applying a secondary packing-flow pack bag with inert gas for storing multilayered blisters.

**Keywords:** blister foil; flow pack bag; inert gas; dietary supplement; stability study.


1. **INTRODUCTION**

In the last several decades, probiotic products have been increasingly used for the prevention of certain gastrointestinal and urogenital disorders, to improve the general condition of the human body, and as a supplement to pharmacological therapy. They are most often registered as dietary supplements, and less often as drugs. They are produced as solid pharmaceutical forms (capsules), but also in the forms of powders and solutions.

In 2001, the definition of probiotics was introduced by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), formulated as: “live microorganisms, which when administered in adequate amounts confer a health benefit on the host” [1].

Probiotics are microorganisms - bacteria and fungi, which contribute to the health of the host by stimulating the growth of beneficial bacteria, suppress pathogens by inhibiting mucosal adhesion and stimulate the production of antimicrobial agents [2].

Probiotic products are formulated through a careful selection of strains with probiotic potential followed by strength standardization in order to produce a commercially viable product [3]. In order to obtain a high-quality, safe and effective probiotic product the following is required: an appropriate selection of documented and clinically proven probiotic strains as an Active Pharmaceutical Ingredient (API) and optimization of technological processes that will ensure the probiotic characteristics of the finished product. After the selection of strains, it is necessary to ensure that the therapeutic effect of probiotics is demonstrated in a daily dose of $10^8$ to $10^{10}$ probiotic cells [4].

Stability of a probiotic product is affected by light, moisture, and the presence of oxygen. Oxygen is one of the key factors that affect the viability (activity of live strains) and the stability of the probiotic strain, whether it is a bacterial...
or a yeast strain. The effect of oxygen is causally related to the technological process of production, which must ensure the viability of probiotic strains during all phases of the production process and during the shelf life [5].

Optimization of technological processes aims to maintain probiotic characteristics while adhering to all criteria during production to ensure quality, bioavailability and the optimal therapeutic effect of the product [3,6].

Preventing the effects of oxygen on the encapsulated probiotic strain allows a stable probiotic product to be maintained during its predicted shelf life. Packaging materials, both primary and secondary, have the most important role in preserving the encapsulated form of the probiotic strain from the harmful effect of oxygen, decreasing the viability of probiotic strains [7,8]. The information displayed on the declaration of the probiotic product has to correspond to the characteristics of the product, which is the responsibility of the manufacturer [9]. In order to maintain the probiotic viability, it is necessary to use such a packaging material that represents an absolute barrier to oxygen. This prerequisite is necessary, and this research confirms that the adequate primary packaging material (primary protection of the product) provides the maximum protection for probiotic strains, in this case yeast Saccharomyces boulardii. This strain was chosen because of its clinically confirmed probiotic properties. The aim of this study was to select an adequate packaging material for blisters and for the flow pack bags of the encapsulated probiotic product Bulardi that will fulfill the requirements of probiotic viability. Therefore, we have tested a hypothesis that it is necessary to choose the right packaging material for blisters and flow pack bags that will provide stability to the encapsulated probiotic strain, yeast, and thus improve efficiency during the shelf life of the product. For flow pack bags, the influence of inert gas as an additional factor that reduces the effect of oxygen on the probiotic product, was also considered.

2. Materials and Methods

2.1. Active ingredients and excipients

Saccharomyces boulardii DBVPG was purchased from Gnosis (Switzerland). Used excipients: magnesium stearate (Magnesia, Germany), corn starch (Ingredion, USA) and microcrystalline cellulose (Newgreen Pharmchem Co Limited, China). Hypromellose capsules, of plant origin, manufactured by Capsugel, France, were used for the encapsulation process.

2.2. Packaging material

Polyvinyl chloride (PVC) foil was purchased from Cetinka, Serbia, aluminium foil from Budis, Serbia, PVC/polyvinylidene chloride/polyethylene (PVC/PVdC/PE) foil from Perlen, Switzerland, and polyethylene terephthalate/aluminium/transparent polyethylene (PET/Al/TPE) foil from Comex, Serbia. A combination of a PVC foil and aluminium foil was used as the primary packaging material and the following samples were prepared:

a) Sample 1: blister combination of a PVC foil and aluminium foil;

b) Sample 2: blister combination of a PVC/PVdC/PE foil and aluminium foil;

c) Sample 3: blister combination of a PVC/PVdC/PE foil and aluminium foil sealed in a flow pack bag at atmospheric conditions;

d) Sample 4: blister combination of a PVC/PVdC/PE foil and aluminium foil sealed in a flow pack bag filled with inert gas (nitrogen).

Two types of primary (inner) packaging material were used for blisters:

a) PVC foil 25 μm thick and

b) PVC/PVdC/PE foil 383 μm thick.

For samples 3 and 4, secondary packing - flow-pack bags were used as an additional packing material for samples of capsules blistered in a PVC/PVdC/PE foil (triplex foil). A PET/Al/TPE foil was used for flow pack bags in which samples 3 and 4 were packed. There was only one difference between the samples 3 and 4: inert gas (>99.999 % nitrogen) was injected instead of air, while closing the flow pack bag of the sample 4.

2.3. Production of capsules, blisters and flow pack bags

The technological process of making capsules, blisters and flow pack bags is shown in Figure 1.
The production of capsules begins with the weighing of the active substances and excipients. The active component is measured in such a way as to provide a minimum of $5 \times 10^9$ yeast cells in each capsule. The excipients are weighed, mixed with the active substance, and an encapsulating mass is formed in a cubical type of mixer. The mixture is divided into individual doses - capsules by using an automatic encapsulation machine (Macofar, Italy).

The capsules are blistered in the next phase by using a blister machine (Uhlmann, Germany), which combines two types of foils: PVC and aluminium foil (sample 1) and PVC/PVdC/PE and aluminium foil (samples 2, 3, 4). During blistering, the capsules are placed inside the formed depressions and both foils are fused together to form blisters containing 10 capsules each. The PVC/PVdC/PE and Al blisters for preparation of the sample 3 were packed inside flow pack bags, and for the sample 4 the PVC/PVdC/PE and Al blisters were packed in flow pack bags with injected inert gas.

All phases of production of capsules and blisters are performed in strictly controlled ambient conditions: $22 \pm 2$ °C temperature and $30 \pm 5$ % humidity. Using the described production procedures, samples were prepared for analysis. A Bulardi blister with ten capsules each as well as a flow pack bag are shown in Figure 2.

Figure 1. Flowchart of capsule and blister production of probiotic product samples containing Saccharomyces boulardii DBVPG

Figure 2. Photographs of a) blister with sample capsules (top and bottom); b) flow-pack bag with sample blisters (for samples 3 and 4)
2.4. Counting viable probiotic yeast cells

The number of viable cells of probiotic yeast was determined in capsules from all four samples (3 capsules per sample). The number of yeast cells was determined in the microbiological laboratory by the method of decimal dilutions and then the mean values of the three counts were determined using the following method.

The initial sample was one gram of the active substance from the capsules dissolved in the nutrient medium Tripton soy broth (Himedia, India) in order to obtain the initial dilution of $10^{-2}$, and then $1$ cm$^3$ of this suspension was diluted by decimal dilutions in peptone water (Himedia, India) to the final dilutions of $10^{-8}$ and $10^{-9}$.

An aliquot ($1$ cm$^3$) of the final dilutions was placed in yeast glucose - chloramphenicol agar (YGC agar, Himedia, India) as a nutrient medium for the cultivation of yeast $S. boulardii$. The nutrient agar with the aliquots was incubated in Petri dishes at $33 \pm 1$ °C for $48$ h. After incubation, colonies (CFU-Colony forming units) were counted and the mean final number of yeasts per capsule (CFU/capsule) was calculated from the two final dilutions. This procedure was repeated for all analyzed samples.

This method of determining the number of yeast cells was applied immediately after the production of all four samples. The samples were then stored at a temperature of $25\pm2$ °C and air humidity of $55\pm5$ % RH and this procedure was repeated at the end of the shelf life, i.e. after 24 months.

2.5. Statistical analysis

The data were analyzed using one-way ANOVA followed by Tuckey’s multiple comparisons tests for means comparison between the groups. Data are expressed as a mean ±SD. A $p$-value <0.05 was considered statistically significant. All statistical analyses were performed by using the IBM SPSS version 24 (SPSS Inc., USA).

3. RESULTS

In accordance with the mentioned technological aspects, we have derived the hypothesis that for the formulation of $5\times10^9$ living cells of $Saccharomyces boulardii$ per capsule, i.e. for the recommended daily probiotic dose in one capsule, the packaging material in blisters and flow pack bags should be properly selected in order to ensure the preservation of probiotic viability during the product shelf life. The requirement is that the declared number of living cells ($5\times10^9$ CFU/caps) has to be preserved until the end of the products shelf life by using the adequate packaging material that will be an effective protection for living cells. The content (the number of $Saccharomyces boulardii$ DBVPG 6763 yeast) was determined in four different primary packaging materials in blisters immediately after the production of samples and after 24 months (the expiry date) stored at the temperature of $25\pm2$ °C and air humidity level of $55\pm5$ % RH. The results are presented in Table 1.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Probiotic yeast cell number $\times 10^9$, CFU / capsule</th>
<th>Survival, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immediately after production</td>
<td>24 months after production</td>
</tr>
<tr>
<td>Sample 1</td>
<td>14.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>12.4</td>
<td>0.26</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>13.3±0.85</td>
<td>0.22±0.11</td>
</tr>
<tr>
<td>Sample 2</td>
<td>14.5</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>13.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>13.8±0.66</td>
<td>5.3±1.01</td>
</tr>
<tr>
<td>Sample 3</td>
<td>15.2</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>13.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>14.4±0.85</td>
<td>6.8±0.95</td>
</tr>
<tr>
<td>Sample 4</td>
<td>14.8</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>13.6</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
<td>7.7</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>14.3±0.62</td>
<td>7.2±0.76</td>
</tr>
</tbody>
</table>
Values with different superscript letters inside the column of the Table 1 with the results after 24 months are significantly different. $p < 0.05$ (ANOVA followed by the post hoc Tukey's multiple comparison tests).

At the start of the shelf life, the analysis of the number of living cells in all tested samples has shown around $13\times10^9$ cells per capsule (Table 1), which was in accordance with the predefined content of probiotic yeasts. The obtained results from all tested samples indicate that the manufacturing process of encapsulated probiotic, containing yeast, confirms to the defined technological procedures and to the prescribed requirements of the probiotic viability at the start of the shelf life.

The analysis of all four samples at the end of the stability study (after 24 months) revealed that the number of yeasts during the shelf life in the three-layer PVC/PVdC/PE foil was higher than that in the blister of PVC foil indicating better protection in the first case. With the application of this multi-layered foil as a primary packaging material, the viability of probiotic cells was preserved by 38.3% in relation to the preserved 1.7%. using the single-layered PVC foil (Table 1).

After the testing of blister samples 3 and 4, it was proven that blisters in flow pack bags were better protected, and the probiotic viability was significantly higher than in the blisters left without flow pack bags (samples 1 and 2, Table 1). In this research, the highest viability of probiotic cells, in relation to the start was shown with the use of a multilayer PVC foil (PVC/PVdC/PE) packed within a flow pack bag filled with nitrogen as an inert gas (50.3%) (Table 1).

Thus, the combination of flow pack bags together with the multi-layered blister material provided additional protection to the product in blisters.

The obtained result of 50% viability retention of *S. boulardii* probiotic is satisfactory. In this research, the hypothesis was that the probiotic cell survival could be increased if oxygen and moisture were eliminated from the cell environment, which was confirmed in Sample 4. The authors did not find comparable scientific data in literature in terms of preserving the viability of *S. boulardii*. Nitrogen, as an inert gas, was used in Sample 4 in order to further eliminate the presence of oxygen from the atmosphere in flow pack bags and consequentially, from the contents of blisters and capsules. The inclusion of additional packaging materials as well as the inert gas in the production of probiotic products slightly increases the price, but it contributes greatly to the stability and viability of probiotic microorganisms and increases the market competitiveness. Flow pack bags that were not filled with nitrogen, as well as blisters which were made from a multilayer PVC foil also met the specified requirements of the cell number (more than $5\times10^9$ cells per capsule), but the largest number of cells was observed in Sample 4 with the addition of nitrogen. Thus, the best combination of blister packaging materials to preserve the viability of the probiotic strain *Saccharomyces boulardii* is a three-layer PVC/PVdC/PE blister inside flow pack bags, which ensures that the number of living yeast cells does not fall below the declared $5\times10^9$ yeast cells per capsule until the expiry date.

### 4. CONCLUSION

The increasing use of probiotic products obliges manufacturers to carefully choose an effective probiotic strain and to optimize production parameters in order to preserve the viability of probiotic cells. The pharmaceutical production of probiotics thus satisfies the needs of users for a safe and effective probiotic product in a comfortable encapsulated form that can be stored at a temperature of up to 25°C.

This research confirms the importance of the application of proper packaging materials to preserve the activity of the chosen probiotic strain. The initial hypothesis was proven that the primary packaging blister material PVC/PVdC/PE in combination with additional protection in the form of flow-pack bags preserve the viability of probiotic cells in capsules for two years during the shelf life. Better protection is noticeable in the case of application of PVC/PVdC/PE blister foil as compared to the PVC blister foil because it probably provides a better barrier to moisture and oxygen from the environment.

The effects of preserving the viability of probiotics by protection against external conditions were enhanced with the use of additional laminate foil-secondary packaging in the form of a flow pack bag. This research confirms that the flow pack bag additionally protects the blisters from external influences, with the best results being obtained by combining PVC/PVdC/PE blisters inside flow pack bags filled with the current of inert gas instead of air.
The process of optimizing the conditions of production and packaging of probiotics is a continuous process and a constant challenge for pharmaceutical production because it involves constant monitoring and control of process parameters as well as examination and introduction of improved, modern packaging materials.

REFERENCES


SAŽETAK

Unapređenje vijabilnosti i stabilnosti probiotskog proizvoda sa *Saccharomyces boulardii* DBVP

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(Sručni rad)

Probiotski proizvodi se poslednjih decenija sve više koriste za prevenciju određenih gastrointestinálnih i urogenitalnih poremećaja, za poboljšanje opšteg stanja organizma i kao dopuna farmakološke terapije. Najčešće su registrovani kao dijetetski suplementi, a ređe kao lekovi u obliku kapsula, praškova i rastvora. Optimizacija tehnoloških procesa proizvodnje i pakovanje ovih proizvoda ima za cilj održavanje probiotskih karakteristika uz istovremeno pridržavanje svih kriterijuma u toku proizvodnje koji osiguravaju kvalitet, biološku raspoloživost i optimalno terapijsko dejstvo U ovom radu razmatran je značaj izbora primarnog i sekundarnog materijala za pakovanje sa ciljem očuvanja vijabilnosti probiotskih ćelija u kapsulama tokom dve godine tj. tokom roka upotrebe. Poređenjem primene PVC (polivinil hlorid) i PVC/poliviniliden hlorid/polietilen (PVC/PVdC/PE) blister folija uočena je bolja zaštita probiotskih ćelija od kisenika i vlage primenom višeslojene folije. Dodatne efekte zaštite probiotskih ćelija u ovom radu potvrđene su primenom dodatne zaštite blistera upotrebom sekundarnog pakovanja (engl. *flow pack*) kесica sa inertnim gasom.

**Ključne reči:** blister folija; „flow pack” kесica; inertni gas; dijetetski suplement; studija stabilnosti