Low moisture starch for improved viability and stability of new probiotic *L. plantarum* 299v preparation

Davor J. Korčok*, Olivera Čolić, Nada Tršić-Milanović, Bogdan Mitić

Abela Pharm d.o.o., Viline Vode b.b., Belgrade, Serbia

Abstract

Probiotic pharmaceutical preparations are more and more popular because of the increasing level of evidence of their beneficial effect on human health. The goal of this study was to determine and develop the encapsulated probiotic formulation with the optimal filling amount of active ingredient - *Lactobacillus plantarum* that would, in combination with other active ingredients: iron, vitamin C and excipient starch, fulfil requirements for therapeutic action while maintaining process parameters' requirements of manufacturing as well. The optimal formulation of a multicomponent probiotic-based formulation that fulfils requirements for sustaining all active ingredients while respecting technological process requirements, will enable a routine pharmaceutical manufacturing that could yield both efficient and safe dietary products.

Keywords: *probiotic product;* Lactobacillus plantarum*; iron; vitamin C; starch*.

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1. INTRODUCTION

We have been witnessing for the past few decades a growing trend in probiotic use. Following pharmaceutical forms are used in the production of probiotic food supplements: capsules, powders, drops etc. In the pharmaceutical industry, the most prevalent are capsulated probiotics marketed as dietary supplements. Multidisciplinary teams are tasked with connecting requirements of optimal formulation that will benefit the host's flora with the technological demands in probiotic manufacturing process.

According to the definition from the World Health Organisation (WHO), probiotics represent live organisms that, if applied in optimal amounts, contribute to human health [1]. Probiotics are microorganisms - bacteria and yeast that contribute to the host's health by stimulating the growth of beneficial bacteria, suppressing pathogens via inhibition of mucosal adherence and production of antimicrobials [2].

Microorganisms with probiotic potentials are usually from the genus of Lactobacilli, Bifidobacterium and yeasts [3,4]. Probiotic products are formed via careful selection of strains with the probiotic potential, and dosage standardization process in order to create a commercially viable product [4].

Requirements for a successful probiotic product are a selection of well documented and clinically proven probiotic strain as an Active Pharmaceutical Ingredient (API) and the optimisation of technological procedures that will ensure probiotic characteristics of the probiotic product.

The next three key factors that determine probiotic action are: viability, dose and influence on the human health. First two factors share a direct link with the technological procedure used that must ensure probiotic viability during all stages of manufacturing, during product storage, its shelf-life and its transit through the gastrointestinal tract of the patient [3]. Technological process optimisation has as its goal maintaining probiotic characteristics while adhering to all criteria of a pharmaceutical manufacturing process that ensures quality, bioavailability and optimal therapeutic action [3,5].

Optimization of the manufacturing process of probiotic capsules is always followed after the analysis of the water activity (water activity - aw) of all components. Water activity is a requested parameter of analysis for probiotic formulations because it is proportional to the free water, which is a potential source of chemical instability and microbiological contamination of raw materials and also the possible source of decreasing viability of probiotic cells [6]. For the purpose of sustaining probiotic viability, it is necessary to use all components exhibiting low water

Correspondence: Davor J. Korčok, Abela Pharm Ltd., Viline vode bb, 11000 Belgrade, Serbia
E-mail: davorkorcok@abelapharm.rs
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activity. The knowledge of moisture interactions with APIs is crucial for better understanding of the manufacturing process and the prediction of the product's stability and its shelf life. The use of excipients that bind free-water from the capsule, reduces its potential interaction with API [7]. The viability of dried *Lactobacillus* probiotic is less dependent on the excipients, but strongly dependent on the water activity [8]. This precondition is necessary because the literature states and this research confirms that the maximal level of water activity in probiotic capsules is ≤ 0.25. The aw value greater than 0.536 is critical, because that is the point when *Lactobacillus* probiotic cells degrade more quickly [9,10].

In addition to the active components, it is also important to select right excipients, which support probiotic activity in finished product formulation (the most frequent choice is usually maize starch) [11,12].

Another goal of this study was to test technological aspects of multicomponent probiotic formulation design - *Lactobacillus plantarum*, iron and vitamin C (enables improved iron absorption) that will be beneficial in balancing the gut microflora, prevention or treatment of mild cases of iron deficiency in population suffering from low iron absorption rate.

Also, the goal was to determine optimal probiotic formulation that will fulfil probiotic viability requirements, as well as a repeated process of probiotic product manufacturing regarding the used technological steps [13]. Probiotic product development includes viability and stability studies [14]. However, to ensure realisation of the key probiotic product parameter that is viability, the most influencing factor is the presence of minimal amount of moisture in raw material and a low ambient humidity during each stage of capsule production (aw value is requested to be ≤ 0.25 in probiotic capsules and ambient humidity during the manufacture process ≤ 35%) [8,9,10]. A constructed hypothesis showed the necessity for low-moisture starch use in optimal probiotic formulation, and to prove it, two different kinds of starch were used.

2. MATERIALS AND METHODS

2.1. Active ingredients

Lactobacillus plantarum 299v (DSM 9483) is procured as a lyophilized powder form from Probi AB (Probi AB, Lund, Sweden). This strain is chosen due to its confirmed probiotic traits as well as its influence on enhancement of iron absorption [15,16]. The requested aw value for this probiotic component is ≤ 0.04.

A sucrosomial iron formulation acquired from Alesco S.R.L. Italy was used in this study; a form with high bioavailability and without irritating effects on the gastrointestinal tract. Vitamin C was acquired from Shandong Luwei Pharm Co. China. The requested aw value for this active component is ≤ 0.04.

Three formulations were defined: A) Formulation 1 consisted of 50 mg of lyophilized *L. plantarum* 299v powder, 10 mg of sucrosomial iron and 15 mg of vitamin C per capsule; B) Formulation 2 consisted of 80 mg of lyophilized *L. plantarum* 299v powder, 10 mg of sucrosomial iron and 5 mg of vitamin C per capsule; C) Formulation 3 consisted of 100 mg of lyophilized *L. plantarum* 299v powder, 10 mg of sucrosomial iron and 15 mg of vitamin C per capsule. The requested aw value for all three formulations- capsulated mass samples were ≤ 0.07. In case this request is fulfilled it is possible to obtain the aw value ≤ 0.25 in the finished product- capsule.

Blistering process was performed on all three formulations immediately after the capsulation process finished, enclosing the capsules into a PVDC/aluminium foil in order to fully protect them from the outdoor environment - air, light and humidity. During sampling, chosen blisters were opened and capsules from within were analysed: determining the number of probiotic cells, mass variations and water activity.

2.2. Excipient and capsules

For test samples of formulations, a maize starch with up to 5 % moisture was used (Roquette, France). Another type of maize starch with 2 % moisture (UniPure FL, Germany), was used as an excipient in all analysed encapsulated formulations. Both starch types containing low percentage of moisture were chosen in order to reduce the influence of moisture on the probiotic cells inside formulations. Test samples labelled PU1-5%S, PU2-5%S and PU3-5%S for all three formulations were prepared with the starch containing up to 5 % moisture, while samples with starch that contains 2 % moisture for all three formulations were labelled as: U1-2%S, U2-2%S and U3-2%S.

For the capsule filling process, hypromellose based capsules of herbal origin were chosen, from the manufacturer Capsugel, France. These capsules are suitable for moisture sensitive products [17]. Capsule size was chosen to be number 1.

2.3. Manufacturing of capsules and blisters

Technological processes of capsule and blister pack manufacturing are shown in Figure 1.

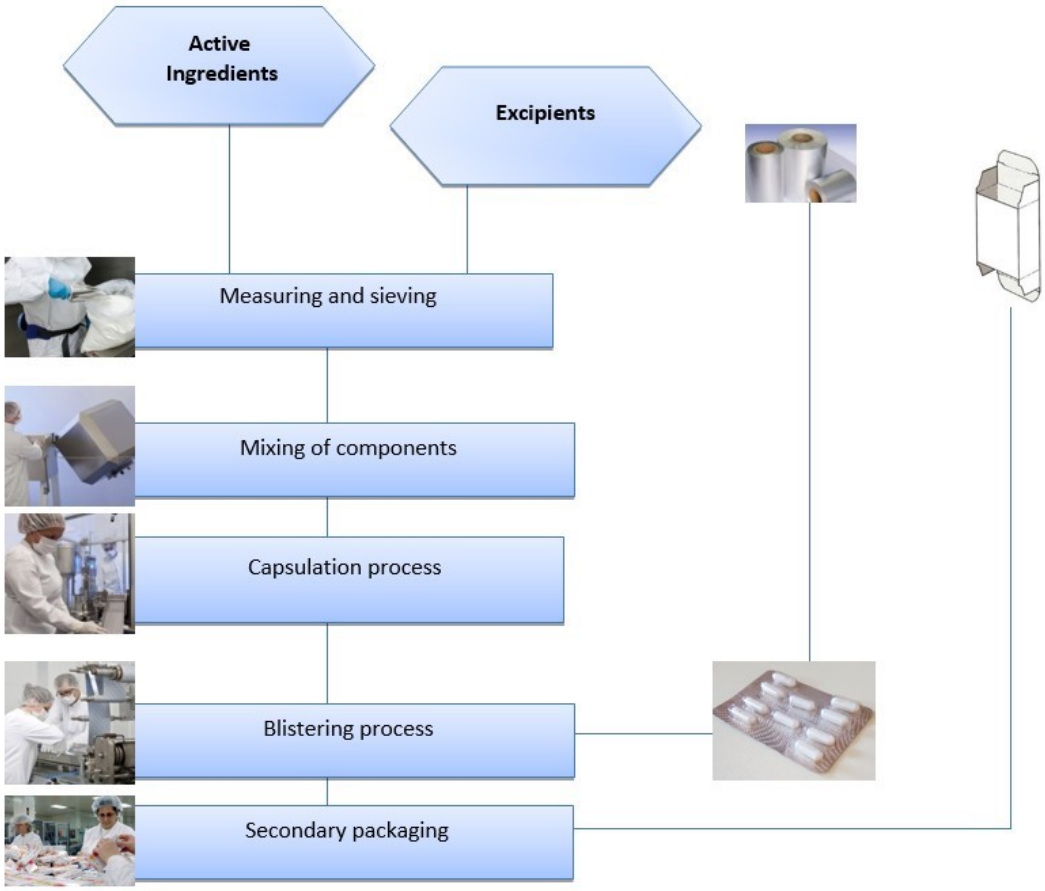

Figure 1. Manufacturing process of capsules and blisters of the probiotic product containing Lp 299v and iron

The chosen active substances and excipient were measured, mixed together in the mixing machine and the mass for capsulation was formulated. The mass was then encapsulated using an automatic powder-capsule-dosing machine (Macofar, Italy).

Next, the capsules were put into the blistering process using the machine (Uhlmann, Germany) that connects two types of foil: PVDC and aluminium foils. The PVDC foil passes through the forming stations. The capsules were placed inside the formed indentations and both foils attached together to form blisters containing 10 capsules each.

All stages of production and capsule blistering were conducted in strictly controlled environment: 22 ± 2 °C for temperature, and 30 ± 5 % for air humidity.

By using the described manufacturing procedures first test samples were prepared- test samples containing starch with 5% moisture (PU), and test samples containing starch with 2 % moisture (U).

2.4. Blisters with capsules

Immediately after capsulation process was performed, all three formulations were blister packed into PVDC/aluminium foil (10 capsules each), as described above, in order to fully protect capsules from external conditions- air, light and humidity. Chosen blisters were opened and capsules analysed for probiotic cell count, during sampling.

2.5. Technological procedure for encapsulated formulation manufacturing

Manufacturing of probiotic encapsulated formulation was performed in three stages: measuring and mixing of components, capsulation process and blistering process as described above. Blisters containing capsules were packed together with leaflets, inside individual cardboard boxes.

2.6. Viable probiotic cell count

Following the recommendations from the manufacturer, the probiotic strain *Lactobacillus plantarum* 299v was cultivated inside MRS broth (Biokar Diagnostics) [18]. Probiotic cell count was determined using *agar plate* method

(NMKL method, 2007, Probi). Probiotic strain samples in the form of powder (capsule content) were rehydrated inside sterilized sodium-chloride solution (Sigma Aldrich, USA), peptone (Sigma Aldrich, USA) and purified water and then diluted in serial solutions. Sampling was performed by using a pipette, the aliquot of 0.1 ml from the last two diluted solutions and agar plates were covered with these solutions. The agar plates were incubated under anaerobic conditions, at the temperature of 37 °C for two days. After the incubation period, colonies formed on the plates were counted and the results are presented as colony forming units or CFU, per powder mass or the capsule content. The above-mentioned procedure was performed on all three counting tests and the results are shown as mean values of the three counts.

2.7. Minimum moisture request

During production and storage of probiotic products, it is necessary to assure minimal amounts of moisture in used raw materials, as well as humidity in the work environment. In the case of active components with aw value ≤0.04 and if the moisture content in starch equals 2 %, the production goal is achieved that is to yield capsules with nominal water activity level not exceeding 0.25 [8,9,10].

2.8. Stability of the encapsulated formulation

Samples for three selected formulations labelled U1, U2 and U3 were placed into a set of conditions for stability testing. Condition set scheme for determination of stability using microbiological analysis was taken from the requirements for long term stability testing proposed by EMA, listed in the available sources [19,20]. Testing was performed in real time set of conditions, and at the temperature of 25±2 °C, with a relative humidity of 60 ± 5 %. Stability testing of the three formulations' samples was done in prearranged and defined time intervals.

2.9. Capsule mass measuring

Capsule mass measuring was performed using Pioneer PA214CM analytical scales (Ohaus Corp., USA). A mean value was determined for capsule mass by the measurements of three samples. The maximal allowed variation is ± 7.5% from the mean powder mass inside the capsules (320 mg) according to Ph. Eur 8.0 Vol. [21, 22].

2.10. Water activity determination inside encapsulated samples

Water activity was determined using LabSwift Novasina measuring device (Novasina, Switzerland). A mean value for mass of 20 capsules was determined for all three formulations, the samples labelled -U, by measurements of three samples.

3. RESULTS

In accordance to mentioned technological aspects, a hypothesis was derived for a formulation which ensures a recommended daily probiotic dose in just one capsule (10^9 CFU/day), that also contains recommended micronutrient doses- of iron and vitamin C and maize starch which supports the product viability [12,23]. Another goal of this study was to create encapsulated product containing optimal number of probiotic cells, with tolerable mass variations and minimal water activity, produced in conditions of optimal technological aspects that maintain required parameters during its shelf life. The results of other studies have been confirmed which state the aw value ≤0.25 as a criterion for preservation of probiotic viability [9]. Stability study was conducted to meet the requirement of a minimum 2 years shelf-life formulation, which will maintain its probiotic stability for its entire shelf-life.

An optimal quantity of the active substance in the formulation was chosen- a lactobacilli strain *Lactobacillus plantarum* Lp 299v in the amount of 50 mg/caps., with a nominal water activity value of 0.15 during the shelf life.

Due to unsatisfactory results for water activity in test samples with starch with moisture content of 5 % (PU samples), another samples were prepared using starch containing up to 2 % moisture (U samples), which were additionally tested in stability studies.

The course of stability testing indicated that the chosen formulation maintained a viability which equalled 10^9 CFU/caps., and showed a relatively small increase in water activity value after 24 and 30 months (from the nominal value of 0.15 after 24 months and up to 0.19 per capsule at the end of the study).

3.1. Determining water activity in the chosen formulation

First stages of the study included testing of water activity inside samples so that newly manufactured capsule samples that were filled with different quantities of the active ingredient Lp 299v (50, 80 and 100 mg Lp299v) should not exceed the value of 0.25.

Table 1 presents the results of water activity determination in capsule samples.

Table 1. Water activity (aw) in newly manufactured capsule samples containing starch with 5 % moisture content -PU and samples with starch containing 2 % moisture-U

Sample label	50 mg Lp299v aw value	80 mg Lp299v aw value	100 mg Lp299v aw value
PU- 5 % moisture starch	0.031	0.032	0.031
U- 2 % moisture starch	0.015	0.015	0.017

Formulation that contained starch with 2 % moisture (U) has fulfilled the requested aw value and therefore this formulation was chosen for further water activity, viability and stability testing.

For the water activity and sample viability, a time period of sampling was chosen as follows: for the first 6 months- monthly sampling, after first 6 months- one sampling every 6 months until the completion of the study (Figure 2).

Water activity testing in all three formulations has shown that the lowest value of water activity was for the sample containing 50 mg of API. Also, water activity value was measured to be 0.15 after 2 years of stability testing, 0.19 after 30 months, which both satisfy the condition ≤ 0.25 aw.

3.2. Probiotic cell viability testing

Samples with the chosen formulation U, having starch with 2 % moisture content were tested for viability in three groups of samples- containing 50, 80 and 100 mg Lp 299v over the period of 30 months (Figure 3).

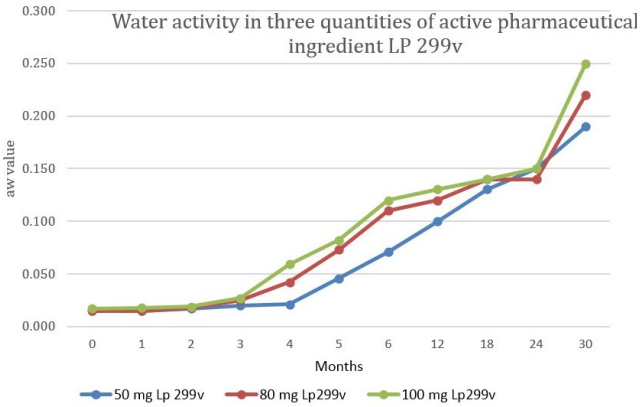

Figure 2. Water activity (aw) in capsule samples with starch containing 2 % moisture-U, during long term stability testing on 25 ± 2°C and relative air humidity of 60±5 %

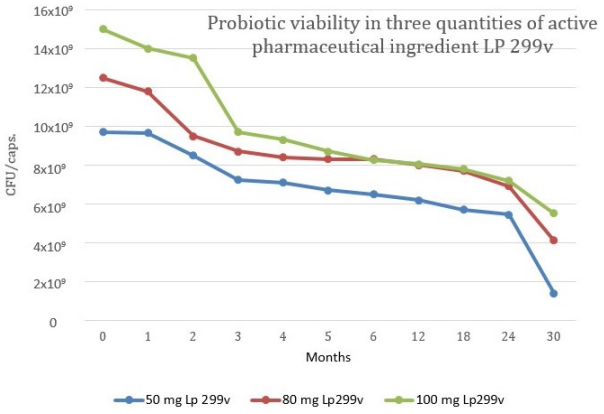

Figure 3. Probiotic viability during the course of stability testing at the temperature of 25 ± 2 °C and relative air humidity of 60±5 %

3.3. Mass variation in three formulations of capsule samples

During optimisation of the chosen parameters, 20 capsule samples were examined for mass variations for all three subgroups of the U formulation. The results are shown in Figures 4 and 5. The results have shown that mass variations in all three formulations complied with specified limits for encapsulated dosage products according to the European Pharmacopoeia [21].

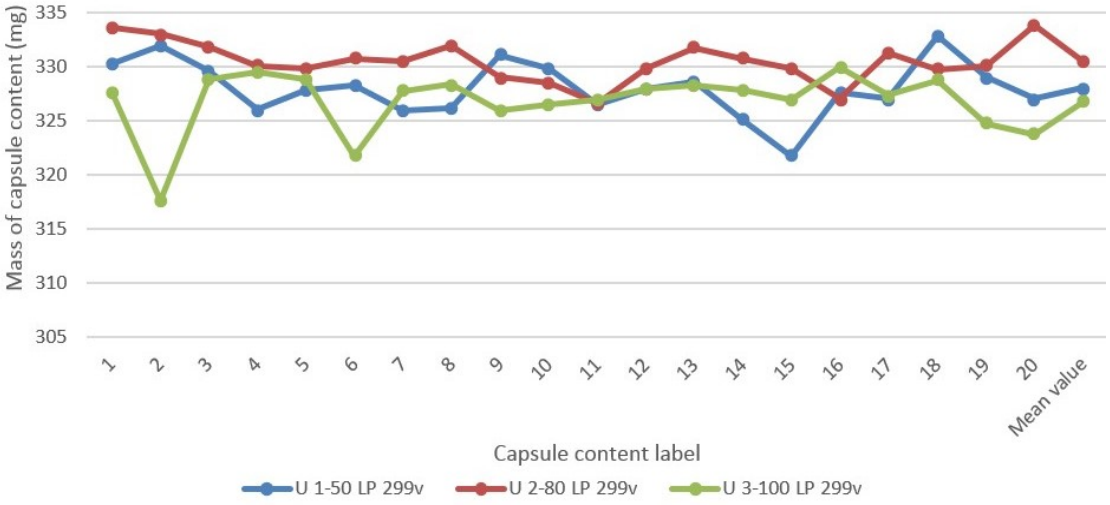

Figure 4. Mass variations in the U capsule content for three formulations (50, 80 and 100 mg Lp299v)

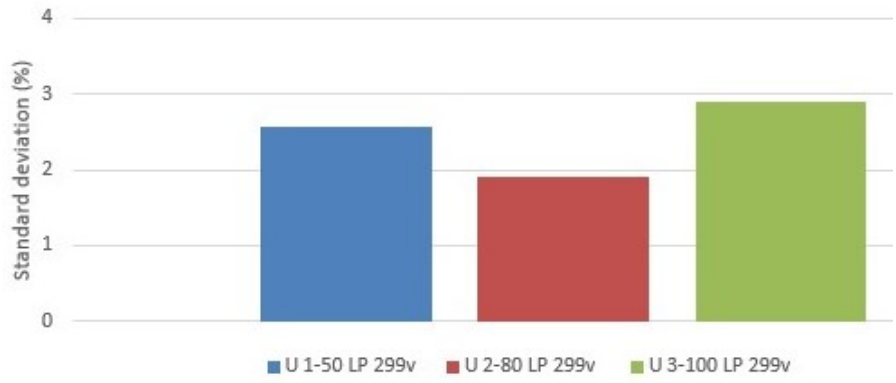

Figure 5. Standard deviations for mass variations. % in the U capsule content for three formulations (50, 80 and 100 mg Lp299v)

4. CONCLUSION

In this work, we have shown formulation and process parameter optimization during the production of an encapsulated probiotic product. A hypothesis was postulated meaning that the amount of used micronutrient did not vary-vitamin C and iron, while the amount of lactobacilli varied, together with the moisture content in starch (5% and 2% of moisture content).

Excipient optimization led to the choice of starch containing 2 % moisture. It showed that a minimal active ingredient quantity of 50 mg/caps maintained the therapeutic action while sustaining basic requested parameters - mass uniformity and the water activity value. Mass uniformity represents a measure of sample homogenization, while low water activity value represents the product prerequisite for stability over time.

The course of stability testing indicated that the chosen formulation maintained a viability which equalled to 10^9 CFU/caps, and showed a relatively small increase in water activity value after 24 and 30 months (from the nominal value of 0.015% up to 0.19% per capsule at the end of the study).

The addition of micronutrients- Vitamin C and iron to the probiotic strain has improved the therapeutic action while maintaining the required stability of the probiotic culture during the study period. Above mentioned methods of analysis for both the formulation and manufacturing parameters of encapsulated probiotic products have practical and theoretical values. The next research direction is confirmation of the clinical action for the chosen formulation.

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SAŽETAK

Skrob sa niskim sadržajem vlage za unapređenje vijabilnosti i stabilnosti novog probiotskog preparata sa *L. plantarum* 299v

Davor J. Korčok, Olivera Čolić, Nada Tršić-Milanović, Bogdan Mitić

Abela Pharm d.o.o., Viline Vode b.b., Beograd, Srbija

(Stručni rad)

Probiotski farmaceutski preparati su sve popularniji jer su sve prisutniji dokazi njihovog blagotvornog efekta na zdravlje ljudi. Cilj ovog rada je razvoj formulacije kapsuliranog probiotka sa optimalnom količinom aktivne komponente-probiotika Lactobacillus plantarum koja uz dodatak ostalih aktivnih komopnenti: gvožđa i vitamina C i ekscipijensa skroba, zadovoljava zahteve terapeutske doze, a istovremeno ispunjava zahteve procesnih parametara. Optimalna formulacija višekomponentnog probiotskog proizvoda sa zahtevom očuvanja svih aktivnih komponenti uz istovremeno ispunjenje zahteva tehnoloških postupaka proizvodnje omogućava rutinsku farmaceutsku proizvodnju efikasnog i bezbednog dijetetskog proizvoda.

Ključne reči: probiotski proizvod, Lactobacillus plantarum, gvožđe, vitamin C, skrob.