Regular Article

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Development of Probiotic Formulation for the Treatment of Iron Deficiency Anemia

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Probiotics are increasingly more present both as functional foods, and in pharmaceutical preparations with multiple levels of action that contribute to human health. Probiotics realize their positive effects with a proper dose, and by maintaining a declared number of probiotics cells by the expiration date. Important precondition for developing a probiotic product is the right choice of clinically proven probiotic strain, the choice of other active components, as well as, the optimization of the quantity of active component of probiotic per product dose. This scientific paper describes the optimization of the number of probiotics cells in the formulation of dietary supplement that contains probiotic culture *Lactobacillus plantarum* 299v, iron and vitamin C. Variations of the quantity of active component were analyzed in development batches of the encapsulated probiotic product categorized as dietary supplement with the following ingredients: probiotic culture, sucrosomal form of iron and vitamin C. Optimal quantity of active component *L. plantarum* of 50 mg, was selected. The purpose of this scientific paper is to select the optimal formulation of probiotic culture in a dietary supplement that contains iron and vitamin C, and to also determine its expiration date by the analysis of the number of viable probiotic cells.

Key words *Lactobacillus plantarum* 299v; iron; development; anemia; stability

During the past decade probiotic products have become very popular in the category of functional foods and dietary supplements because of their wide range of preventative and therapeutic possibilities, as well as, their general lack of side effects. According to Food and Agriculture Organization (FAO) of the United Nations and WHO, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[1]. Probiotics are microorganisms, such as bacteria and yeast, that benefit the host by stimulating growth of preferred microorganisms, and antagonizing pathogens by inhibiting their mucosal adherence and by producing antimicrobial compounds.[2,3] The most common probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*. Lactobacilli and bifidobacteria are used in both functional foods, and in dietary supplements. In commercial aspect, in order to be taken into consideration for the development of marketable product, probiotics must fulfill several criteria including safety and functional and technological characteristics. Good viability, activity and survival of probiotics in food products during processing, storage and consumption, are considered essential for optimal functionality.[4–7] Also, probiotic bacteria in a particular product must be present in sufficient number, because its activity depends directly on the number of viable cells. Standard condition for achievement of probiotic effects is the minimal number of 10^6 colony forming unit (CFU) per gram of viable bacteria until expiration date. A minimum therapeutic level is considered to be 10^8–10^9 cells/d.[8–10]

Iron deficiency, specifically iron deficiency anemia, is an important Public Health issue in both developing and in industrial countries. According to WHO, anemia affects over 2 billion people worldwide, with 50% of cases being secondary to iron deficiency. Strategies for preventing iron deficiency are based on combination of dietary improvement, iron fortification of food, and iron supplementation.[11] Among these strategies, supplementation can be cost effective in various vulnerable population groups.[12] There is also some evidence that probiotic can enhance bioavailability of iron. Human studies have been performed to discover underlying mechanism of probiotic effect on iron status, such as creating an acidic environment in the intestinal tract which makes iron more absorbable, making iron biologically available by producing iron-chelating ligands, or degrading mineral complexing phytic acid from food.[13–15] Therefore, making probiotics a part of a diet plan, can prevent and ameliorate iron deficiency anemia in humans. One of the probiotics strain hypothesized to have a positive effect on iron absorption is *Lactobacillus plantarum* 299v.[13,16,17] Iron deficiency is one of the most frequent micronutrient deficiencies in the world. For that reason, supplementation strategy can be cost effective in prevention of iron deficiency. Additionally, clinical studies show that the use of *L. plantarum* 299v can contribute to the improvement of the iron absorption.[13,16,18] Based on this knowledge and market demand, the probiotic formulation was developed using the *L. plantarum* 299v strain with addition of sucrosomal iron and vitamin C. Based on this knowledge and market demand, the probiotic formulation was developed using the *L. plantarum* 299v strain with addition of sucrosomal iron and vitamin C. In clinical studies, this strain has been shown to colonize gastrointestinal tract and was found to have many positive effects on health, including reducing intestinal gas problems and producing pain in people with irritable bowel syndrome, as well as exhibiting anti-inflammatory and cholesterol lowering effects.[19–21]

The objective of the presented work was to analyze a design of probiotic formulation in capsules also containing iron and

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vitamin C (that also contributes to absorption of iron), which would be used in the prevention and treatment of mild cases of iron deficiency in the population of people with reduced absorption of iron. A daily dose of 10^9 CFU/d capsule formulation was chosen. This type of probiotic product design would be innovative to the Serbian market. Main purpose of this scientific paper was to select the optimal number of probiotics cells in the finished product, and to determine its expiration date by analyzing the number of viable probiotic cells.

Final confirmation of the activity of the selected formulation is the improvement of blood status of iron in clinical studies.

Experimental

Active Ingredients *L. plantarum* 299v (DSM 9483) strain was purchased in lyophilized form from Probi AB (Probi AB, Lund, Sweden). Sucrosomal iron was purchased from the company Alesco S.R.L., Italy. This is the form of iron that has increasing bioavailability and which does not develop irritations of the gastrointestinal tract.

Vitamin C is obtained from the manufacturer Shandong Luwei Pharm Co., China.

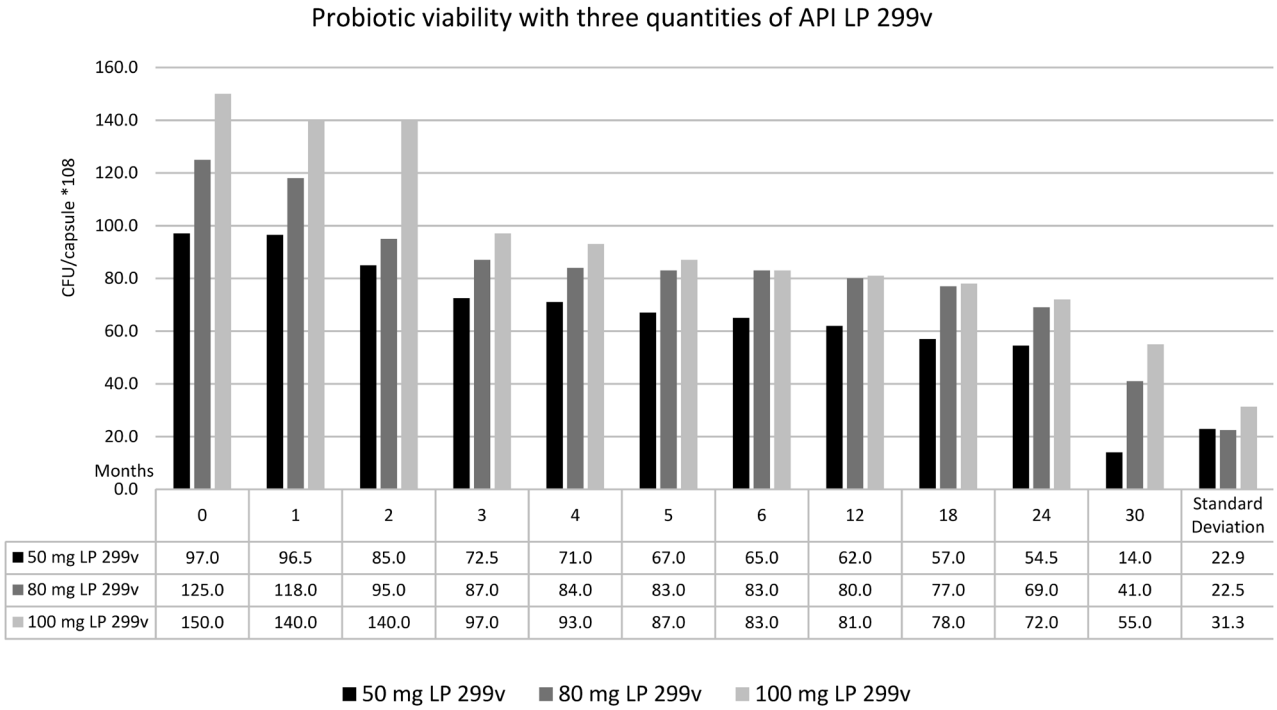
Excipients and Capsule Maize starch (UniPure FL, Germany) with 2% moisture was used as the excipient in all the analyzed formulations of the product. Reduced amount of moisture was used in order to minimize the effect of moisture on the probiotic cells in the formulations.

Encapsulated Formulations Hypromelose capsules were used and they were manufactured by the company Capsugel, France. Capsules size 1 were selected.

Three formulations were defined: a) Formulation 1 included 50 mg of lyophilized powder of *L. plantarum* 299v, 10 mg of sucrosomal iron and 15 mg of vitamin C, per capsule; b) Formulation 2 included 80 mg of lyophilized powder of *L. plantarum* 299v, 10 mg of sucrosomal iron and 15 mg of vitamin C per capsule; c) Formulation 3 included 100 mg of lyophilized powder of *L. plantarum* 299v, 10 mg of sucrosomal iron and 15 mg of vitamin C per capsule.

All three formulations were blistered inside polyvinylidene chloride (PVdC)/Al foil immediately after encapsulation for the purposes of complete protection of capsules from the influences of external environment (air, light and moisture). During sampling, blisters with the selected formulations were opened, and the capsules were analyzed to determine the number of probiotic cells.

Counting the Viable Number of Probiotic Cells According to the recommendations of the manufacturer, the probiotic strain *L. plantarum* 299v was replicated in the MRS broth (Biokar Diagnostics, U.S.A.).[22] The number of probiotic cells was determined by using agar plate method (Nordic Committee on Food Analysis or in Norwegian: Nordisk Metodikkomité for Næringsmidler (NMKL) method, 2007, Probi). The samples of the probiotic culture in the form of powdered content of the capsule were rehydrated in the sterile solution of sodium chloride (Sigma-Aldrich, U.S.A.), peptone (Sigma-Aldrich) and purified water. After that, by using the method of decimal dilutions the samples were diluted in the series of decimal dilutions. A 0.1 mL of the last two samples was taken with the pipette and they were added to the agar plates. Colonized substrates were incubated on 37°C in the anaerobic conditions during two days. After incubation the number of colonies was counted on the agar surface and the result was expressed as the number of colonies or CFU in powder or capsule. Presented method was completed three times per one analysis, and results were expressed as the average values of all three counts±standard deviation (S.D.).



Months	0	1	2	3	4	5	6	12	18	24	30	Standard Deviation
50 mg LP 299v	97.0	96.5	85.0	72.5	71.0	67.0	65.0	62.0	57.0	54.5	14.0	22.9
80 mg LP 299v	125.0	118.0	95.0	87.0	84.0	83.0	83.0	80.0	77.0	69.0	41.0	22.5
100 mg LP 299v	150.0	140.0	140.0	97.0	93.0	87.0	83.0	81.0	78.0	72.0	55.0	31.3

Fig. 1. Graphic Representation of the Three Average Values of the Number of Probiotic Cells in Capsules with Three Quantities of *L. plantarum* 299v under the Set of Ambient Conditions A (25±2°C Temperature and 60±5% Relative Humidity)

Number of experiments: 99. Active pharmaceutical ingredient (API)-*Lp* 299v-*L. plantarum* 299v.

Stability of the Encapsulated Formulations The samples for the selected formulations were duplicated because the stability studies were also completed in two different sets of temperature and humidity conditions (A and B set of conditions). Sets of conditions were selected from the long-term conditions and intermediate stability regime that was proposed by the European Medicines Agency (EMA).[23–25] Stability testing was performed in real time. First, stability study under set of conditions A was selected and after that, the selected formulation with 50 mg of *L. plantarum* 299v was analyzed under the set of conditions B. Set A of ambient conditions included the temperature of 25±2°C and the relative humidity of 60±5% (results shown on the Fig. 1), whereas the set B of ambient conditions included the temperature of 30±2°C and relative humidity of 65±5% (results shown on the Fig. 2).

Long-term studies should be carried out under conditions simulating the storage conditions recommended on the product label (25±2°C). Long-term studies should extend at least as long as the desired shelf-life claim (expiration date) for that product.

Clinical Study of the Effects of the Selected Formulation The effects of the selected formulation of 50 mg of *L. plantarum* 299v on the absorption of iron, were monitored in clinical study with target population of healthy women from 20 to 40 years of age, which represent a population that is prone to iron deficiency. The hypothesis of this study was that the absorption of iron was enhanced by the simultaneous oral administration of *L. plantarum* 299v and vitamin C. Formulation that included 50 mg of *L. plantarum* 299v, 10 mg of sucrosomal iron and 15 mg of vitamin C was used in conducted clinical study in comparison to the oral administration of 10 mg of sucrosomal iron and 15 mg of vitamin C. The clinical study was conducted during the October, 2017, and both groups had 10 subjects. Both groups took their morning therapy of one capsule on empty stomach for 7 d. A capsule containing *L. plantarum* 299v, sucrosomal iron and vitamin C was taken by 10 subjects in study group and capsule containing sucrosomal iron and vitamin C was taken by 10 subjects in control group. Ten to twelve days after the therapy was completed, iron status of subjects in blood was analysed in the morning, on empty stomach. Iron was determined using the spectrophotometric method with ferrosin (Roche Hitachi apparatus).

Results

Results of probiotic cells count in the capsules containing three variations of mass of the active probiotic component *L. plantarum* (*Lp*) 299v (50, 80, 100 mg/capsule) under the set of conditions A are shown in Fig. 1. Long term stability study was performed in the time period of 30 months (ambient conditions were 25±2°C temperature and 60±5% relative humidity) in the time limits defined in the Table 1.

Samples were analyzed and the number of probiotic cells was followed in time with results expressed in the units CFU/capsule.

Results of the uniformity of mass for probiotic product with three quantities of active substance *L. plantarum* 299v (50, 80,

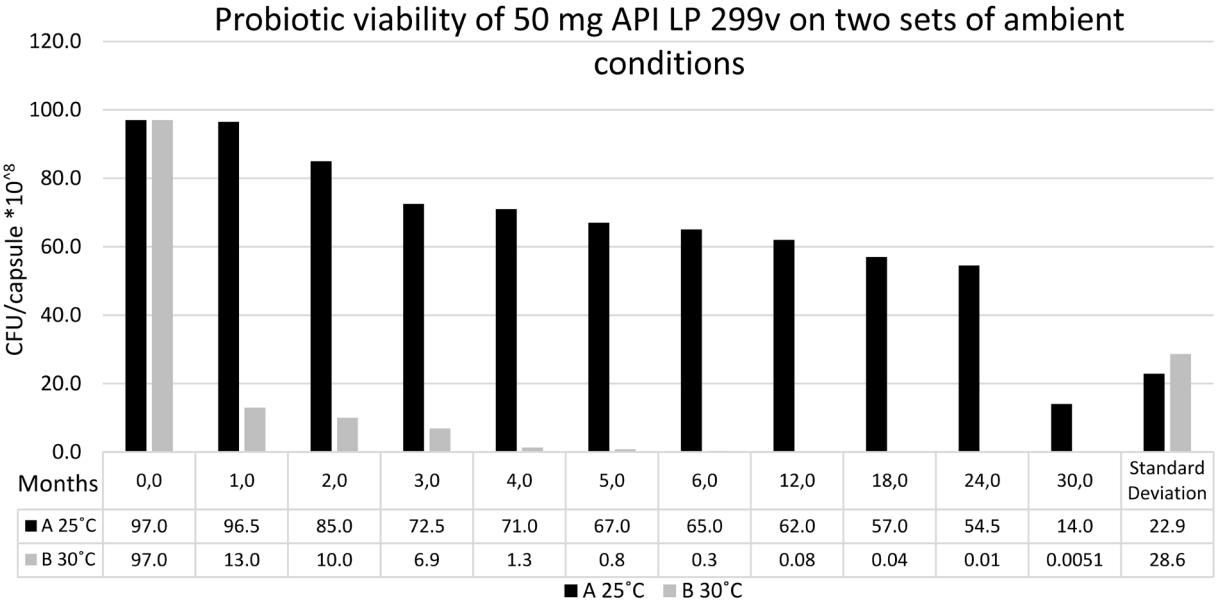


Fig. 2. Comparison of the Three Average Values of the Number of Probiotic Cells in Capsules with the Selected Formulation of 50 mg *Lp* 299v in the Following Ambient Conditions: 25±2°C (Set of Conditions A) and 30±2°C (Set of Conditions B)

Lp 299v-*L. plantarum* 299v; Number of experiments: 66.

Table 1. Sampling Time Intervals under the Set of Conditions A and B

Months										
Start of stability testing-preparation of samples	Sampling set of conditions A/set of conditions B									
0	1	2	3	4	5	6	12	18	24	30

100 mg/capsule) are depicted in Table 2. Mass of the content of 20 capsules was measured and mean value and S.D. was calculated.

Second part of the stability study is shown in Fig. 2. According to the results from Fig. 1, formulation with 50 mg of active component *Lp* 299v was selected. This formulation has fulfilled all the conditions of the number of probiotic cells during the time period of two years, which was the reason why it was selected to be analyzed in the intermediate analysis in order to confirm the suggested expiration date of two years. Also, in Fig. 2. are shown comparative data of the number of probiotic cells in the formulation 1 with 50 mg of *Lp* 299v under the ambient conditions of the temperature of 25±2 and 30±2°C.

Data from the study group and control group of subjects, whose blood was analyzed 10–12 d after the therapy had been completed, is presented in Table 3. The obtained results showed that the blood status of iron in the group of subjects who received iron and vitamin C with *Lp* 299v was increased by 11% due to increased absorption, compared to the group of subjects who used only iron and vitamin C which is graphically depicted in Fig. 3.[16)]

Table 2. Uniformity of Mass-Capsule Content for Three Formulations (50, 80, 100 mg *Lp* 299v)

Capsule sample label	U 1-50 *Lp* 299v (mg)	U 2-80 *Lp* 299v (mg)	U 3-100 *Lp* 299v (mg)
1	330.30	333.67	327.65
2	331.95	333.05	317.68
3	329.67	331.87	328.86
4	326.08	330.08	329.53
5	327.86	329.83	328.82
6	328.34	330.75	321.78
7	325.95	330.55	327.75
8	326.15	331.95	328.38
9	331.1	329.05	325.96
10	329.82	328.55	326.55
11	326.5	326.65	326.95
12	327.95	329.85	327.94
13	328.6	331.77	328.28
14	325.09	330.76	327.88
15	321.75	329.87	326.95
16	327.66	327.05	329.95
17	327.07	331.35	327.36
18	332.85	329.75	328.75
19	329.08	330.09	324.78
20	327.07	333.85	323.79
Sum	6560.84	6610.34	6535.59
Mean value	328.042	330.517	326.7795
Deviation %	2.51	3.28	2.12

Discussion

In this study, three formulations were analyzed during the defined storage conditions by monitoring the number of probiotic cells. During the analysis, all three formulations have proved the required technological characteristics of the encapsulated probiotic-viability of probiotic strain, the uniformity of the mass of the capsule, complete closing of capsule, and the required properties of the foil and sealing of foil during

Table 3. Data from the Study Group and Control Group of Subjects (Mean Values and S.D.)

	Study group (n=10)		Control group (n=10)	
	Mean	S.D.	Mean	S.D.
S-Fe (μmol/L)	19.4	4.1	17.5	3.6
Ferritin (ng/mL)	44.0	9.2	41.9	7.2
TIBC (μg/L)	69.6	6.8	64.7	5.6
Hb (g/L)	138.9	8.5	135.3	5.6
Age (year)	29.8	5.6	28.3	5.0
BMI (kg/m^2)	21.6	1.7	21.5	1.8
CRP (mg/L)	<5		<5	

S-Fe: serum iron concentration; TIBC: total iron binding capacity; CRP: C-reactive protein; BMI: body mass index; Hb: hemoglobin.

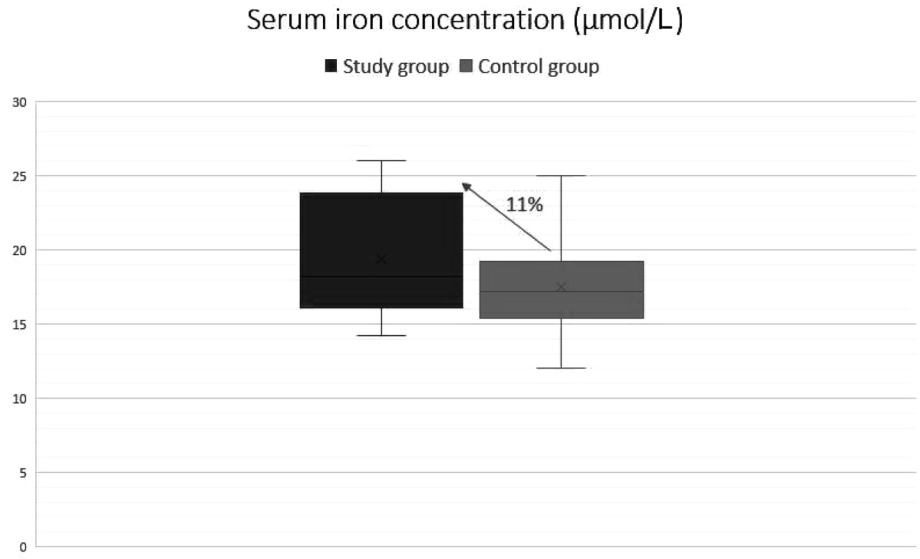


Fig. 3. Serum Iron Concentration of the Study and Control Group of Subjects

The bars and whiskers represent means with their values corresponding maximum and minimum values of serum iron.

blistering process.

In the first part of the study, three formulations with different quantities of the active component *Lp* 299v (50, 80, 100 mg/capsule), were selected. They all contained equal quantities of iron and vitamin C, and the addition of maize starch as excipient. The results of the first part of the study (results shown on the Fig. 1), show that after defined time period and set of ambient conditions A, the number of probiotic cells decreases in time, as it was expected. The decrease of the number of probiotic cells is greater in the formulations that possessed larger quantities of active component (80, 100 mg of *Lp* 299v), than in the formulation with 50 mg *L. plantarum* 299v. This is another evidence that shows that a greater number of probiotic cells is not a prerequisite for maintaining of the required cell viability over time.[9] After two years, under the conditions set A, a formulation with 50 mg of active component had the minimum required number of probiotic cells, *i.e.*, 10^9 CFU per capsule. For these reasons, this formulation was chosen for the second part of the study, where the expiration date of the formulation was confirmed.

In the second part of the study (Fig. 2), the obtained results confirmed that the expiration date of two years can be declared for the followed product under the storage conditions of room temperature. In addition to this, it was confirmed that the number of probiotic cells of *Lp* 299v was maintained in the range of 9.7 at start to 5.45×10^9 CFU/capsule during the time period of two years under the temperature of 25± 2°C. At this temperature at the end of the study, after two and a half years, the selected formulation contained 1.4 billion probiotics cells (CFU/capsule), which is at the very bottom of the required dose, and therefore was not considered sufficient for further consideration for the expiration date to be two and a half years. During the set of conditions B, the selected formulation kept the number or 10^9 viable cells per capsule during the period of two months, which confirmed that this formulation can be stored for the shorter time period even in those temperatures (high outdoor temperatures and high humidity during summer or when traveling to warmer areas).

These results confirmed the stability of the selected probiotic formulation at the recommended storage temperature of finished product (room temperature). Samples were tested after the completion of all technological phases of production of finished product and during the stability study.[26,27]

Final confirmation of the efficacy of the selected formulation is the result of a clinical study that showed the increased blood status of iron due to its higher absorption in the group of subjects who took *L. plantarum* together with iron and vitamin C, than in the group of subjects who took only vitamin C and iron.

Conclusion

This study has shown the possibility of the design of capsules with *L. plantarum* 299v, sucrosomal iron and vitamin C, with condition that the viability of the probiotic cells secures the minimal daily therapeutic dose of at least 10^9 probiotic cells per capsule during two years of storage on the temperature of 25±2°C. The proposal of this design is innovative, because it contains *L. plantarum* 299v, a probiotic strain that possesses many clinically confirmed positive effects on health, including reducing intestinal gas problems and pain in people with irritable bowel syndrome, and it also possesses anti-inflammatory and cholesterol-lowering effects. A probiotic product with this design has combined effects, and when taken regularly, it contributes to the balance of intestinal flora, whereas the addition of iron improves the level of this micronutrient. The effects of the selected formulation were confirmed by the clinical study that presented better iron absorption in the subjects who used capsules with *L. plantarum* 299v with iron and vitamin C.

Due to the reasons mentioned above, this formulation can be very effective in people who beside iron deficiency suffer from underlining symptoms of the mentioned gastrointestinal tract diseases. Directions for the future research will be the analysis of other parameters of this probiotic product, such as: additional microbiological parameters, heavy metal content, parameters of the blister seal and further clinical confirmation of its action on the selected population.

Conflict of Interest The authors declare no conflict of interest.

References

1) FAO and WHO, "Report of joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria." Cordoba, Argentina, 2001, pp. 19–20.
2) Salminen S., Ouwehand A. C., Isolauri E., *Int. Dairy J.*, **8**, 563–572 (1998).
3) Szajewska H., Mrukowicz J. Z., *Paediatr. Drugs*, **7**, 111–122 (2005).
4) Das S., Bhattacharjee D., Manna A., Basu S., Chowdhury S., Mukherjee S., Bhattacharayya B. K., *International Journal of Pharmaceutical Sciences and Research*, **5**, 1830 (2014).
5) Mattila-Sandholm T., Myllärinen P., Crittenden R., Mogensen G., Fondén R., Saarela M., *Int. Dairy J.*, **12**, 173–182 (2002).
6) Mortazavian A. M., Mohammadi R., Sohrabvandi S., "Delivery of probiotic microorganisms into gastrointestinal tract by food products." INTECH Open Access Publisher, 2012.
7) Solanki H. K., Mistry A. Z., Shah D. A., Prajapati V. D., *Asian Journal of Pharmaceutical Research and Health Care*, **4**, 42–51 (2012).
8) Moumita S., Goderska K., Johnson E. M., Das B., Indira D., Yadav R., Kumari S., Jayabalan R., *Lebenson. Wiss. Technol.*, **77**, 460–467 (2017).
9) Kosin B., Rakshit S. K., *Food Technol. Biotechnol.*, **44**, 371–379 (2006).
10) Patel A. K., Deshattiwar M. K., Chaudhari B. L., Chincholkar S. B., *Bioresour. Technol.*, **100**, 368–373 (2009).
11) Organization W. H., "Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia." 2008.
12) Balgir P. P., Kaur B., Kaur T., *International Journal of Fermented Foods*, **3**, 45 (2014).
13) Bering S., Suchdev S., Sjøltov L., Berggren A., Tetens I., Bukhave K., *Br. J. Nutr.*, **96**, 80–85 (2006).
14) Scholz-Ahrens K. E., Ade P., Marten B., Weber P., Timm W., Açil Y., Glüer C. C., Schrezenmeir J., *J. Nutr.*, **137** (Suppl. 2), 838S–846S (2007).
15) Sazawal S., Dhingra U., Hiremath G., Sarkar A., Dhingra P., Dutta A., Menon V. P., Black R. E., *J. Pediatr. Gastroenterol. Nutr.*, **51**, 341–346 (2010).
16) Hoppe M., Önning G., Berggren A., Hulthén L., *Br. J. Nutr.*, **114**, 1195–1202 (2015).
17) Bering S., Sjøltov L., Wrisberg S. S., Berggren A., Alenfall J., Jensen M., Højgaard L., Tetens I., Bukhave K., *Br. J. Nutr.*, **98**, 991–997 (2007).
18) Balamurugan R., Mary R. R., Chittaranjan S., Jancy H., Shobana

Devi R., Ramakrishna B. S., *Br. J. Nutr.*, **104**, 931–934 (2010).
19) Goossens D., Jonkers D., Russel M., Thijs A., van den Bogaard A., Stobberingh E., Stockbrügger R., *Dig. Liver Dis.*, **37**, 44–50 (2005).
20) Ducrotté P., Sawant P., Jayanthi V., *World J. Gastroenterol.*, **18**, 4012–4018 (2012).
21) Naruszewicz M., Johansson M. L., Zapolska-Downar D., Bukowska H., *Am. J. Clin. Nutr.*, **76**, 1249–1255 (2002).
22) De Man J. C., Rogosa M., Sharpe M. E., *J. Appl. Bacteriol.*, **23**, 130–135 (1960).
23) Klayraung S., Viernstein H., Okonogi S., *Int. J. Pharm.*, **370**, 54–60 (2009).
24) Kaur M., Kaur G., Kaur H., Sharma S., *International Journal of Pharmaceutical, Chemical & Biological Sciences*, **3**, 1231–1241 (2013).
25) Group, I. E. W., Q1A (R2) Stability Testing of New Drug Substances and Products. in International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use, 2003.
26) Gueimonde M., Sanchez B., *Microb. Ecol. Health Dis.*, **23**, 2–5 (2012).
27) CPMP/ICH/2736/99,ICH Q1A (R2), "Stability testing of new drug substances and drug products," European Medicines Agency, 2003.