Chocolates containing encapsulated in cocoa butter microemulsion cells of a probiotic strain *Lactiplantibacillus plantarum*

Iliyan Dobrev [1*], *Bogdan* Goranov[1], *Raina* Hadjikinova[2], *Zapryana* Denkova[1] and *Petya* Ivanova[3]

[1]University of Food Technologies - Plovdiv, Department of Microbiology, 26 Maritza Blvd., 4002, Plovdiv, Bulgaria
[2]University of Food Technologies - Plovdiv, Technology of tobacco, sugar, vegetable and essential oils, 26 Maritza Blvd., 4002, Plovdiv, Bulgaria
[3] Institute of Food Preservation and Quality, 4000 Plovdiv, Agricultural Academy - Sofia, Bulgaria

Abstract. Chocolates were prepared with the probiotic strain *Lactiplantibacillus plantarum* 13/20 and a prebiotic component of blackcurrant, cherry and blueberry flours. The probiotic cells were introduced by encapsulation in cocoa butter and the prebiotic components by dissolution in an alginate gel. The rheological performances of the resulting chocolates were investigated. It was shown that the addition of prebiotic in the amount of 3.5% negatively affected the rheological properties as well as the organoleptic performance. The candies were characterized by higher moisture content. It has been shown that the size of the gel beads significantly affected the rheology of the chocolate products. The larger the diameter of the gel beads, the stronger their influence on the rheological properties of the chocolates. The sample with gel pearls and blueberry at 2,5% had the best organoleptic characteristics. The microbiological status of the chocolates was determined. All chocolates met the standard requirements for microbiological safety.

1 Introduction

Chocolate products are becoming increasingly popular as a carrier for probiotics to the gastrointestinal tract [1, 2]. In turn, they are divided into three main groups: chocolate bars, chocolates and chocolate figurines. The main semi-finished material in their production is the chocolate mass. It is an almost anhydrous dispersed system obtained with the participation of sugar, cocoa products (cocoa mass, cocoa butter and cocoa powder) and, in the case of milk chocolate mass, with different types of dehydrated milk [3, 4]. The main chocolate types are dark, milk and white chocolate and they are distinguished by their content of cocoa solids, milk fat and cocoa butter [4]. Cocoa powder contains a complex structure of proteins, polysaccharides and lipids [5, 6], which can form a good encapsulating mixture together with alginate or FOS. The characteristic taste of chocolate products is determined by combining the specific taste characteristics of cocoa products with the sweet taste of sugar [7]. Enrichment of the composition of different types of chocolate with probiotics is essential for the health of organisms and is associated with the increase in market demand [8].

Probiotics are defined as "live microorganisms that, when ingested in adequate amounts, are beneficial to the host [9]. To exert their preventive role they must contain a high concentration of viable cells (above 10^6-10^7 CFU/cm^3/g product [FDA], [10,11]. Microencapsulation has been applied to extend the survival and shelf-life of probiotics under stressful conditions [12]. Encapsulation of probiotic bacteria in cocoa powder has been accomplished [13].

The production of chocolate products involves a number of technological operations, the main ones being: mixing, grinding, conching, tempering, forming and cooling. To define a product as chocolate it must also meet the requirements set out in Directive 2000/36/EC [14].

The aim of the present work was to prepare chocolates with cells of the probiotic strain *Lactiplantibacillus plantarum* 13/20 encapsulated in cocoa butter and to determine the rheological performance of the finished products.

2 Materials and methods

In this work, a lyophilizate of the probiotic strain *Lactiplantibacillus plantarum* 13/20 with a viable cell concentration of $4,3.10^{14}$ cfu/g was used.

2.1 Preparation of suspension of *Lactiplantibacillus plantarum* 13/20.

1 g of the lyophilized concentrate was incorporated and homogenized in 100 g of cocoa butter (Mixture I). Additionally, prebiotic components from blackcurrant, cherry and blueberry flours were added. One gram of each of the three powder preparations was extracted and

* Corresponding author: iliyan_dobrev@uft-plovdiv.bg

homogenized with 80 cm^3 of 1% alginate solution (Mixture II).

2.2 Encapsulation of viable cells of *Lactiplantibacillus plantarum* 13/20 in cocoa butter.

The two mixtures (Mixture I and Mixture II) were mixed, homogenized for 30 min at a stirring rate of 600 rpm.

200 cm^3 of cooled 2% $CaCl_2.2H_2O$ solution were placed in a vessel and the above mixture was fed to it in a thin stream using a peristaltic pump with continuous stirring at 600 rpm for 1 h. The resulting gel beads were separated from the liquid phase, washed with 0.85% saline and fed to the chocolate mass for incorporation. The concentration of viable cells in the gel beads was above 10^{10} cfu/g.

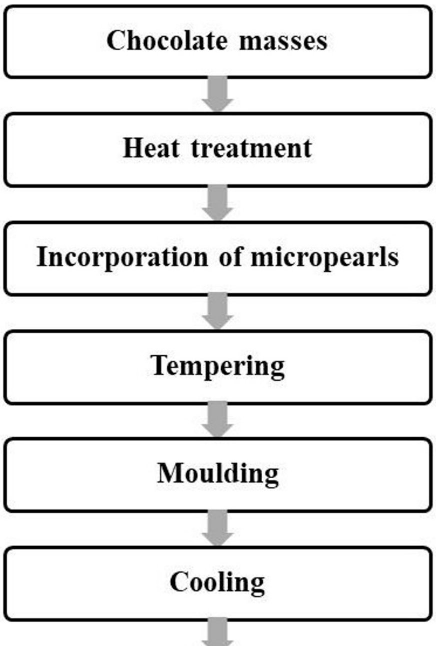


Fig.1. Technological scheme for the preparation of chocolate candies with microemulsion

2.3 Preparation of chocolates with microencapsulated probiotic cells

The chocolates were prepared in the laboratory according to the process flow chart reflected in Fig. 1. For the preparation of the candies, chocolate mass from the company 'Belcolade' (with a cocoa mass content of 55 %), which by its composition meets the requirements laid down in Directive 2000/36/EC, was used, and the prepared microemulsion was added at a quantity of 2,5 g/100 g chocolate mass and 3,5 g/100 g chocolate mass.

The chocolate mass was melted to a temperature of 45-50 °C and the specified amount of microemulsion was dosed into the melted mass. The chocolate mass was tempered to a final temperature of 31 °C and cast into pre-tempered moulds. The moulds were then cooled to a temperature of 8 °C. The cooled candies were removed from the moulds.

For the purpose of the analysis, 7 samples were obtained and analysed The were designated as follows: control (obtained without the participation of micro-beads) - C; chocolates obtained with the participation of 2.5% and 3.5% of gel beads with the participation of blueberry flour – $B_{2.5}$, $B_{3.5}$, cherry – $V_{2.5}$, $V_{3.5}$, and blackcurrant - K2.5, K3.5. The resulting chocolates are shown in Fig. 2.

Fig.2. Photos of the obtained microemulsion chocolates

2.4 Technological analysis

To determine the influence of the microbeads included in the chocolates, their wetting kinetics were monitored at a relative humidity of φ=75% A saturated NaCl solution was used to maintain a constant relative humidity at t=20˚C [15].

The values of the dry matter content and the pH were determined according to [16].

The permanganate method according to Petrov, 1976 [17] was used to determine the content of water soluble tannins in chocolates.

The rheological behavior of chocolate masses was determined with a rheoviscometer "Rheotest 2", (Germany). The Casson plastic viscosity of the chocolate masses was determined according to the method of the International Organisation of Cocoa and Chocolate [18]. The Casson plastic viscosity (ηsa) and Casson limit stress (τsa) values were defined by a computer program [19].

The hardness of the chocolates was determined using a Höpler's consistometer.

The particle size in the melted chocolate masses used to obtain the candies was determined using a microscope "Boeco", Germany and computer programs "Scope photo" and "Image tool".

The organoleptic evaluation was carried out according to BS 5313, point 2.1.

2.5 Microbiological analysis: microbiological parameters:

Coliforms according to ISO 4831, ISO 4832;

Escherichia coli, according to ISO 7251, ISO 16649-1.3;

Salmonella sp., according to ISO 6579;

Coagulase-positive staphylococci, according to ISO 6888-1-3;

Sulfite-reducing clostridia, according to ISO 15213;

Spores of microscopic fungi, according to ISO 7954;

Yeasts, according to ISO 7954.

3 Results and discussion

Table 1 and Table 2 present the values of the determined parameters of the analyzed chocolates.

Table 1. Values of indicators of chocolates

Indicators	Sample type			
	C	$B_{2.5}$	$B_{3.5}$	$V_{2.5}$
Dry content, %	99,45 ±0,02	98,47±0,10	98,27±0,18	98,39±0,21
Tannins,% DM	1,29±0,07	1,18±0,08	1,10±0,10	1,25±0,05
pH value	6,17±0,05	6,10±0,08	6,04±0,04	6,05±0,10

Table 2. Values of indicators of chocolates

Indicators	Sample type			
	C	$V_{3.5}$	$K_{2.5}$	$K_{3.5}$
Dry content, %	99,45±0,02	98,03±0,09	98,19±0,15	97,87±0,04
Tannins,% DM	1,29±0,07	1,18±0,10	1,24±0,12	1,22±0,10
pH value	6,17±0,05	6,11±0,03	6,07±0,04	6,10±0,08

From the data in Table 1 it is evident that no significant differences were observed between the samples in terms of the analyzed parameters. To a small extent, a difference was observed with regard to the dry matter content, which was highest in the control.

Chocolate products are characterised by a smooth, melting consistency when eaten, which is largely determined by the particle size of the finished product. The particle size of the chocolates analysed was therefore determined. The percentage distribution of particles by size group and the average size are shown in Table 3 and Table 4. Some of the microscopic images used for the analysis are shown in Fig. 3

The wetting kinetics of the chocolates was investigated as a function of the microbead content, at a relative humidity of φ=75%. The results obtained are shown in Fig. 4.

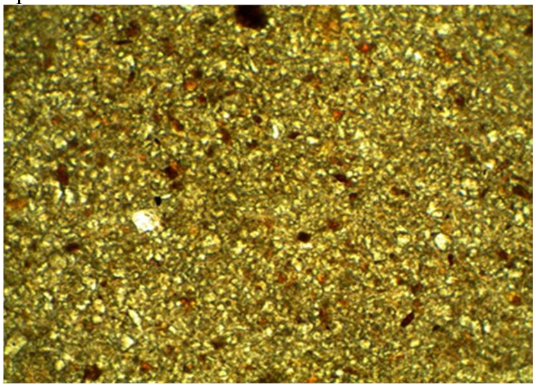
Table 3. Particle size distribution, %

Size groups, μm	Sample type			
	C	$V_{3.5}$	$K_{2.5}$	$K_{3.5}$
0 – 20	56	52	50	53
21 – 30	40	34	32	31
31 – 50	4	13	15	16
51 – 80	0	1	3	0
over 80	0	0	0	0
Average size, μm	17,58±1,05	19,23±1,11	20,05±1,07	20,18±1,31

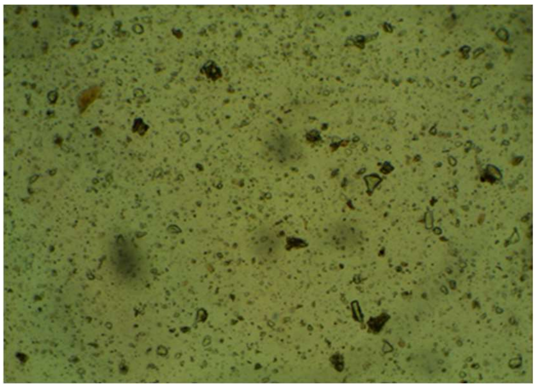
Table 4. Particle size distribution, %

Size groups, μm	Sample designation			
	C	$V_{3.5}$	$K_{2.5}$	$K_{3.5}$
0 – 20	53	52	52	53
21 – 30	31	31	33	29
31 – 50	16	16	14	16
51 – 80	0	1	1	2
over 80	0	0	0	0
Average size, μm	20,18±1,31	21,02±1,09	21,12±1,23	21,15±1,07

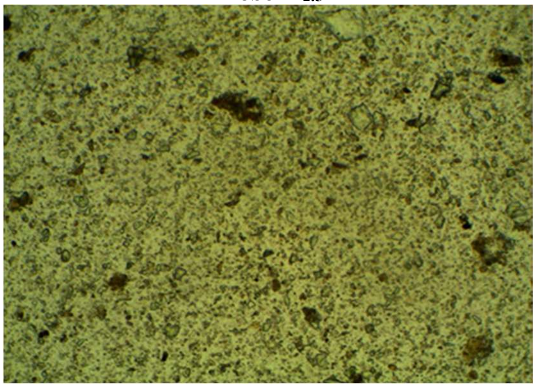
The graphic shows that the moisture content of the samples increases over time. Sample $K_{3.5}$ has the highest degree of wetting and the control the lowest. After 240h (10 days), the moisture content of sample $K_{3.5}$ has increased by 0.041% and that of the control by 0.025% compared to the initial moisture content.



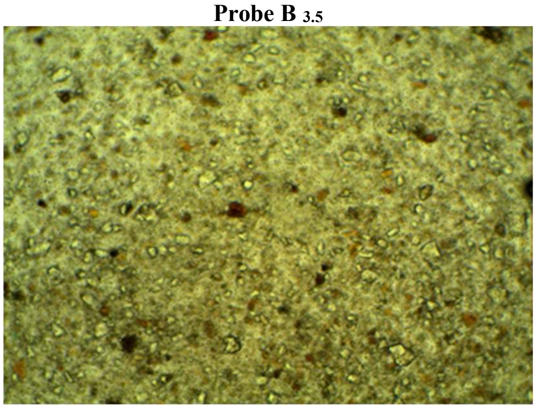
Probe C



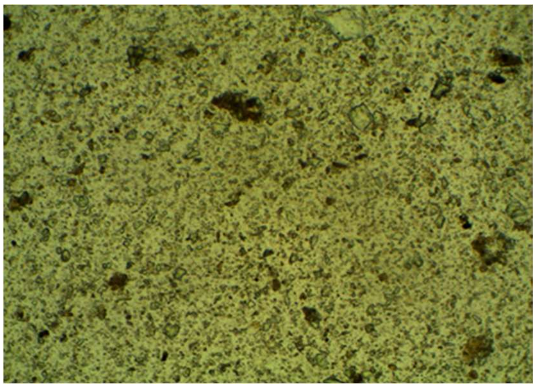
Probe B $_{2.5}$



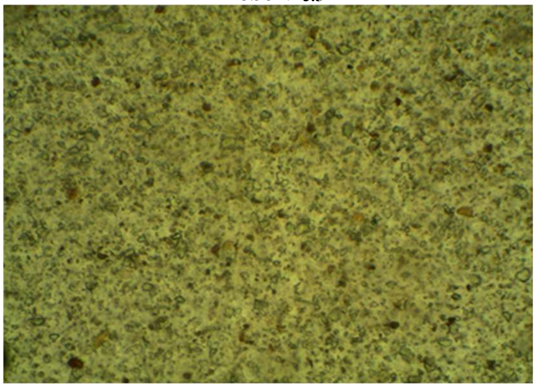
Probe B $_{3.5}$



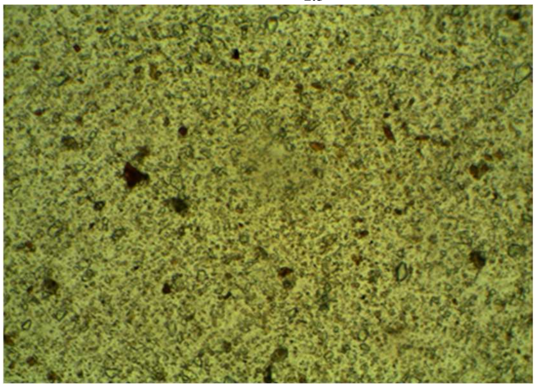
Probe V $_{2.5}$



Probe V $_{3.5}$



Probe K $_{2.5}$



Probe K $_{3.5}$

Fig.3. Photos used to determine the particle size in chocolates

The main rheological parameters that characterize the quality of the melted chocolate masses are viscosity and static boundary stress. The International Organisation of Cocoa and Chocolate (OICC) has adopted the expression of the viscosity of chocolate masses by the Casson plastic viscosity value. For this purpose, the Casson limit stress and the Casson plastic viscosity were defined. The rheograms of the analyzed samples in coordinates $(1+a)\sqrt{D}$ s;$(1+a)\sqrt{\tau}$, (where a=0.81) at a temperature of 40 ^{0}C, where Ds, velocity gradient, s^{-1}; τ, tangential stress, Pa, are shown in Fig. 5.

It can be seen from the dependence plotted in Fig. 5 that rheologically all samples had similar behavior. Their rheograms represented straight lines and satisfied the requirement of Casson's plastic viscosity calculation method. In Table 5 the values of the Casson boundary stress (τsa) and Casson plastic viscosity (ηsa) calculated with a computer program are reported [19].

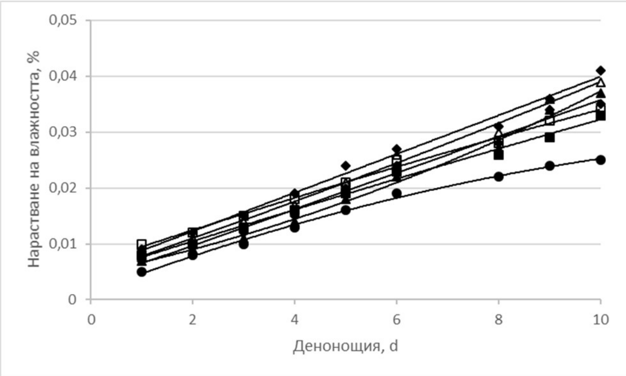


Fig.4. Wetting kinetics of chocolates depending on the content of microbeads

Type of powder: ●-C; ■-$B_{2.5}$; □-$B_{3.5}$; ▲- $V_{2.5}$; Δ- $V_{3.5}$; ♦-$K_{2.5}$; ◊- $K_{3.5}$

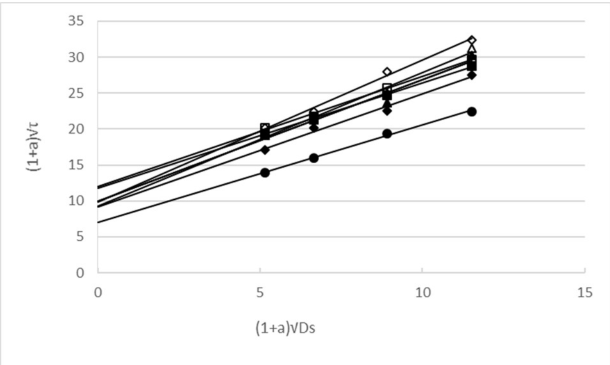


Fig. 5. Reograms of chocolate tables used for the preparation of chocolates, in coordinates (1+a)√Ds;(1+a)√τ at 40˚C

Type of powder: ●-C; ■-$B_{2.5}$; □-$B_{3.5}$; ▲- $V_{2.5}$; Δ-$V_{3.5}$; ♦- $K_{2.5}$; ◊- $K_{3.5}$

The data presented in Table 3 show that the values of the boundary stress (τsa) and the plastic viscosity (ηsa) of the control were lower than those of the chocolate mass with microbeads included in its composition. The limit stress of the control was about 63% lower than sample $K_{3.5}$, which had the highest value of this parameter. As the concentration of microbeads increased, the Casson boundary stress and viscosity increased for all samples. More likely, this dependence was due to the interaction of the imported gel beads with the chocolate matrix. According to Todorova [20] the appropriate Casson viscosity values, for moulding by casting, for a natural chocolate mass is 2.95 Pa.s. All samples analysed met this requirement except sample C3.5.

The chocolates were evaluated organoleptically by 10 independent tasters for appearance, texture, aroma, taste, sweetness and aftertaste. The results of these tests are shown in Fig. 6.

All prepared chocolates had properties similar to the control sample. The tasters gave the highest score to the blackcurrant and cherry samples at 3,5 %, followed by blackcurrant at 2,5 % and blueberry at 2,5 % (Fig. 6). The microbiological status of the chocolates was determined. The experimental data are shown in Table 6. All samples met the standard requirements for microbiological safety.

Table 5. Casson values of boundary stress (τsa) and plastic viscosity (ηsa)

Sample type	τca , Pa	ηca, Pa.s
C	13,44	1,22
B 2.5	32,79	2,15
B 3.5	35,46	2,37
V 2.5	21,09	2,83
V 3.5	25,50	3,06
K 2.5	20,83	2,48
K 3.5	36,98	2,66

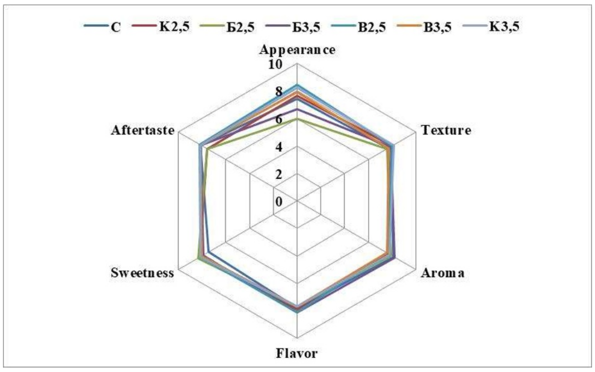


Fig. 6. Organoleptic evaluation of the chocolate candy samples compared to the control sample

4 Conclusion

An innovative approach was used to incorporate a probiotic strain encapsulated in cocoa butter into a chocolate matrix. The high concentration of viable cells in the gel matrix provided the required amount of active cells and their beneficial effect on the organism. The introduction of blueberry, blackcurrant and cherry flours enriches the chocolate matrix with substances with antioxidant properties. The functionality of chocolate products is thus enhanced. It has been shown that the smaller the imported gel beads, the better the rheological characteristics of the prepared chocolate products.

Table 6. Microbiological status of the chocolate candies

Sample type	Microbiological parameters							
	Enterobacteria	Fungi	Yeast	Sulfitreducing clostridia	*Salmonella* sp.	Coliforms	Coagulase-positive staphylococci	*E.coli*
0 – 20	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10
21 – 30	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10
31 – 50	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10
51 – 80	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10
over 80	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10
Average size, µm	< 10	Not found	Not found	< 10	Not found	< 10	< 10	< 10

References

1. A. H. Rad, M. M. Roudbaneh, V. G. Tabrizian, M. Javadi, N. Harati, H.N. Rad, Z. Kasaie, Chocolates as a probiotic carrier food: a review. International Journal of Probiotics and Prebiotics, 11, 37–42, (2016).
2. M. Hossain, C. Ranadheera, Z. Fang, S. Ajlouni, Healthy chocolate enriched with probiotics: a review. Food Science and Technology, 40(4), 1–13, (2020).
3. A. Zumbe, A. Lee, D. Storey, Polyols in confectionery: the route to sugar-free, reduced sugar and reduced calorie confectionery, British Journal of Nutrition 85, Suppl. 1, pp.31-45, (2001). https://doi.org/10.1079/BJN2000260
4. S. Possemiers, M. Marzorati, W. Verstraete, T. Van de Wiele, Bacteria and chocolate: a successful combination for probiotic delivery. International Journal of Food Microbiology, 141(1-2), 97-103, (2010) DOI: https://pubmed.ncbi.nlm.nih.gov/20452073/
5. J. Oracz, E. Nebesny, D. Zyzelewicz, G. Budryn, B. Luzak, Bioavailability and metabolism of selected cocoa bioactive compounds: a comprehensive review. Crit. Rev. Food Sci. Nutr. 60 (12), 1947–1985, (2020). https://doi.org/10.1080/10408398.2019.1619160
6. V. Sorrenti, S. Ali, L., Mancin, S. Davinelli, A. Paoli, G. Scapagnini, Cocoa polyphenols and gut microbiota interplay: bioavailability, prebiotic effect, and impact on human health. Nutrients 12 (7), 1908, (2020). https://doi.org/10.3390/nu12071908
7. G. Marshalkin, Technology of confectionery products; Pischevaya Promyshlennost 445 pp. (in Russian), (1978).
8. M. Min, C. R. Bunt, S. L. Mason, M. A. Hussain, Non-dairy probiotic food products: an emerging group of functional foods. Critical Reviews in Food Science and Nutrition, 59(16), 1-16, (2019). http://dx.doi.org/10.1080/10408398.2018.1462760
9. Food and Agricultural Organization of the United Nations and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations [online], (2002).
10. Q.Y. Dong, M. Y. Chen, Y. Xin, X. Y. Qin, Z., Cheng, L. E. Shi, Z. X., Tang, Alginate-based and protein-based materials for probiotics encapsulation: a review. Int. J. Food Sci. Technol. 48 (7), 1339–1351 (2013). https://doi.org/10.1111/ijfs.12078
11. G. Frakolaki, V. Giannou, D. Kekos, C. Tzia, A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods. Crit. Rev. Food Sci. Nutr. 61 (9), 1515–1536, (2020). https://doi.org/10.1080/10408398.2020.1761773
12. N. V. Braber, L. Vergara, Y. Rossi, C. Aminahuel, A. Mauri, L. Cavaglieri, & M. Montenegro, Effect of microencapsulation in whey protein and water-soluble chitosan derivative on the viability of the probiotic Kluyveromyces marxianus VM004 during storage and in simulated gastrointestinal conditions. LWT—Food Science and Technology, 118, 108844 (2020). https://doi.org/10.1016/j.lwt.2019.108844
13. N. M. Hossain, C. S. Ranadheera, Z. Fang, S. Ajlouni, Impact of encapsulating probiotics with cocoa powder on the viability of probiotics during chocolate processing, storage, and in vitro gastrointestinal digestion. J. of Food Science; 86, 1629–1641, (2021). DOI: https://pubmed.ncbi.nlm.nih.gov/33822381/
14. Directive 2000/36/EC of the European Parliament and of the Council concerning cocoa and chocolate products intended for human consumption (2000).
15. L. Robert, Jr. Bradley, Moisture and total solids analysis In: Food analysis, Springer, New York, pp. 85-104, (2010).

16. I. Lurie, L. Skokan, A. Tsitovich, Technochemical and microbiological control in confectionery production, Moscow, Koloss, 415 pp.(in Russian), (2003).
17. K. P. Petrov, Practicum on biochemistry of food and vegetable raw materials, ed. Pischevaya Promyshlennost, Moscow (in Russian), (1976).
18. OICC - Untersuchungamethoden des office International die Cacao et die Chocolat viskositat von Schokolade-Viskositat, Analytische Mathoden, Blat 10-D, (1970).
19. B. Milenkov, M. Hadjikinova, D. Hadjikinov, D. Hrusavov, A computer program for calculating the plastic viscosity of chocolate mass, Journal of Food and Packaging Science, Technique and Technologies, 5, pp.69-72, (2014).
20. Z. Todorova, Investigations into the use of Bulgarian soy phosvolepiden concentrate and other surface-active substances in the production of chocolate masses. PhD thesis, Institute of Food and Vegetable Chemistry (UFT), Plovdiv, 166 pg, (1982).