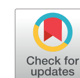
Electronic Journal of Biotechnology 48 (2020) 62–71

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Survival of commercial probiotic strains and their effect on dark chocolate synbiotic snack with raspberry content during the storage and after simulated digestion



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A R T I C L E I N F O

Article history:
Received 30 December 2019
Accepted 11 September 2020
Available online 18 September 2020

Keywords:
Beads
Bioactive compounds
Chocolate
Functional food
Maltitol
Polyphenols
Prebiotics
Probiotic bacteria
Pro-health foods
Raspberry

A B S T R A C T

Background: A key challenge for manufacturers of pro-health food containing active probiotic microorganisms is to develop a product with attractive sensory features along with maintenance of declared number of microorganisms during storage and transfer by alimentary tract.
Results: The highest concentration of polyphenols was observed in snacks without an additive of probiotics as well as those with an additive of *L. rhamnosus* and *B. animalis* bacteria and concentration of these compounds increased by 9.5% during six months of storage. None of the products distinguished itself in the sensorial assessment although each was assessed positively. The number of microorganisms was stable and comparatively high during six months of storage at a room temperature and in cooling conditions (10^8 cfu/g). In the digestion model, an influence of aggressive digestion conditions was examined in the alimentary tract on the number of microorganisms, which allowed to arrange strains from the most resistant (*S. boulardii*) to the most sensitive (*B. breve*). It must be noted that currently on the market there is no available snack containing probiotic yeast as well as there is no literature data on works on such formulation of food.
Conclusions: In the newly developed snack made of chocolate, in which sugar has been replaced with maltitol, a raw material was added in the form of raspberry, prebiotic in the form of inulin and a strain of probiotic bacteria, including the unprecedented so far *S. boulardii*, which stands a high chance to occupy a good place on the market of functional food.
How to cite: Cielecka-Piontek J, Dziedziński M, Szczepaniak O, et al. Survival of commercial probiotic strains and their effect on dark chocolate synbiotic snack with raspberry content during the storage and after simulated digestion. Electron J Biotechnol 2020;48. https://doi.org/10.1016/j.ejbt.2020.09.005.

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1. Introduction

The history of chocolate dates back to over 4000 years. Chocolate originated in the areas of today's Mexico where the cocoa bushes were discovered first. It is produced from cocoa beans, from which cocoa powder and cocoa butter are first obtained and used as its basic ingredients [1]. Cocoa butter is rich in fatty acids, including the saturated and unsaturated ones, free fatty acids, antioxidants, mineral salts, and vitamins [1,2,3]. Chocolate is also a source of tryptophan, serotonin, and dopamine and positively affects the cerebral metabolic processes and endorphin production [4]. Taking all these into account, probiotic chocolates can be considered as an interesting product that meets the criteria of functional foods rich in nutrients and health-promoting compounds and the expectations of consumers, especially children. Currently, the number of recognized chocolate products that are of premium quality and possess pro- and prebiotic properties is few. Therefore, novel chocolate products should be developed to be used as functional foods. Infectious diarrhea is a serious and very common problem among adults who travel frequently (so-called travel diarrhea), and persons with reduced immunity (e.g. HIV carriers, elderly, people who underwent chemotherapy) [5]. Probiotic microorganisms that are of particular importance in preventing and alleviating the effects of infectious diarrhea are bacterial strains of the genus *Lactobacillus* and Bifidobacterium and the bacterial species

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.09.005
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Streptococcus thermophilus, as well as the yeast species *Saccharomyces boulardii* [6].

Lactobacillus rhamnosus GG is one of the best examined probiotic bacteria strains, also in clinical conditions [7]. It demonstrates a capability of colonizing the mouth cavity and is characterized by anti-microorganism properties, inter alia, towards the *Streptococcus sobrinus* species which is the major reason for children's caries. *Bifidobacterium animalis* subsp. lactis belongs to psychobiotics due to the capability of generating GABA neurotransmitter [8]. It demonstrates anti-inflammatory properties in particular in case of intestine inflammation by bacteria from the Enterobacteriaceae family [9]. The research showed that daily consumption of *B. animalis* subsp. lactis improves indicators referred to as obesity biomarkers [10]. Moreover, a positive influence of *B. animalis* on neonates' body was proven, inter alia, in the aspect of reducing the risk of infection of respiratory tract [11]. *Bacillus coagulans* GBI-30, 6086 strain has a short history of use in the prevention of diarrhea [12]. This strain produces lactic acid, which supports the growth and multiplication of the beneficial bacteria present in the intestine. In this way, it helps maintain a proper balance of the intestinal microbiome and reduces the risk of diarrhea [12].The consumption of chocolates containing *B. coagulans* GBI-30 promoted the mobilization of the cells of the immune system to more effectively fight bacteria and viruses that are harmful to human health [13]. The results showed that the strain allows for more efficient digestion of vegetable proteins, thus preventing the production of gasses by fermentation in the large intestine [14,15]. *Saccharomyces boulardii* is one of few species classified to probiotics. Apart from the fact that it demonstrates features typical of pro-health microorganisms, its importance is significant in prevention of secondary infections with *Clostridium difficile*, it contributes to elimination of pathogenic *Helicobacter pylorii* bacteria, thanks to which it prevents acute inflammatory states of the alimentary system, mainly of stomach and intestines [16,17,18]. The use of *S. boulardii* is advisable in treatment of blastoctoza [19]. Bifidobacterium breve is also an important and effective species of probiotic bacteria. There is literature data which shows that its use supports treatment of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [13,20]. Moreover, it has been noted that the use of Bifidobacterium combined with *Lactobacillus* allows to increase effective treatment of infections caused by *H. pylori* [21] twice. The literature data also indicates the participation of *B. breve* in the relation between bowels and brain and in prevention of tendencies to obesity [22,23]. Although a lot of mechanisms of probiotics effect are not known due to the increasingly numerous scientific evidence indicating their positive influence on body, it is advisable to consume them a few times per week [24].

The objective and at the same time novelty of this work consists in preparation of a biofunctional snack containing the core consisting of lyophilisate of raspberry fruits and inulin as well as an external coat comprising dark chocolate and one of five species of probiotic microorganisms. At the same time, it must be noted that currently on the market there is no product that is so complex and rich in pro-health substances, containing probiotic yeast.

2. Materials and methods

2.1. Materials

The raw samples of chocolates were purchased from “BARS” Halina Kalemba (Wloszakowice, Poland), a semi-industrial-scale factory of confectionery products. The obtained masses and bars of dark and white chocolates were examined and enriched in a lyophilizate of live cells of the following probiotic species: *L. rhamnosus* GG (5.0×10^{11} cfu/g), *B. breve* DSM 16604 (2.5×10^{11} cfu/g), *Bifidobacterium animalis* subsp. *lactis* (2.0×10^{11} cfu/g), *S. boulardii* (2.0×10^{10} cfu/g), and *B. coagulans* GBI-30, 6086. *Bacillus coagulans* strain was purchased from Cornelius Poland (Warsaw, Poland), while the other microbial strains were purchased from Probiotical S.p.A. Import-BART Sp. z o.o. Sp. K (Słupno, Poland) in powdered form, with granules ranging from 100 to 200 μm. Freeze-dried raspberry was obtained from WPPH “Elena” (Kokanin, Poland), and long-chain inulin from NVC (Sulejówek, Poland).

2.1.1. Chocolate beads preparation

Probiotic chocolate snacks enriched in fruits were prepared with two layers: a core made of white chocolate with inulin, as a prebiotic and fruit fraction (lyophilized raspberry); and an outer layer made of dark chocolate with probiotic microorganisms (bacteria or yeasts) (Table 1). The white chocolate core was enriched in bee honey to improve plasticity. The dose of probiotic microorganisms was chosen in such a way that their numbers were even for each experimental variant and amounted to 10^8 cfu/g. The probiotic mass fraction constituted 5% of the product core. The beads prepared with probiotic microorganisms were named as follows: BLr - *L. rhamnosus*, BBb - *B. breve* DSM 16604, BBa - *B. animalis* subsp. *lactis*, BSb - *S. boulardii*, and BBc - *B. coagulans* GBI-30, 6086. The first stage of work involved the development of a recipe for a functional chocolate-fruit snack. The main requirement for preparation was that the snack should be in the form of a ball with the core (inner layer) containing a inulin and the shell (outer layer) containing a probiotic in the form of a lyophilizate. First, the number of probiotic microorganisms in particular lyophilisates was marked. Afterwards, it was calculated what dose (g) of lyophilisate will correspond with 10^8 cfu/g. The dose determined in grams was added to liquefied dark chocolate at 50°C.

Accordingly, five variants of double-layered beads were prepared, containing various probiotic microorganisms at an equal number of 1.0×10^8 cfu/g and 5% inulin, and a control without a probiotic. The newly developed products were analyzed after the production process and during storage for physicochemical features such as pH value and water activity, as well as the content of polyphenols, including flavonols, phenolic acids, and catechins. In addition, the products were tested in an in vitro model imitating the digestive process in the stomach to assess the impact of the aggressive conditions of the digestive tract on the number of probiotic microorganisms.

2.2. Methods

2.2.1. Chemical composition of prepared beads

The chemical composition of the samples included lipid, protein, ash, fats and fatty acids, saccharose, polyols, and cholesterol which was determined using the AOAC method [25]. Fiber content was found

Table 1
The nutritional value of the developed product.

Nutritional value	Per 100 g product	Per portion (30 g)	% DRI[a] per portion (30 g)
Energy [kcal/kJ]	454.9/1901	136/570	6.8
Fats, incl. [g]	27.0	8.1	11.6
Saturated fatty acids [g]	16.2	4.9	24.3
Monounsaturated fatty acids [g]	8.6	2.6	–
Polyunsaturated fatty acids [g]	1.0	0.3	–
Cholesterol [mg]	8.1	2.4	–
Carbohydrates, incl. [g]	55.8	16.7	6.4
Starch [g]	1.5	0.5	–
Monosaccharides [g]	28.5	8.5	9.5
Lactose [g]	0.0	0.0	–
Saccharose [g]	0.5	0.1	–
Polyols [g]	18.2	5.5	–
Proteins [g]	5.3	1.6	3.2
Fiber [g]	6.4	1.9	–
Iron [mg]	7.0	2.1	15
Calcium [mg]	92.1	27.6	23

[a] DRI—Dietary Reference Intake.

using a method described by Dziedzic et al. [26]. Carbohydrates content constituted the difference of 100 and the sum of protein, fat, water and ash. Minerals content was estimated according to the PN-R-04014:1991 standard [27]. Energy value and the recommended daily intake (RDI) were calculated according to the Regulation of the Minister of Health on nutritional value labeling and European Parliament and Council Regulation (UE) [27,28].

2.2.2. pH and water activity (aw) measurement

The pH changes in the three types of probiotic chocolates analyzed were determined using a pH-meter (Mettler-Toledo, USA). Water activity (a_w) was determined using the Thermoconstanter Novasina RTD 33 TH-1 a_w-meter (Novasina, Lachen, Germany). These parameters were analyzed at the beginning of the experiment and during the storage period at measurement points T2-T4.

2.2.3. Extraction

The chocolate samples were defatted three times using 2.0 g of chocolate material per 10 mL of n-hexane (POCh, Poland, Warsaw). The defatted cocoa solids were air-dried for 24 h to remove the residual organic solvent. The cleaned and defatted samples (2.0 g) were extracted two times with 10 mL of aqueous methanol (40:60) (700 g/L) for 30 min in an ultrasonic bath to obtain the extract. After each extraction, the mixture was centrifuged for 10 min at 3000 × *g* and the supernatant was decanted. The supernatants were combined and filtered to remove the residual particles.

2.2.4. Catechin content

Catechins were identified by the HPLC method using the RP-C18 column (150 × 4.6 mm; 5 μm) (Agilent, USA), a photo-diode detector (λ = 279 nm), and a gradient elution system. As an eluent, a mixture of 0.01 M phosphate buffer and methyl alcohol was used at a flow rate of 1 mL min^{-1}. The references used were as follows: (-)-epicatechin (min. 90%; Merck-Sigma, Poland, Warsaw), (+)-catechin (min. 98%; Merck-Sigma, Poland, Warsaw), and (-)-epigallocatechin gallate (min. 95%; Merck-Sigma, Poland, Warsaw). The relationship between the resultant peaks and the concentrations of the three studied compounds was analyzed. Calibration curves of the studied compounds were generated for seven concentration levels and five independent measurements for each of them. The concentrations of (+)-catechin and (-)-epicatechin in the analyzed products were presented in mg per 100 g d.w., and the concentration of (-)-epigallocatechin gallate in g per 100 g d.w.

2.2.5. Composition of phenolic acids and flavonols

The percentage composition of phenolic acids was analyzed using an Agilent UPLC system (Perlan Technologies, Poland, Warsaw) equipped with a Bin Pump Infinity DAD 1290 detector (λ = 260 and 310 nm). A nonlinear concentration gradient was used to analyze the dry extract. The gradient consisted of H_3PO_4 buffer (solvent A, pH 2.7), which was adjusted using acetonitrile-water mixture (solvent B, 1:1 (v/v)). The gradient profile decreased from 95% of solvent A at 1 min to 50% of solvent B at 52 min, and the run time was 58 min. The volume of samples injected after filtration (using a PTFE (polytetrafluoriethylene) filter; 0.45 μm) was 10 μL. Phenolic acids were identified using the standards dissolved in methanol (caffeic, chlorogenic, ferulic, and p-coumaric acids). The extracted flavonols were separated and identified with the Agilent UPLC system (Perlan Technologies, Poland, Warsaw) using a Nova-Pak C18 reversed-phase column (3.9 × 150 mm, particle size 5 μm; both from Waters, Milford, MA, USA). A solution of 0.3% (v/v) HCOOH in H_2O was used as solvent A, while 100% CH_3CN (Honeywell, United Kingdom) was used as solvent B. The flow rate was maintained at 1 mL min^{-1}. The gradient profile was as follows: 85% of solvent A at 0 min and 25% of solvent B at 40 min. Chromatograms were obtained using a UV–Vis detector at λ = 370 nm. The separated flavonol compounds were determined as isoquercetin, quercetin, and kaempferol, and the percentage composition of individual flavonols was calculated.

2.2.6. Sensory profiling

The profile of the dark probiotic chocolate samples tested was assessed in the sensory laboratory. To determine a broad range of sensory characteristics, that is, sensory profiling, a quantitative method was used. This was performed by a 16-member team who had been trained for this purpose. The sensory sensitivity of the members was confirmed before analysis. As a part of sensory profiling, the following qualitative determinants were marked: color (brown, gray, crème, and general preference), odor (yeast, off, seasoning, metallic, and general preference), taste (salty, spicy, yeast, off, metallic, chemical, bitter, and general preference), and consistency (moisture and crispness). The intensity of each qualitative mark was determined using a structured 10-cm-long line scale with adequate edge captions (intense/not intense). The obtained results were converted into numerical values and presented in points. Trials were encoded and given to the examiners, guaranteeing the anonymity of the first, by placing them in white, odorless, lockable vessels. All trials were performed at 21 ± 2°C, and the number of trials was chosen to guarantee the possibility of testing the products several times.

2.2.7. In vitro digestion model

To determine the kinetics of changes in bacterial count in the probiotic chocolates containing microorganisms, the in vitro digestion model was applied. The experimental set up was a fermenter of 1-L volume (Sartorius, Poland, Warsaw). The temperature of the fermenter was set at 37°C. Two experimental trials were conducted in this study. For the first trial which was the control run, the bacterial lyophilizate was dissolved in phosphate-buffered saline (PBS) (pH 7.4) (Merck-Sigma, Poland, Warsaw). The second trial was carried out with milk chocolate with probiotic microorganisms at concentrations of 1.0×10^6, 1.0×10^7, and 1.0×10^8 cfu/g. In the initial step, a mixture of chocolate samples in PBS buffer or a selected nutrient matrix was prepared. This prepared sample was subjected to the first step of in vitro digestion, which was designed for simulating the conditions in the stomach (P1). A model gastric juice was prepared with 300 U/mL pepsin (Merck-Sigma, Poland, Warsaw) and lowering the pH to 4.0 using 1 M HCl. This step was performed for 4 h at 37°C (P2). Peristaltic movements were simulated by stirring the mixture with a magnetic stirrer. In the next step, digestion in the small intestine was imitated by changing the pH to 6.0 using 1 M $NaHCO_3$ and adding 10 mL of pancreatic intestinal extract (Merck-Sigma, Poland, Warsaw) (P3). In the next step, the pH was increased to 7.4 by adding 1 M $NaHCO_3$, following which a standardized inoculum (MSA-2006, ATCC, VA, United States) of the intestinal microbiome (10^6 cfu/mL of the food content) isolated from human stool was introduced. The digestion process was carried out for 2 h at 37°C (P4). To simulate the passage of the product through the large intestine, the pH was increased to 8.0 with 2 M $NaHCO_3$, and further digestion was carried out under anaerobic conditions for 18 h (P5).

2.2.8. Bacterial count determination

The number of microorganisms was determined using serial dilution method. To determine the number of *Lactobacillus* bacteria, MRS-agar medium (Biocorp, Warsaw, Poland) was used [29]. The incubation was carried out for 48 h at a temperature of 35°C. To determine the number of *Bifidobacterium* bacteria, BSM medium was applied (Merck-Sigma, Warsaw, Poland), and the incubation was carried out at 37°C for 48 h under absolutely anaerobic conditions. To determine the number of yeast, the potting method was used with PD medium containing chloramphenicol (Biocorp, Warsaw, Poland). The incubation was carried out at a temperature of 30°C under aerobic conditions for 48–72 h. For the determination of the number of *Bacillus* bacteria, the number analysis method was used according to

the manufacturer's instructions. Briefly, 1 g of the sample was dissolved in 199 mL of peptone water and mixed for 5 min in a stomacher (Bax Mixer, Bionovo, Poland, Legnica), and the pH of the suspension was adjusted to 8.5. Then, 20 mL of the suspension was incubated at 75°C for 30 min. Following incubation, the suspension was immediately cooled to a temperature below 40°C. In the next step, 1.0 mL of the cooled suspension was transferred to a sterile tube containing 9 mL of peptone water. The microbial count in the prepared suspension was 0.5×10^3 cfu/g of product. Then, further decimal dilutions were performed. After dilutions, 1.0 mL of the prepared sample was spread on the surface of the media, and at least two Petri dishes for each medium were used for each dilution of the sample. To determine the total count of *Bacillus* bacteria, a *Bacillus* Selective Agar Base (Merck-Sigma, Warsaw, Poland), without supplement, liquefied with medium was used. The Petri dishes were incubated for a minimum of 48 h at 40°C.

2.2.9. Storage test

All chocolate variants, as well as the bacterial lyophilizate, were stored for 24 months both at room temperature (20°C) and under refrigeration condition (4°C). Similar to the determination of the bacterial count, the bacterial viability was analyzed at the following time points: postproduction (T1) and one (T2), three (T3), and six (T4) months postproduction.

2.2.10. Statistical analysis

The experiment was repeated three times, and all measurements were obtained in duplicate. The STATISTICA PL 13.1 (StatSoft, Inc., Krakow, Poland) software was used to calculate the values of means and standard deviations in principal component analysis, and to evaluate the significance of differences between means. The analysis of variance was conducted for a completely randomized design experiment. Tukey's test was used for comparing the mean values at a significance level of $p \le 0.05$. The slope of the regression curve (coefficient A/24 h) was determined in the Excel 2007 program to present the dynamics of the measured factors (i.e. physical changes) and to show the changes in the polyphenols content during storage. The data were expressed as means and standard deviations of independent measurements for four specimens (n = 4). The relationship between the factors and the storage periods for each factor can be described using [Equation 1].

$$Y = A \times X + B \qquad [1]$$

where y is the dependent variable, x is the independent variable, A is the independent variable coefficient per slope of the line, and B is the intercept.

3. Results

3.1. Physicochemical indicators: pH and water activity

Among a number of physicochemical indicators describing the quality of food products, pH and water activity were assessed in this work. The analysis of the pH values after six months of storage (Table 2) showed that the pH of all samples was similar (ranging between 4.43 and 4.75) and increased after the storage period. The R^2 parameter was determined for each sample to assess the relationship between the pH value and storage time. All the R^2 coefficients were in the range of 0.0152–0.9694, which indicated the disproportionate increase in pH in relation to storage time. It was noted that the addition of the probiotic strain had no impact on the pH value of the samples.

During storage, the water activity of the samples was also monitored. It was observed that after six months of storage A_w decreased by 9.2–21.8% on average. The R^2 coefficient ranged between 0.9243 and 0.9688, which indicated the proportionate decrease in a_w during the storage of the tested symbiotic products.

Table 2
Changes in pH, A_w values, and total content of polyphenols at different storage times. The dynamics of changes is expressed as r^2.

	T1	T2	T3	T4	R^{2*}
pH, n = 5					
Bc	$4.59^{ab} \pm 0.07$	$4.77^{c} \pm 0.11$	$4.72^{c} \pm 0.14$	$4.67^{b} \pm 0.10$	0.0152
BLr	$4.67^{a} \pm 0.05$	$4.68^{a} \pm 0.02$	$4.72^{b} \pm 0.04$	$4.72^{b} \pm 0.15$	0.7854
BBb	$4.43^{a} \pm 0.02$	$4.52^{b} \pm 0.12$	$4.47^{a} \pm 0.09$	$4.55^{b} \pm 0.05$	0.5227
BBa	$4.62^{a} \pm 0.04$	$4.69^{a} \pm 0.14$	$4.71^{b} \pm 0.05$	$4.75^{b} \pm 0.10$	0.8372
BSb	$4.76^{a} \pm 0.03$	$4.77^{a} \pm 0.11$	$4.74^{a} \pm 0.11$	$4.74^{a} \pm 0.08$	0.6367
BBc	$4.68^{a} \pm 0.04$	$4.71^{a} \pm 0.10$	$4.73^{a} \pm 0.02$	$4.77^{a} \pm 0.04$	0.9694
Aw, n = 5					
Bc	$0.275^{b} \pm 0.04$	$0.266^{b} \pm 0.07$	$0.283^{b} \pm 0.09$	$0.244^{a} \pm 0.04$	0.4639
BLr	$0.284^{d} \pm 0.04$	$0.261^{c} \pm 0.01$	$0.242^{b} \pm 0.03$	$0.222^{a} \pm 0.02$	0.9369
BBb	$0.281^{c} \pm 0.01$	$0.283^{c} \pm 0.06$	$0.265^{b} \pm 0.02$	$0.255^{a} \pm 0.08$	0.9243
BBa	$0.277^{c} \pm 0.02$	$0.271^{c} \pm 0.01$	$0.255^{b} \pm 0.07$	$0.221^{a} \pm 0.03$	0.9867
BSb	$0.291^{c} \pm 0.04$	$0.288^{c} \pm 0.04$	$0.253^{b} \pm 0.05$	$0.231^{a} \pm 0.06$	0.9589
BBc	$0.282^{c} \pm 0.07$	$0.280^{c} \pm 0.06$	$0.273^{b} \pm 0.08$	$0.255^{a} \pm 0.02$	0.9688

Bc - lack of probiotic organisms, BLr - *Lactobacillus rhamnosus* GG, BBb - *Bifidobacterium breve* DSM 16604, BBa - *Bifidobacterium animalis* subsp. *lactis*, BSb - *Saccharomyces boulardii*, and BBc - *Bacillus coagulans* GBI-30, 6086; T1 - postproduction, T2 - one month postproduction, T3 - three months postproduction, and T4 - six months postproduction.
a, b, c - mean values marked with different letters in the line differ significantly according to Tukey's test ($p \le 0.05$).
*Linear regression equation [Equation 1], coefficient A/time of storage—change of coefficient A during storage; R^2—coefficient of determination, $p < 0.05$.

3.2. Phenolic acids, flavonols, and catechins content

Tested samples were analyzed for their polyphenols content (Table 3). It was noted that storage affected the composition of analyzed aglycons in the symbiotic products. The prohealth potential of the newly developed products was evaluated by determining the total content of phenols distinguished into flavonols, phenolic acids, and catechins Table 3 shows the total content of polyphenols in the newly developed chocolate-fruit beads during their storage period.

From the data presented in Table 3, it appears that the total content of phenolic compounds increased in all tested samples. The highest content was observed in the control sample, BLr, and BBa, in which phenols content was increased after six-month storage by 9.5% in relation to the content at the first measurement point. The R^2 coefficient ranged between 0.5551 and 0.9950, which indicated that changes in phenolic compounds in the tested samples during storage were proportionate to the storage time.

In the following stage, the developed symbiotic products were tested for their composition of flavonols and phenolic acids (Fig. 1).

Among the analyzed compounds, the content of quercetin was the highest, followed by others in the order: chlorogenic acid > isoquercetin, ferulic acid > kaempferol > *p*-coumaric acid > caffeic acid. It was noted that storage and the species of probiotic strains had no significant impact on the changes in the analyzed polyphenols. Differences in values between the first and last measurement times were also not significant. The chocolate beads were also analyzed for their catechin content (Fig. 2).

Epicatechin constituted the highest fraction in the tested compounds, followed by catechin. The content of epigallocatechin gallate in the chocolate beads was significantly lesser compared to the other flavonoids. No significant changes in the content of these flavonoids were observed after the six-month storage.

3.3. Sensory profile

During the research, the sensory qualities of chocolate beads were analyzed by the sensory panel. The assessed parameters were the

Table 3
Total phenolic content in biofunctional chocolate-fruit beads.

Tested product	Measurement point				R^2
	T1	T2	T3	T4	
	Total content of polyphenols [mg/g]				
Bc	475.33[a] ± 2.42	477.57[a] ± 4.55	525.66[b] ± 3.18	511.31[b] ± 6.44	0.5551
BLr	473.25[a] ± 3.44	479.41[a] ± 6.31	523.12[b] ± 5.72	519.37[b] ± 8.31	0.7310
BBb	489.51[a] ± 6.55	477.71[a] ± 7.21	501.87[b] ± 3.43	521.40[b] ± 3.65	0.8360
BBa	470.31[a] ± 0.02	488.17[b] ± 0.02	499.79[b] ± 0.02	510.61[b] ± 0.02	0.8884
BSb	481.31[a] ± 5.07	492.44[b] ± 1.99	492.61[b] ± 7.09	499.43[b] ± 4.65	0.7737
BBc	485.32[a] ± 8.11	486.66[b] ± 1.61	491.92[b] ± 3.62	499.43[b] ± 7.71	0.9950

Bc - lack of probiotic organisms, BLr - *Lactobacillus rhamnosus* GG, BBb - *Bifidobacterium breve* DSM 16604, BBa - *Bifidobacterium animalis* subsp. *lactis*, BSb - *Saccharomyces boulardii*, and BBc - *Bacillus coagulans* GBI-30, 6086; T1 - postproduction, T2 - one month postproduction, T3 - three months postproduction, and T4 - six months postproduction N = 3.

color of the outer layer, the color of the bead core, aroma, and taste (Fig. 3). The outer and inner layers were evaluated based on five descriptors. Both color and homogeneity of the layers were tested. No significant differences were noticed in the color descriptors between the tested samples. However, higher homogeneity of dark color was observed in the outer layer (9.5) of dark chocolate, compared to the inhomogeneous color in the inner layer of white chocolate (5.0). The outer layer had a dark brown color with shades of gray, while the core of the product was pinkish beige. Chocolate beads possessed a slightly sour chocolate flavor and a mild chocolate aroma. The addition of probiotics did not affect the intensity of chocolate flavor, nor caused off or bitter flavor.

3.4. Microorganisms survivability in biofunctional chocolate-fruit beads

The effect of in vitro digestion process on the viability of microorganisms present in chocolate beads was analyzed (Fig. 4). Analysis was made at all five control points. The first one imitated the gastrointestinal matrix, which was not subjected to the conditions prevailing in the digestive tract. The second one simulated the conditions in the stomach, the third the conditions in the duodenum, the fourth the conditions in the small intestine, and the final stage the conditions in the large intestine. The initial number of probiotic microorganisms was equalized in all the variants tested, and ranged from 1.0 to 1.9 × 10^8 cfu/g. The number of live cells differed in each tested sample and during each digestion stage. The results showed that after the complete digestion process (P5), the highest number of microorganisms was found in the chocolates prepared with *S. boulardii* yeasts. In this sample, the number of yeasts was even at the end of the digestion (7.3 × 10^8 cfu/g) process than at the beginning (1.0 × 10^8 cfu/g). The poorest results were observed for chocolate samples prepared with *Bifidobacterium* bacteria. In both cases, a drop in the number of bacteria from 10^8 to 10^5 cfu/g was noticed. In the case of *L. rhamnosus*, *B. lactis*, and *L. rhamnosus*, a slight decrease in the number of bacteria was observed at the end stage. Hoverer, for all the tested samples, we observed the largest drop in microorganisms number after the digestion step imitating the process in the stomach. Probably, this was caused by the decrease of pH to 2.0 and the addition of high-active proteolytic enzymes. A significant decrease was observed in the number of *Bifidobacterium* bacteria, which did not revert even after the conditions were changed to more favorable ones. The highest survivability in the in vitro digestion conditions was observed for yeasts which are known to possess numerous protection mechanisms activated as a response to multifunctional environmental stress [30], should be added that a question of food matrix is not meaningless. Thus, the rich composition of chocolate and its rheological properties allowed retaining a high number of microorganisms both during the digestion process and storage period [31].

3.5. Assessment of probiotic microorganisms stability during storage period

The beads were stored for six months after the final production process. Two different temperatures were applied during storage (4 and 20°C) in order to test the microbiological stability of the beads. The samples were analyzed after one, three, and six months of storage.

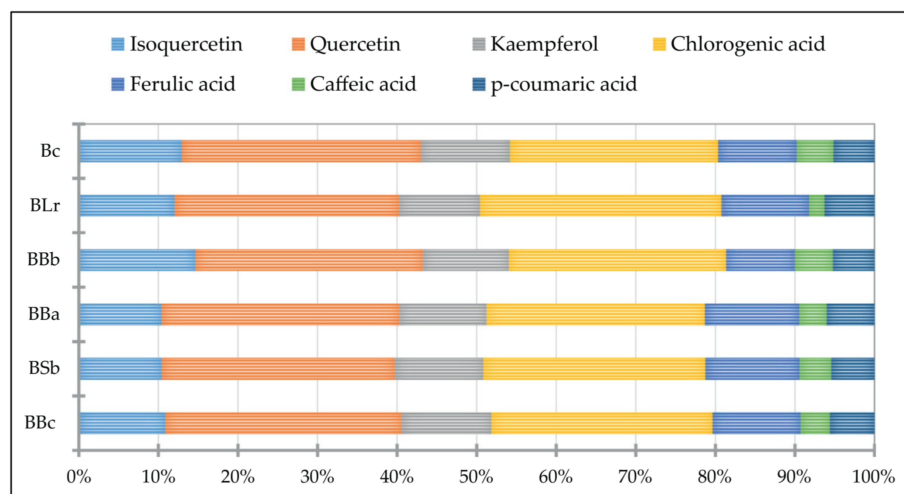

Fig. 1. Concentration (in per cent) of flavonols and phenolic acids in probiotic chocolate-fruit beads. Bc - lack of probiotic organisms, BLr - *Lactobacillus rhamnosus* GG, BBb - *Bifidobacterium breve* DSM 16604, BBa - *Bifidobacterium animalis* subsp. *lactis*, BSb - *Saccharomyces boulardii*, and BBc - *Bacillus coagulans* GBI-30, 6086.

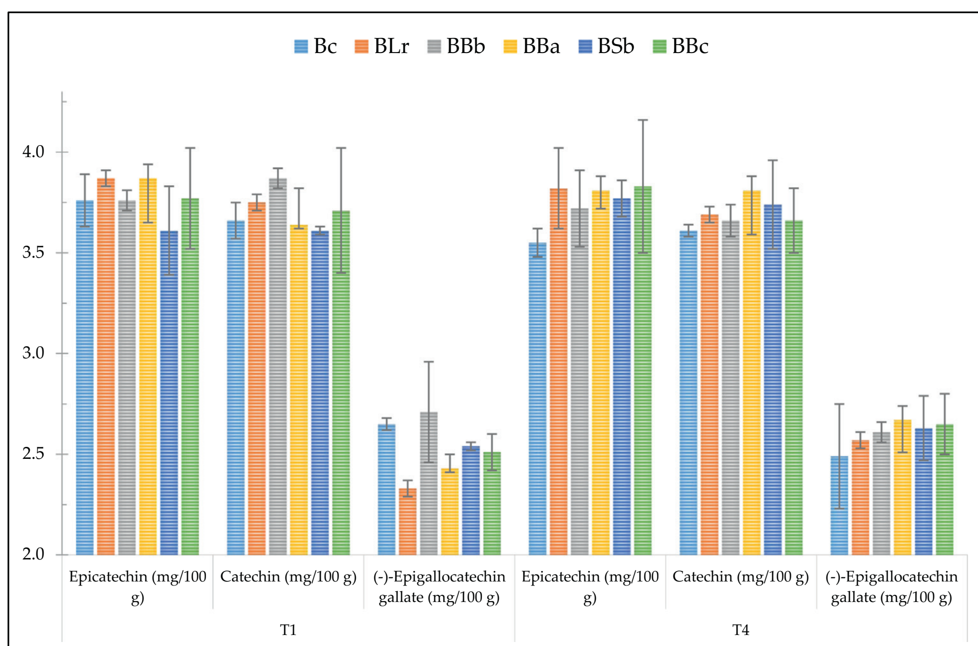

Fig. 2. Catechin content (mg/100 g) in tested chocolate beads. Bc - lack of probiotic organisms, BLr - *Lactobacillus rhamnosus* GG, BBb -*Bifidobacterium breve* DSM 16604, BBa - *Bifidobacterium animalis* subsp. *lactis*, BSb - *Saccharomyces boulardii*, BBc - *Bacillus coagulans* GBI-30, 6086; T1- postproduction, T4 - six months postproduction.

From the results shown in Table 4, an optimistic conclusion can be drawn that the six-month storage period did not negatively affect the number of microorganisms. In all the tested samples, the total number of microorganisms was high, similar to the initial level (Table 4, T1 measurement point), irrespective of the temperature applied. The only variant for which a decrease in the number of microorganisms was observed was chocolate-fruit beads containing lactic acid fermentation bacteria *L. rhamnosus* GG. The number dropped from the initial 1.9×10^8 to 2.0×10^7 cfu/g during the sixth month of storage at the ambient temperature. However, in the same beads stored at 4°C, no decrease in the number of *L. rhamnosus* GG bacteria was observed.

4. Discussion

Probiotic chocolates are currently considered as a dynamically developed group of food products. Although a significant number of patents on probiotic chocolates including our functional dessert have been registered, there is still space for innovation [32,33,34].

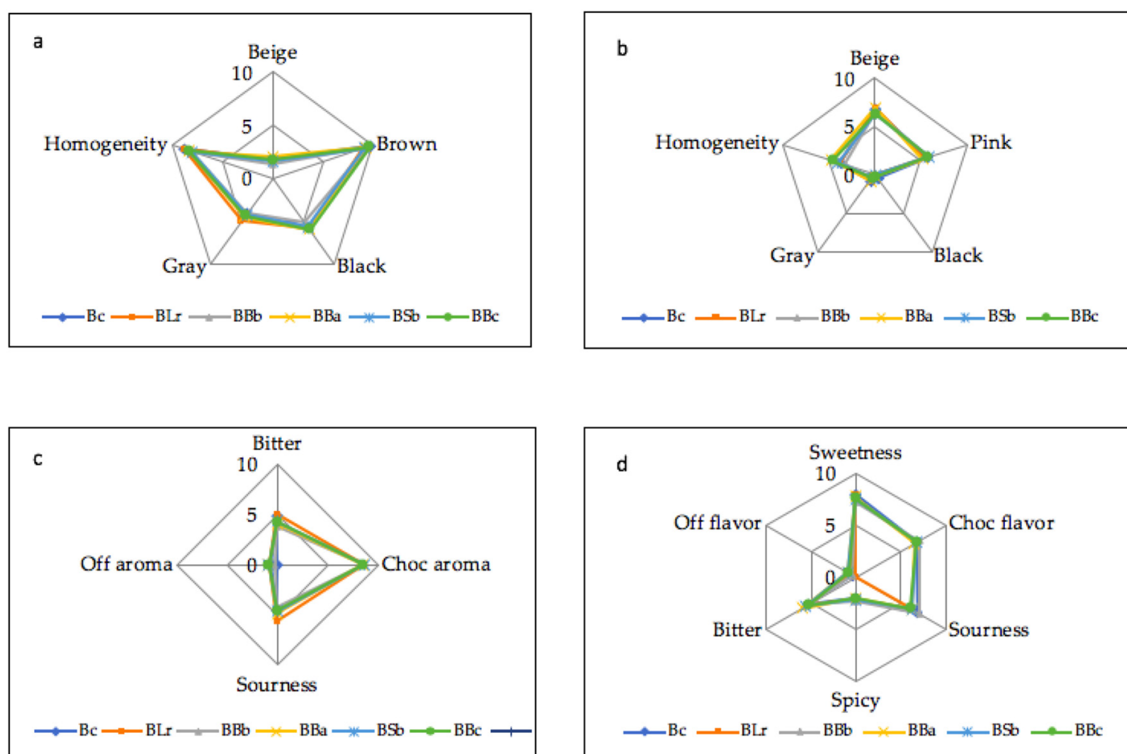

Fig. 3. Sensory profile of symbiotic chocolate beads: (a) outer color, (b) inner color, (c) aroma, and (d) taste.

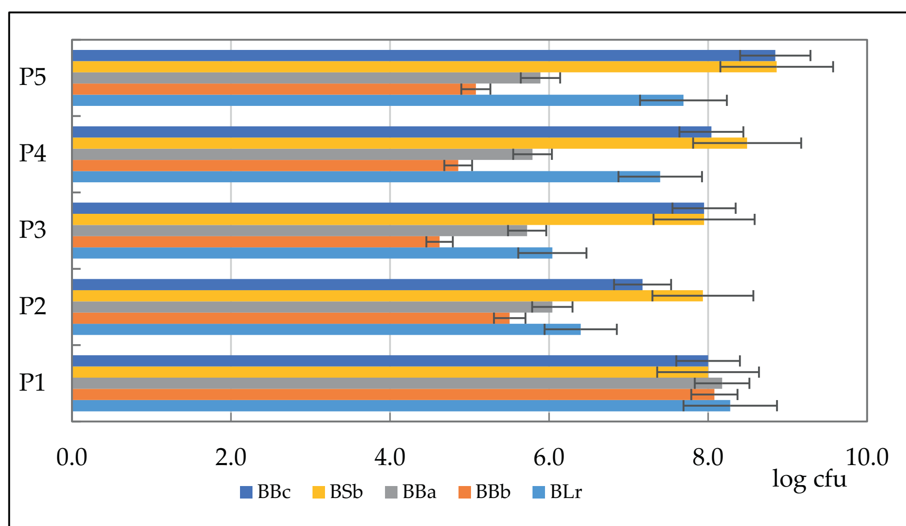

Fig. 4. Number of probiotic microorganisms, which are the biofunctional additive, in chocolate-fruit beads after simulated gastric digestion in vitro (P1-P5, as in the "*In vitro* digestion model" section).

Biofunctional pro- and prebiotic chocolates can be an interesting snack not only for children but also for adolescents. A number of conscious consumers choose novel products with confirmed biofunctional properties. However, in addition to ingredients, parameters that clearly indicate that a product possesses prohealth potential and commonly acknowledged properties should be assessed. Water activity is one of the basic product determinants. This parameter is related to the factors affecting the product stability - growth and enzymatic activity of microorganisms, rate of nonenzymatic browning reaction, and rate of oxidation of fats and colorants. As a result, these associations were linked with a trend of changes in the mechanical properties of food, which affect the sensory profile [35]. In the case of chocolate beads with prebiotics, water activity was significant in the context of not only microbial safety but also the stability of probiotic strains. A minimum water activity that is needed for the growth of different groups of microorganisms is 0.9; for a majority of bacteria, $a_w = 0.8$, and for a majority of yeasts or molds, $a_w = 0.7$. It is commonly stated that microorganisms cannot grow in food with $a_w < 0.6$ [28]. Low water activity of the prepared chocolates likely affected the stability of the probiotic strains. It also had an impact on the sensory attractiveness, as enzymatic reactions, which change the profile of taste and aroma, usually require the presence of water [36].Chocolate contains a high amount of fat (Table 1), which is labile to oxidation processes, often occurring at dry conditions with less water activity (minimal oxidation rate of fats at an a_w range of 0.1–0.3). The highest stability of food products is reached at a water activity equal to the water content in monolayer, that is, a_w of 0.07–0.35 corresponding to a water content of 2–15% [37]. Therefore, changes in these indicators determine many of the properties of the chocolate beads. The changes in pH and a_w could also affect the content of phenolic compounds. In this study, an increase in the total content of polyphenols was observed during storage, while the content of specific catechins and the ratio of the tested polyphenols remained constant. This phenomenon can be explained by the specificity of the methods used. In the Folin-Ciocalteau method, interferences may be induced by different nonphenolic compounds such as reducing sugars, aromatic amines, sulfuric dioxide, ascorbic acid, sorbic acid, ferrous irons, and others. The effect observed was feasibly related to the changes in the chocolate matrix, which either caused or were the effects of changes in the water activity and pH of the product. As mentioned, the production processes are usually considered to affect negatively the total phenolic content. Such results were previously shown for raw materials and manufactured products [36]. Not only processing but also long-term storage can accelerate the processes of enzymatic and chemical oxidation of polyphenolic ingredients, and the degree of these changes depends on raw material or environmental conditions (e.g. temperature, pH, water activity, time, and oxygen concentration).

A previous study showed the high antioxidative potential of dark chocolate enriched in probiotics [31]. The addition of lyophilized raspberry should have reduced the total antioxidant capacity of the

Table 4
Number of probiotic microorganisms during storage of chocolate-fruit beads at temperatures of 20 (a) and 4 °C (b).

Measurement point	Incubation time (month)	BLr	BBb	BBa	BSb	BBc
		Microorganisms number (cfu/g)				
(a)						
T1	0	$1.9 \times 10^8 \pm 0.1$	$1.2 \times 10^8 \pm 0.2$	$1.5 \times 10^8 \pm 0.2$	$1.0 \times 10^8 \pm 0.2$	$1.0 \times 10^8 \pm 0.3$
T2	1	$8.3 \times 10^8 \pm 0.2$	$6.3 \times 10^8 \pm 0.1$	$5.6 \times 10^8 \pm 0.2$	$2.6 \times 10^8 \pm 0.2$	$6.6 \times 10^8 \pm 0.2$
T3	3	$5.1 \times 10^8 \pm 0.1$	$8.2 \times 10^8 \pm 0.4$	$5.0 \times 10^8 \pm 0.2$	$8.8 \times 10^8 \pm 0.2$	$1.9 \times 10^8 \pm 0.2$
T4	6	$2.0 \times 10^7 \pm 0.2$	$1.0 \times 10^8 \pm 0.2$	$2.2 \times 10^8 \pm 0.2$	$2.1 \times 10^8 \pm 0.2$	$5.4 \times 10^8 \pm 0.2$
(b)						
T1	0	$1.9 \times 10^8 \pm 0.2$	$1.2 \times 10^8 \pm 0.2$	$1.5 \times 10^8 \pm 0.2$	$1.0 \times 10^8 \pm 0.2$	$1.0 \times 10^8 \pm 0.2$
T2	1	$8.1 \times 10^8 \pm 0.2$	$5.4 \times 10^8 \pm 0.2$	$5.1 \times 10^8 \pm 0.2$	$5.9 \times 10^8 \pm 0.2$	$2.6 \times 10^8 \pm 0.3$
T3	3	$5.0 \times 10^8 \pm 0.2$	$2.2 \times 10^8 \pm 0.2$	$5.0 \times 10^8 \pm 0.2$	$1.0 \times 10^8 \pm 0.2$	$5.0 \times 10^8 \pm 0.2$
T4	6	$7.5 \times 10^8 \pm 0.1$	$9.1 \times 10^8 \pm 0.2$	$1.1 \times 10^8 \pm 0.2$	$7.1 \times 10^8 \pm 0.3$	$2.7 \times 10^8 \pm 0.3$

Bc - lack of probiotic organisms, BLr - *Lactobacillus rhamnosus* GG, BBb - *Bifidobacterium breve* DSM 16604, BBa - *Bifidobacterium animalis* subsp. *lactis*, BSb - *Saccharomyces boulardii*, BBc - *Bacillus coagulans* GBI-30, 6086; T1 - postproduction, T2 - one month postproduction, T3 - three months postproduction, and T4 - six months postproduction.

final product, as extra polyphenolic compounds had been added with the freeze-dried raspberry. Flavonoids are considered to regulate the activity of enzymes, which are responsible for the generation of reactive oxygen species (ROS). They also possess metal-chelating properties, and hence, they prevent Fenton reaction, which is responsible for the ROS synthesis [38]. Moreover, polyphenols, including catechins, can regulate the growth of microorganisms. In this work, the content of individual fractions was significant, as the number of probiotic strains determined the nutritional quality of the developed product. The results of previous work overlapped with prior proceedings, in which it was noted that polyphenols present in chocolates had no impact on the number and survivability of *B. coagulans* bacteria [31]. In addition to the defined and desirable biofunctional properties, consumers expect specific sensory stimuli from chocolate-based products, and therefore, probiotic additive would be undesirable if it affected the sensory and physicochemical properties of the product. Differences in the sensory properties of chocolate beads can be related to the addition of differences in cocoa varieties, proportions of ingredients, mixing techniques, and preparation methods. It is known that saccharose with sugar alcohols negatively affects the rheological properties and hence the storage conditions and the quality of chocolates [39]. In previous works, it was reported that maltitol allowed obtaining chocolates with similar rheological properties as those prepared with saccharose, and so it can be recommended as a good alternative to saccharose in chocolate preparations [40,41]. Nebesny et al. [42] analyzed chocolates sweetened with isomalt. The chocolates were prepared with live cells of lactic acid fermentation bacteria *S. thermophilus* MK-10 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 151. The cells were freeze-dried beforehand in yogurt matrix and added to chocolates in powder form. The authors determined the physicochemical and sensory properties of chocolates and also the survivability of cells during six-month storage at temperatures of 4 and 18°C. Milk chocolates containing isomalt scored slightly higher mean values in sensory assessment (4.82–4.90 points) than the control chocolates prepared without saccharose (4.83–4.87 points). Dark chocolates containing saccharose attained lower scores (4.73–4.75) than their equivalents prepared without yogurt (4.82–4.86).

Another research group developed a method for the production of milk chocolates with live cells of probiotic bacterial strains, *L. casei* and *Lactobacillus paracasei* [43]. According to the authors, milk chocolates exhibited an identical sensory profile as reference chocolates prepared without probiotics, while in the former the total number of live bacterial cells remained at a functional level between 10^6 and 10^8 cfu/g after 12-month storage, regardless of the temperature [42]. The highest number of probiotic bacteria survived in the chocolates stored at a temperature of 4°C, which is consistent with our results (Table 4). According to Nebesny et al. [42], the sensory attributes of dark chocolates and the chocolates enriched with live cells of two bacterial strains, *L. casei* and *L. paracasei*, did not differ from the control chocolates prepared without probiotic bacteria.. The chocolate beads developed in this study received very positive note from consumers (Fig. 3). Addition of probiotic microorganisms did not negatively affect the sensory feelings of the responders. Prohealth properties were induced in the chocolate beads by their composition and sensory properties. However, whether adequate number of probiotic bacteria will be maintained at different stages of the production process, at the end of the process, during long-term storage, and also in the human digestive tract remains a question. Indicators such as number and survivability of microorganisms are pivotal for the biofunctionality of the final product. A key element of the research on the prohealth features of probiotic chocolate is conducting in vitro tests, simulating the conditions of the digestive tract (Fig. 4). Similar tests on the biofunctionality of probiotic chocolates were conducted by Ramakrishna et al. [44]. The authors prepared milk chocolates substituting defatted powdered milk by a powdered yogurt at the ratios of 50% and 100%. Microbiological analysis of chocolates showed the presence of bacteria of the genus *Lactobacillus* at a level of 3.37 log cfu/g. Possemiers et al. [45] confirmed in their study that chocolate is a good matrix for the oral delivery of microcapsuled mix of *Lactobacillus helveticus* CNCM I-1722 and *Bifidobacterium longum* CNCM I-3470 bacteria. The authors applied an in vitro model in their work so as to determine the effect of the simulated conditions of digestion process on the survivability of probiotic bacteria, dispersed in three food matrices - dark chocolate, milk chocolate, and milk. The authors noted that both chocolate matrices offered excellent protection for the microorganisms. Moreover, the authors tested a simulation model of ecosystem of the human intestinal microflora so as to determine the effect of long-term delivery of product containing the aforementioned probiotic bacteria. Denaturating electrophoresis in gel gradient analysis and quantitative polymerase chain reaction showed that both probiotic species survived until the end of the experiment, and their quantity was at a satisfactory level in the colon. A fact is that storage of products containing probiotic microorganisms often results in a decrease in the number of these microorganisms, especially at the end of the shelf-life. A general cause can be the temperature applied during storage. Nebesny and Zyzelewicz [46] analyzed dark chocolates prepared with probiotic strains of two species, *L. casei* and *L. paracasei*. Lyophilizates added to the chocolate mass contained 7.9×10^9 cfu/g. The authors of the study tested the effect of product storage for 12 months at different temperatures (4, 18, and 30°C). They noted that the ratio between the cell number after storage period and the initial cell number was highest for the product stored at 4°C (89–94%) than that stored at 18°C (80–87%) and finally at 30°C (60–67%). Moreover, in the case of product stored at 30°C, the number of probiotic bacteria was at a level that can prevent the biofunctional effect of the bacteria. Similar findings about the effect of temperature on maintaining the number of probiotic bacteria were described in the other paper of the authors [42]. In our study, as the chocolates were stored at ambient temperature, only the information about the number of bacteria at 18°C can be referred.

Silva et al. [47] informed that survivability of bacteria from the species *B. animalis* subsp. lactis and *L. acidophilus* in food matrix i.e. chocolate is very high equal to 10^8 CFU/g. Storage for 120 d at 25°C and digestion in in vitro model just like in our research (Fig. 4 and Table 4) did not cause a considerable decrease in liveliness of the bacteria. For comparison, in vitro digestion of lyophilisate of *B. animalis* subsp. lactis and *L. acidophilus* contributed to reduction in the number of live cells. Furthermore, adding live probiotic microorganisms to a confectionery i.e. chocolate is not easy due to high temperature at different stages of processing. However, the well-thought-of technological process allows to deliver microorganisms to the place of action i.e. large intestine [48]. Another possibility is selection of appropriate probiotic strains. For example, the strain of *L. plantarum*-LRCC5193 (LP-LRCC5193) bacteria isolated from fermented vegetables demonstrated a much higher degree of tolerance for high temperature, gastric juice and bile acids. Similarly, good results were obtained by other authors for the compilation of three micro-capsuled probiotic strains, *B. breve* BR2, *L. acidophilus* LH5 and *S. thermophilus* ST3, the number of which remained at a stable and high level during 360 d of storage at 4°C and 20°C [49].

5. Conclusion

The snack made of dark chocolate, in which sugar has been replaced with maltitol, enriched by a vegetable raw material in the form of raspberry, prebiotic in the form of inulin and a selected strain of probiotic bacteria stands a high chance to occupy a good place on the market of biofunctional food. Our research showed a high nutrition and pro-health value of the newly developed chocolate snacks. High content of flavonoids and phenolic acids demonstrating high anti-oxidative activity and selected, resistant to adverse environmental conditions, probiotic microorganisms are the basic advantages of the

developed snack. It is important that the use of the matrix in the form of chocolate allows to secure microorganisms against an adverse impact of the digestion process as well as at the stage of storage, which additionally increases quality of the newly developed product.

Conflict of interest

The authors declare no competing interests.

Financial support

This work was supported by National Center for Research and Development in Poland, grant no. POIR.04.01.02-00-0059/17.

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