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Functional, rheological and sensory properties of probiotic milk chocolate produced in a ball mill

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Abstract

The aim of this study was to investigate the survival of probiotics (*Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019) in milk chocolate masses prepared at temperatures 35 °C and 40 °C. The influence of probiotics and preparation temperature on rheology, particle size distribution and sensory properties of chocolates, was examined during the 6 months of storage at 20 ± 2 °C.

Inoculation temperature of 40 °C significantly improves the rheological and sensory properties of probiotic chocolate, as well as surviving of *L. acidophilus* NCFM and *L. rhamnosus* HN001 strains. After 6 months of storage, the survival of these strains was above 90%, with viable cell count of about 8.1 log (CFU/g). Inoculation temperature of 40 °C provides higher scores of overall sensory quality (4.52-4.68), higher quality category (excellent), lower maximal viscosity (for 1.2 Pa·s) of chocolates, than temperature 35 °C. Compared to the chocolate without probiotics, those inoculated at 40 °C achieved less increase in volume weighted mean diameter distribution (average 0.8%) than chocolates inoculated at 35 °C.

Based on the results reported in this paper, seeding of the probiotics in industrial conditions can be done in the mixing tank (at 40 °C) before the phase of chocolate shaping. Addition of probiotics at this stage facilitates the manufacturing process, improves the overall quality of chocolate and preserves the probiotics as key component of this type of product.

***Key words**: milk chocolate; ball mill; probiotics; sensory analysis; rheological properties.*

1. Introduction

Food products containing probiotics are one of the largest markets of functional foods, and the most accessible are dairy products. Probiotics are usually lactic acid bacteria naturally present in food. Development of the food industry and various hygienic treatments used during food processing, leads to significantly reduced contact of humans with microorganisms, which can be one of the reasons of a growing number of allergies.[1] Expanding knowledge about nutrition increased the demand for healthy food, and introduced probiotics as desirable food components. There are nearly 20 known bacterial species, which beneficially affect the balance of more than 400 different microorganisms that naturally inhabit the human digestive system.[2] Various types of probiotic bacteria include *Lactobacillus* and *Bifidobacterium* as the most used species.[3] Many of these have already been successfully included in the production of fermented dairy products, but their use in confectionery industry is still a challenge. Numerous studies conducted in this area lead to the discovery of new probiotic strains. Recently, a few new strains, identified as *Lactobacilus acidophilus* NCFM, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019, were characterized to have immune-modulating and anti-infection properties as well as being contributors to the overall bowel health.[4-6] These strains do not degrade gastric mucin in vitro[7], nor do they express toxic or pathogenic effects on humans.[6, 8-10]

Applying probiotics to confectionery products may offer a good alternative to dairy products. Beside the necessary recommended dose of probiotics of at least 10^6-10^7 CFU per gram[11], the viability of probiotics during storage of confectionery products is a special issue that needs to be dealt with considering the sensitivity of these

73 microorganisms to aerobic conditions. As these products are often exposed to oxygen,
74 researchers have so far studied survivability of probiotic strains during storage of
75 confectionery products, especially chocolate, at various temperatures. Nebesny et al.[12]
76 examined the viability of *Lactobacillus casei* and *Lactobacillus paracasei* strains in dark
77 chocolate with isomalt and aspartame as sweeteners. After 12 months storage of
78 chocolate at various temperatures, strains survival was 89-94% at 4 °C, 80-87% at 18 °C
79 and 60-67% at 30 °C. Based on the findings reported by Aragon-Alegro et al.[13], during
80 the short storage period of 28 days, as well as the influence of prebiotic inulin, increase in
81 viable cell count of *Lactobacillus paracasei* strain incorporated in chocolate mousse
82 could be achieved.

83 Chocolate is a complex rheological system. It can be described as a suspension
84 consisting of nonfat particles (sugar, cocoa solids and milk powder particles) dispersed in
85 cocoa butter as a continuous fat phase.[14] The chocolate mass is a non-Newtonian fluid,
86 defined by plastic flow, characterized by yield stress necessary to suppress inner
87 resistance so that the chocolate mass can start flowing and also represents the inner
88 resistance of the system in further flow.[15] In addition, the chocolate mass belongs to
89 pseudoplastic materials, showing thixotropic and rheopectic properties.[16,17] Increasing
90 shear rate leads to a gradual destruction of the chocolate mass suspension structure i.e.
91 bond breaking in crystal packing. Rheological properties of chocolate such as Casson
92 plastic viscosity, shear stress and yield stress depend on the content of water and fat in
93 the chocolate mass, concentration and structure of emulsifiers, particle size distribution
94 and their type and concentration, temperature, conching time, tempering conditions and
95 thixotropy.[18-20]

The composition of chocolate has a significant influence on its rheology. Study conducted by Afoakwa et al.[21] proved that the increase of an average particle size results in decrease of Casson plastic viscosity, shear stress, yield stress and apparent viscosity. This reduction is more obvious in lower fat contents, while it is not registered in fat contents of 30% and more. In addition, Schantz and Rohm[22] determined the effects of varied mixtures of lecithin and polyglycerol polyricinoleate (PGPR) on the flow parameters of melted chocolate in order to obtain the optimum emulsifier blend. They found that the PGPR : lecithin ratio in dark chocolate should be 50:50, while in milk chocolate it should be 25:75. The study of Sokmen and Gunes[23] reported that maltitol increases yield stress, isomalt increases plastic viscosity while xylitol increases flow index. Farzanmehr and Abbasi[24] evaluated the effects of sugar substitutes on rheological properties of prebiotic milk chocolate. They showed that sugar substitutes in chocolate recipes lead to reduced hardness and increased moisture. Beside the above mentioned, probiotics are additional ingredients that significantly affects the properties of chocolate. According to the literature reports[25,26], incorporation of lyophilised probiotics increases the rheological parameters of chocolate and negatively influences its flow properties. In addition, due to the grinding stage, friction, and high temperatures that can affect the viability, probiotic bacteria can not be added during composing the milk chocolate - at the beginning of the industrial process. Therefore, the achievement of satisfactory rheology without probiotics damage, demands the incorporation of probiotic bacteria at the process stage that allows full homogenization of the chocolate mass. Due to the above mentioned, seeding of bacteria in industrial conditions could be done in the mixing tank

(where the temperature is 40 °C) before the phase of chocolate shaping or in the tempering machine where the chocolate mass is mixed at 35 °C.

Therefore, the aim of this study was to examine the surviving of probiotic strains *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019, inoculated in milk chocolate at temperatures 35 and 40 °C, after 6 months of storage at 20 ± 2 °C. The impact of probiotics on particle size distribution, rheological and sensory properties of milk chocolate was also examined through a comparative review of milk chocolate with and without probiotics. Comparison the qualitative parameters of probiotic milk chocolates, when the probiotics were seeded at 35 °C and 40 °C, and determination the exact stage of production to carry out the probiotics inoculation, provides important information on the production of probiotic chocolate with improved quality.

2. Material and methods

2.1 Material

2.1.1 Milk chocolate mass

Raw materials used in the production of chocolate mass were: sugar 41.5% (Crvenka AD, Serbia), dairy milk powder 25.4% (fat 25%, protein 28%) (Imlek, Serbia), cocoa butter 18.9% (Theobroma, The Netherlands), cocoa mass 10.4% (Cargill, Ghana), skimmed milk powder (fat 1.7%, protein 35%) (Imlek, Serbia), lecithin 0.5% (Soyaprotein AD, Serbia) and flavoring 0.06% (Etol, Slovenia). The milk chocolate mass was 1.1 ± 0.06% moisture.

2.1.2 Probiotic microorganisms

Concentrated and freeze-dried probiotic strains *Lactobacillus acidophilus* NCFM (Howaru® Dophilus), *Bifidobacterium lactis* HN019 (Howaru® Bifido) and *Lactobacillus rhamnosus* HN001 (HOWARU® Rhamnosus) were obtained from Danisco, (Madison, WI, USA). The lyophilized strains were inoculated in the proportions 2.5 DCU/kg, 2.5 DCU/kg and 5.0 DCU/kg (respectively), according to the manufacturer recommendations, to obtain a functional level of probiotics (at least 10^6-10^7 CFU/g).

2.2 Methods

2.2.1 Production of milk chocolate mass

The chocolate mass was produced in the laboratory ball mill with a homogenizer (capacity 5 kg). The raw materials were measured and simultaneously dosed into the homogenizer (except 10% of cocoa butter, which is dosed 10 min before taking out the mass from the ball mill) and mixed for 20 min at a temperature of 50 ^{o}C and mixer rotation speed of 50 rpm. The homogenous mass was then transferred into the ball mill (ball diameter 9.1 mm; ball mass 30 kg; mixer rotation speed 50 rpm; mill inner diameter 0.250 m; height 0.31 m.; volume of space provided for the balls and 5 kg of chocolate mass is 0.0152 m^3). Applied refining time in the mill was 90 min.

In order to avoid exposure of probiotic bacteria to high temperatures and harmful effects of mechanical shear during milling in the ball mill, probiotics were introduced into the chocolate mass after milling, i.e. before the pre-crystallization and molding phases, when the temperature was reduced. The probiotics were added at temperatures 35 and 40 °C, in the concentrations recommended by the manufacturer. The temperature of the chocolate mass with probiotics was sustained in the mixer in a water bath for 15 min, by mixing with a minimal number of rotations of the blender.

After the probiotics addition the pre-crystallization process was carried out. Pre-crystallization of the chocolate mass was performed in the laboratory precrystallizer, a modified Brabender farinograph.[27,28] The process of pre-crystallization was controlled indirectly by the changes of the mass resistance during mixing, which was registered on a force/time diagram - the thermorheogram. Torque value is a criterion for the viscous behavior of the chocolate mass and is dependent on the crystallization extent of the mass in question. The pre-crystallization temperature of 28 ^{o}C was applied. The pre-crystallized mass was then molded, cooled and removed from the forms. The final products were packed in aluminium foil and marked in blank paper / cardboard, and then stored at 20 ± 2 °C.

The chemical composition of the final milk chocolate tablets, determined using standard AOACC methods[29], was as follows: 9.41 ± 0.10% protein, 30.85 ± 0.07% fat, 53.82 ± 0.17% sugar and 1.1 ± 0.05% moisture. Symbols of the milk chocolate samples prepared in this study are listed in Table 1.

2.2.2 The distribution of particle size (Mastersizer)

Influence of milling time on particle size distribution in milk chocolate samples was determined by Mastersizer 2000 (Malvern Instruments, England) laser diffraction particle size analyzer equipped with a Hydro 2000 μP dispersion unit. Molten milk chocolate samples were dispersed in sunflower oil at ambient temperature (20 ± 2 °C) and added until adequate obscuration was obtained (10-20%). The results were quantified as volume-based particle size distribution, using Mastersizer 2000 Software.

2.2.3 Rheological properties of the chocolate mass

Rheological properties were determined in the rotation viscometer RheoStress (600 HP, Haake, Germany), according to the IOCCC method[30], at the temperature of 40 ± 0.1 °C. Flow curves were determined using the method of the hysteresis loop within the shear rate interval of 1-60 1/s. Shear rate was increased from 1 to 60 s^{-1} in the period of 240 s, then maintained at the maximum speed of 60 s^{-1} for 60 s, and the decreasing of shear rate from 60 to 1 s^{-1} also lasted for 240 s.

2.2.4 Sensory analyses

Acceptance testing

Sensory analysis of probiotic chocolates was conducted after 180 days of storage according to the method describe by Hemsworth et al.[31], with slight modifications. Sixty untrained panellists (35 being women and 25 men, age between 25 and 55) from the faculty, including teachers, students and staff were randomly selected and invited to participate in the sensory evaluation of probiotic chocolates. The participants were asked to assess the appearance, structure, chewing, taste and odour of the seven different chocolates: A35, B35, R35, A40, B40, R40 and control sample without probiotics marked as 0. Each questionnaire consists of four questions: name, age, sex and appearance, structure, chewing, taste and odour for seven consumed products.

The samples were presented monadically at 20 ± 2 ˚C, in individual packs coded with 3-digit numbers, serving 20 g of samples to each panellist. The participants were given seven samples at a time at room temperature (20 ± 2 °C), a pencil, a questionnaire and a glass of cold water to rinse their mouths between samples. They have been asked to mark an value which best represents how much they liked or disliked each of seven samples with respect to appearance, structure, chewing, taste and odour, using a 5-point

scale ranging from 1 = dislike it very much to 5 = like. The sensory analysis was consisted of 420 questionnaires distributed into 7 sessions (7 samples). The obtained scores of these parameters were multiplied by a defined coefficient of importance (Pajin 2009), and the category of quality was defined based on the total number of points.

Quantitative descriptive analysis (*QDA*)

Detailed sensory analysis of probiotic chocolates was done by conducting quantitative descriptive analysis (QDA) with a trained sensory panel according to the method describe by De Pelsmaeker et al.[32], with slight modifications. The panel consisted of 15 assessors (8 being women and 7 men, age between 25 and 55) selected from a pool of the 60 possible candidates which were included in acceptance testing. Panellists were selected based on their abilities to identify and describe differences in chocolates and their recognizing the presence of different ingredients. They participated in a 3 month training period when the sensory descriptors including texture quality parameters (hardness, brittleness, dryness, stickiness and toughness), as well as melting parameters (melting point, melt rate, cooling, meltability) were chosen, defined and measured. The panellists were trained over a period of 15 h to perform quantitative descriptive analysis.

During the QDA test each panellist received the seven chocolate samples (20 g) at a time, in individual packs coded with 3-digit numbers, in random order, a pencil, a questionnaire and a glass of cold water to rinse their mouths between samples. The samples were presented monadically at 20 ± 2 ˚C. Each questionnaire consists of questions: name, age, sex as well as hardness, brittleness, dryness, stickiness, toughness, melting point, melt rate, cooling, meltability for seven consumed products. The panellists have been asked to mark an value which best represents the tested quality parameter for

each of seven samples, on the 5-point scale ranging from 1 = low to 5 = high. The QDA analysis was consisted of 105 questionnaires distributed into 7 sessions (7 samples).

Prior to serving in both analyses (acceptance testing and QDA) all samples were subjected to counts of yeasts, molds and coliforms to evaluate the hygienic and sanitary conditions of the products.

All experiments were performed in compliance with the Serbian Law on Food Safety ("Official Gazette RS", No 41/09) and Guidelines of Regulation on General and Special Requirements for Food Hygiene in Any Phase of Production, Processing and Distribution ("Official Gazette RS", No 72/10) of Serbian Ministry of Agriculture. The protocol was reviewed and approved by an Accredited Microbiological Laboratory Faculty of Technology, University of Novi Sad. The informed consent was obtained from all subjects. All starter cultures are commercial cultures which safety is confirmed by manufacturer Danisco, (Madison, WI, USA).

2.2.5. Viability of probiotic bacteria

The amount of 1 g of investigated chocolate samples was dissolved in 9 mL of sodium chloride solution (0.85%, w/v) at 40 °C, and mixed uniformly. Subsequent serial dilutions were prepared and viable cell count was determined using pour plate technique on MRS agar.[33] Plates were incubated at 37 °C for 48 h in anaerobic conditions. Probiotic bacteria were enumerated as colony forming units per gram of chocolate and expressed as log (CFU/g). Viability tests were performed after 0, 30, 60, 90, 120, 150 and 180 days of storage at 20 ± 2 °C.

2.2.6 Statistical analysis

All experiments were performed in triplicate. Mean values were analyzed using one-way ANOVA. The Tukey post hoc test was performed for means comparison (OriginPro 8, Origin Lab Co., Northampton, USA). Differences were considered as significant at $P < 0.05$.

3. Results and Discussion

3.1 Viability of probiotic bacteria

Probiotic microorganisms are the most sensitive factor in the process of probiotic chocolate production[34]. Their viability is the crucial parameter related to achieving and maintaining the functional properties of probiotic chocolate. Changes in viable cell count of probiotic bacteria in chocolate samples, prepared at different temperatures, during storage at 20 ± 2 °C, are presented in Fig. 1.

As indicated in Fig. 1, initial viable cell count of probiotic bacteria ranged from 7.0 to 7.97 log (CFU/g). It was at a satisfactory level (≥ 10^6 CFU/g) recommended for probiotic products in all chocolate samples. Gradual decrease of viable cell count was observed in both chocolate samples cultured with strain *B. lactis* HN019, regardless the processing temperature, during the whole storage period. This behavior could be explained by the fact that this strain is highly sensitive to oxygen exposure. The mixing phase (which is characterized by the presence of high oxygen level) applied during the chocolate production could be the reason of its poor viability. It could be concluded that strain *B. lactis* HN019 is capable to survive in chocolate, at a desirable level, for no longer than 60 days of storage at 20 ± 2 °C. Observed results are slightly lower than those reported in literature[25] concerning the viability of *B. lactis* HN019 strain, probably due to the lower initial cell count. Based on these findings, it could be concluded that initial cell

count is a crucial parameter for achieving functionality of probiotic chocolate cultured with the high sensitive *B. lactis* HN019 strain.

On the other hand, viable cell count in samples cultured with strains *L. rhamnosus* and *L. acidophilus* remained at a satisfactory level, even greater than recommended ($\geq 10^6$ CFU/g), during the whole storage period. Viable cell count gradually increases during the 90 days of storage, reaching the maximal viable cell count of about 8.85 log CFU/g in samples A40 and R40. The obtained results are consistent with those reported in literature[25] concerning the viability of *L. acidophilus* NCFM, where the viable cell count remains at the same level of about 8.6 log CFU/g during the whole storage period. It is interesting to note that viable cell count reported in literature[25] gradually decreases during the 90 days in contrast to the result obtained in the present study. It could be explained by the fact that initial viable cell count reported in our study was not at its maximal level, which allowed the subsequent growth of *L. rhamnosus* and *L. acidophilus* strains.

Processing temperature has significant influence on viable cell count in samples A40 and R40. Compared to the other samples, after 90 days of storage, samples A40 and R40 have significantly ($P < 0.05$) greater viable cell count. The same behavior was observed in these samples after 120 days of storage. Also, comparing results related to cell growth in production treatment carried out at temperature 30-32 °C reported in literature[25], it could be said that temperature of 40 °C had significantly positive influence on viability of *L. acidophilus* strain, probably by improving the strain activity.

Based on the observed results, it could be concluded that chocolate cultured with *L. rhamnosus* and *L. acidophilus* strains at 40 °C, exhibits high functional quality, and these strains express a great potential for use in production of probiotic chocolate.

299 **3.2 The distribution of particle size (Mastersizer)**

300 Laser diffraction was applied to measure the particle size distribution (PSD) of the
301 milk chocolate products. Optimization of PSD in chocolate requires consideration of
302 palate sensitivity. For example, chocolates milled to a particle size range of 18-25 μm,
303 will have a smoother mouth-feel and texture as compared to a chocolate with a particle
304 size 30 μm or above, which will be perceived "coarse or gritty" in the mouth. EU
305 chocolate has been described as having a fineness of 15-22 μm making their target
306 consumers more used to a smother mouth-feel.[14] PSD parameters obtained for milk
307 chocolates produced in the present study are shown in Fig. 2 and Table 2.

308 The shape of the histograms (Fig. 2) is in accordance with literature data.[21] Due to
309 the presence of probiotics, which complicate the structure, two expressive peaks of about
310 7 μm and 13 μm have been seen in all chocolate with probiotics compared to milk
311 chocolate without probiotics. Chocolate R35 and B35 have pronounced peaks in relation
312 to the chocolate mass R40 and B40 due to easier mixing in of probiotics at higher
313 inoculation temperatures. Inoculation temperature has greater influence on the shape of
314 the histogram, than the type of probiotic cultures.

315 Given that all the chocolate had the same composition, milling time,
316 concentration of emulsifiers and the same precrystallization temperature, it could be said
317 that difference in particle size distribution is due to the presence of certain probiotics and
318 the temperature at which they were inoculated.

319 As shown in Table 2, the volume weighted mean diameter (D) is the lowest in
320 chocolate without probiotics, 13.26 μm and in chocolate with probiotics it ranges from
321 13.9-16.17 μm. Chocolate A40 has the lowest increase of the volume weighted mean

diameter distribution of 4.8% and the chocolate R40 the highest (21.9%) compared with chocolate without probiotics. On average, chocolates from series 40 have a 15.3% larger volume weighted mean diameter distribution, compared to chocolate without probiotics. The average increase in chocolates A35, B35 and R35 is of 16.1%. The lowest increase was in chocolate B35 (14.12 μm), and the highest in R35 (16.08 μm).

Parameter d (0.5) (Table 2) for chocolate without probiotics is 9.06 μm, meaning that 50% of the volume distribution of samples are smaller than particular d (0.5) value. The value of parameter d (0.5) in the chocolates A40, B40 and R40 is in the range of 9.26 to 10.68 μm and the average increase in relation to chocolate without probiotics is 10.66%. In chocolate series 35 the value of parameter d (0.5) ranges from 9.66 to 11.01 μm and the average increase compared to chocolate without probiotics is 15.12%. The value of parameter d (0.9) in the chocolates A40, B40 and R40 is in the range of 32.27 to 37.27 μm and the average increase compared to chocolate without probiotics is 5.0 μm. In chocolates A35, B35 and R35 the value of parameter d (0.9) ranges from 32.33 to 37.23 μm and the average increase compared to chocolate without probiotics was 5.1 μm. The differences in particle size distribution are minimal in all chocolates, but it was noticed that inoculation temperature has greater influence on particle size distribution than the used probiotic culture.

3.3 Rheological properties of the chocolate mass

Rheological parameters such as Casson viscosity and Casson yield value of chocolate masses are of key importance for the manufacturing technology. In industrial processes, these quantities should be as low as possible to decrease resistance during unit processes like mixing or pumping.[14]

345 Fig. 3 shows the thixotropic loops of the milk chocolate samples. All samples of
346 chocolate with and without probiotics have similar yield value curves. Surfaces of
347 thixotropic loops are larger in all samples of chocolate with probiotics, which indicates a
348 greater complexity and lower homogeneity of the system[17] in relation to the chocolate
349 without probiotics. It is assumed that the technique of probiotics mixing is the reason. In
350 addition, the surfaces of thixotropic loops are larger in samples in with probiotics
351 inoculated at 35 °C compared to the samples with probiotics inoculated at 40 °C. Based
352 on this finding, it could be said that it is much easier to carry out the incorporation and
353 homogenization of probiotics at temperature of 40 °C. This significantly facilitates the
354 inoculation of probiotics to the chocolate mass in industrial conditions, because the
355 chocolate mass, before shaping and tempering, is in a container with a mixer at 40 °C. It
356 is also interesting to note that in chocolate samples R35 and A35 there is no statistically
357 significant difference ($P < 0.05$) in the thixotropic loop, indicating that strains *L.*
358 *rhamnosus* and *L. acidophilus* inoculated at 35 °C, affect the chocolate rheology in the
359 same way.

360 Table 3 shows the rheological parameters determined by static measurements.
361 According to published data[21] yield stress and viscosity by Casson decrease with
362 increasing average particle size, which is in line in the series of probiotic chocolates in
363 which the probiotic cultures were inoculated at 40 °C. In comparison to the chocolate
364 sample without probiotics, chocolate samples A40, B40 and R40 have lower viscosity
365 and yield stress. The range of viscosity in this series of chocolate samples is from 2.25 to
366 2.49 Pa·s, and the yield stress from 19.93-23.71 Pa. It is interesting to note that smallest
367 particle size of the chocolate sample A40 (Table 2) lead to the highest viscosity and yield

stress (Table 3) of the sample, while the largest particle size of the chocolate sample R40 (Table 2) lead to the lowest viscosity and yield stress (Table 3) of sample. In addition, the effect of particle size is much more pronounced on yield stress (an increase of 15.94%) than on viscosity (an increase of 9.63%), which is consistent with literature data.[21]

Chocolate samples A35, B35 and R35 have lower yield stress, and higher viscosity compared to the chocolate without probiotics. The range of viscosity is 3.36-3.69 Pa·s, and yield stress from 15.68-19.93 Pa. The highest yield stress and viscosity were observed in chocolate sample A35, and the lowest in sample B35 (Table 3). However, viscosity of samples with *L. acidophilus* and *B. lactis* inoculated at 35 or 40 °C was lower than viscosity of samples inoculated at 30-32 °C reported in the literature[25]. Based on the observed results, it could be said that temperature increasing positively affects rheological parameters of probiotic chocolate and leads to the flow properties improvement. On the other hand, the lowest volume weighted mean diameter (Table 2) was observed in chocolate sample B35. In contrast to the samples prepared at 40 °C, expected dependence, smallest diameter - largest rheological values, has not been achieved and the reasons should be sought in the manner of incorporation of probiotics at temperature 35 °C.

3.4 Sensory analysis

During the storage of probiotic milk chocolate the basic problem that can occur is post - acidification that leads to impaired sensory properties, as well as cell death by which the product may lose its probiotic property. With post - acidification, there was a sour taste and odour due to the emerging of lactic acid.[35] Due to the possibility that probiotic bacteria disrupt the crystallization of cocoa butter and the possible influence of

391 their granulation on textural properties (chewiness, graininess and meltability of
392 chocolate), the texture of chocolate with probiotics, during this experiment, has
393 thoroughly been assessed. To examine the sensory quality of probiotic chocolate samples
394 after 180 days of storage, the acceptance testing and the quantitative descriptive analysis
395 (QDA) methods were used. Comparative illustration of sensory scores of chocolate
396 samples, obtained in the acceptance testing was shown in Table 4.

397 According to the obtained scores, overall sensory quality of all chocolate samples
398 with probiotics is in the domain of excellent or very good after 180 days of storage at 20
399 ± 2 °C (Table 4). In relation to the chocolate without probiotics, the chocolates with
400 probiotics inoculated at 35 °C have lower scores and are even in the lower quality
401 category (VG). The chocolate samples with probiotics inoculated at 40 °C had
402 significantly ($P < 0.05$) higher scores than the chocolate without probiotics. Chocolate
403 samples, with probiotics inoculated at 40 °C are not statistically significant different ($P >$
404 0.05) in relation to the chocolate without probiotics for surface properties and odour.

405 Rating taste and odour, as the most important sensory quality parameters, is of
406 particular importance, because a major change was expected in these sensory parameters.
407 Values for odour for all chocolate samples deviated in the range of 0.91 to 0.97, actually
408 only 6.18%, while the taste changed within the range of 1.60 to 1.72, or only 6.97%. The
409 greatest variation was in chewing, it was 41.17%, then 25% for chocolate breaking and
410 finally chocolate surface appearance 13.04%. It is interesting that the auditors gave very
411 low marks to samples A35 and R35 for chewing and breaking, and these are actually the
412 chocolate samples that had the highest viscosities by Casson.

The rheology confirmed what the sensory examiners concluded by analysis. Low scores for chocolates A35 and R35 for breaking and chewing affected the overall rating and category. In general overall assessment, the chocolates with probiotics inoculated at 35 °C, achieved the lower grades (average by 6.86%), compared to chocolate without probiotics. The overall rating of chocolates with probiotics inoculated at 40 °C was higher on average by 2.14%, compared to the chocolate without probiotics.

Within the QDA method, the sensory examiners analyzed: hardness, brittleness, dryness, stickiness, toughness, density, melting point, melt rate, cooling, meltability in all chocolate samples, and obtained results are shown in Fig. 4.

It is obvious that regardless of the probiotic strain, the chocolates from the series 35 (blue circle) had lower sensory evaluations for all parameters. Among all samples, chocolate B35 had the lowest parameters for: dryness, stickiness, as well as toughness, melting point, melt rate, cooling, meltability. On the other hand, chocolate R40 had top grades for the parameters: hardness, brittleness, melting point melt rate, cooling, meltability, while the chocolate A40 for dryness and density, and chocolate without probiotics for stickiness and toughness.

Based on the observed results it could be said that the quality parameters are strain and temperature dependant. Temperature 40 °C leads to the preparation of chocolates with higher values of quality parameters than temperature 35 °C.

4. Conclusions

Based on the findings reported in this paper, it can be concluded that chocolate mass can be successfully enriched with probiotic strains *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019.

Chocolate samples prepared with *L. acidophilus* NCFM and *L. rhamnosus* HN001 strains at 40 °C, achieved top grades for the hardness, brittleness, melting point, melt rate, cooling, meltability, dryness, density, survivability and these strains should be selected for high quality probiotic chocolate production rather than *B. lactis* HN019.

The inoculation temperature of 40 °C significantly improves the rheological and sensory properties of probiotic chocolate, as well as the surviving of *L. acidophilus* NCFM and *L. rhamnosus* HN001 strains during the storage. After 6 months, the survival of these strains was above 90% with viable cell count of about 8.1 log (CFU/g). Chocolates with probiotics inoculated at 40 °C have significantly higher scores of overall sensory quality and are in the higher quality category (4.52-4.68, excellent) than chocolates inoculated at 35 °C (3.94-4.15, very good). In addition, compared to the chocolate without probiotics, those inoculated at 40 °C achieved less increase in volume weighted mean diameter distribution (average 0.8%) than chocolates inoculated at 35 °C. Chocolates inoculated with probiotics at 40 °C achieved lower maximal viscosity (for 1.2 Pa·s) than chocolates inoculated at 35 °C, which considerably facilitates further phase of chocolate processing.

Based on the presented results, seeding of the probiotics in industrial conditions can be done in the mixing tank (at 40 °C) before the phase of chocolate shaping. Addition of probiotics at this stage of industrial production facilitates the manufacturing process, improves the overall quality of chocolate and preserves the probiotics as key component of this type of product.

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536 **Figure captions:**

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538 **Fig. 1** Changes in viable cell count of probiotic bacteria in chocolate samples prepared at
539 different temperatures during storage at 20 ± 2 °C. Symbols: ■ *L. acidophilus* NCFM, at
540 35 °C, ● *L. acidophilus* NCFM at 40 °C, ▲ *L. rhamnosus* HN001 at 35 °C, ▼ *L.*
541 *rhamnosus* HN001 at 40 °C, ◀ *B. lactis* HN019 at 35 °C and ▶ *B. lactis* HN019 at 40 °C.

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543 **Fig. 2** Histograms of the particle size distribution of milk chocolate without probiotics (–
544 — 0), with a) *L. acidophilus* NCFM inoculated at 35 °C (—— A35) and 40 °C (—— A40);
545 b) *B.lactis* HN019 inoculated at 35 °C (—— B35) and 40 °C (—— B40); c) *L. rhamnosus*
546 HN001 inoculated at 35 °C (—— R35) and 40 °C (—— R40).

Fig. 3 Flow curves of the chocolate samples

Fig. 4 Comparative illustration of sensory grades of parameters: hardness, brittleness, dryness, stickiness, toughness, density, melting point, melt rate, cooling and meltability, for chocolate samples inoculated with: a) *Lactobacillus acidophillus* NCFM; b) *Lactobacillus rhamnosus* HN001; c) *Bifidobacterium lactis* HN019; and control sample marked as 0.

List of tables:

Table 1. Symbols of the milk chocolate samples

Symbol of the milk chocolate	Probiotic	Inoculation temperature (°C)
A 35	*Lactobacillus acidophillus* NCFM	35
B 35	*Bifidobacterium lactis* HN019	35
R 35	*Lactobacillus rhamnosus* HN001	35
0	Control (without probiotics)	/
A 40	*Lactobacillus acidophillus* NCFM	40
B 40	*Bifidobacterium lactis* HN019	40
R 40	*Lactobacillus rhamnosus* HN001	40

Table 2. Particle size distribution (PSD) parameters of the chocolate samples (μm)

	A 35	B 35	R 35	0	A 40	B 40	R 40
d (0.1)[a]	2.6 ± 0.02	2.71 ± 0.04	2.82 ± 0.03	2.7 ± 0.01	2.65 ± 0.03	2.85 ± 0.05	2.51 ± 0.02
d (0.5)[a]	10.62 ± 0.03	9.66 ± 0.04	11.01 ± 0.02	9.06 ± 0.02	9.26 ± 0.04	10.68 ± 0.02	10.12 ± 0.04
d (0.9)[a]	37.23 ± 0.33	32.33 ± 0.50	36.76 ± 0.25	30.35 ± 0.10	32.27 ± 0.29	36.51 ± 0.47	37.27 ± 0.32
D[b]	15.99 ± 0.48	14.12 ± 0.43	16.08 ± 0.28	13.26 ± 0.23	13.9 ± 0.33	15.82 ± 0.40	16.17 ± 0.23

[a]d (0.1), d (0.5), d (0.9) respectively represent 10%, 50%, and 90% of all particles with this size.
[b]D - volume weighted mean diameter.

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596 **Table 3.** Rheological parameters of the chocolate samples determined by static
597 measurements

Samples	Thixotropic curve area (Pa/s)	Casson yield stress (Pa)	Casson viscosity (Pa·s)
A35	3248[a]	19.93[b]	3.69[a]
B35	2476[b]	15.68[c]	3.36[d]
R35	3268[a]	18.99[d]	3.59[a]
0	2776[c]	23.62[a]	3.16[e]
A40	1751[d]	23.71[a]	2.49[b]
B40	1289[e]	21.08[e]	2.35[bc]
R40	1089[f]	19.93[f]	2.25[c]

598 Values are means of three determinations (n=3)
599 Values in the same column with the same superscript are not statistically different (P > 0.05)
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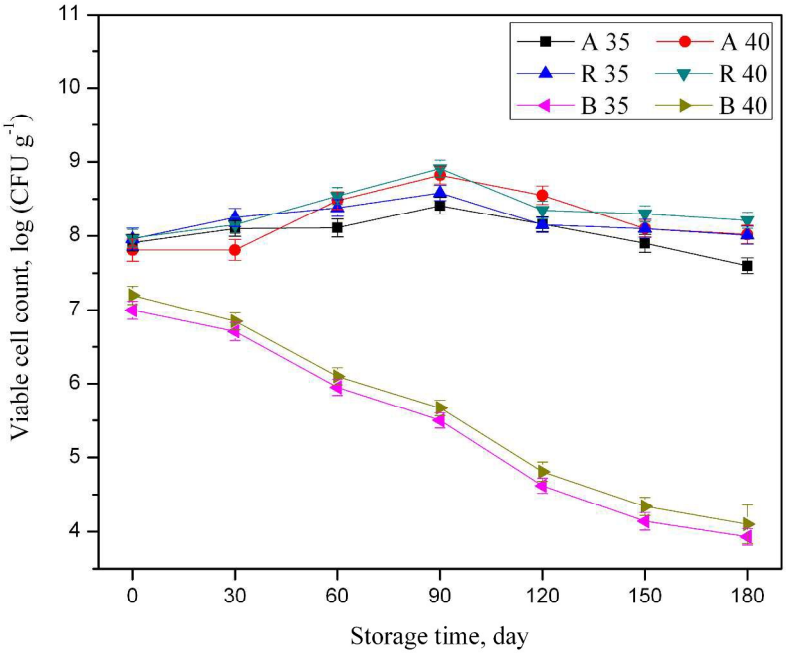
Table 4. Sensory evaluation of the chocolate samples using the scoring procedure

Quality factor	Coefficient of importance	A 35	B 35	R 35	0	A 40	B 40	R 40
Surface properties	0.10	0.40^{ab}	0.41^{ac}	0.41^{bc}	0.45^{deg}	0.46^{dfh}	0.46^{efi}	0.46^{ghi}
Break	0.15	0.48^{a}	0.52^{e}	0.48^{a}	0.64^{bc}	0.68^{f}	0.62^{bd}	0.64^{cd}
Chewing	0.20	0.54^{c}	0.70^{d}	0.57^{e}	0.82^{a}	0.85^{b}	0.82^{a}	0.85^{b}
Odour	0.20	0.91^{ab}	0.92^{acf}	0.93^{bcdgi}	0.95^{dehj}	0.97^{ek}	0.94^{fghl}	0.95^{ijkl}
Taste	0.35	1.61^{ab}	1.60^{ac}	1.62^{bc}	1.65^{e}	1.72^{d}	1.68^{f}	1.72^{d}
The sum of points		**3.94**	**4.15**	**4.01**	**4.51**	**4.68**	**4.52**	**4.62**
Quality category		VG	VG	VG	E	E	E	E

Values are means of three determinations (n=3)
Values in the same row with the same superscript are not statistically different (P > 0.05)
Quality category: E- excellent (4.5-5.0), VG - very good (3.5-4.5), G - good (2.5-3.5), NO (< 2.5) - unsatisfactory



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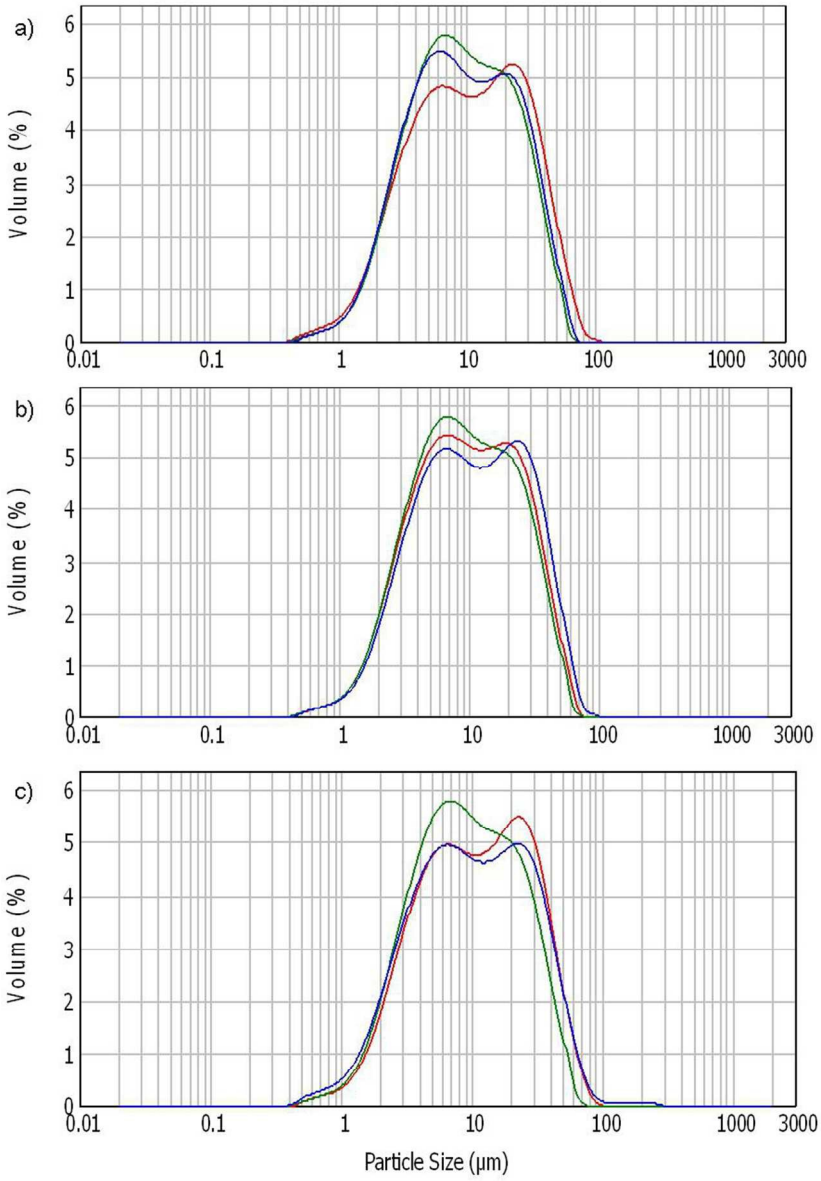

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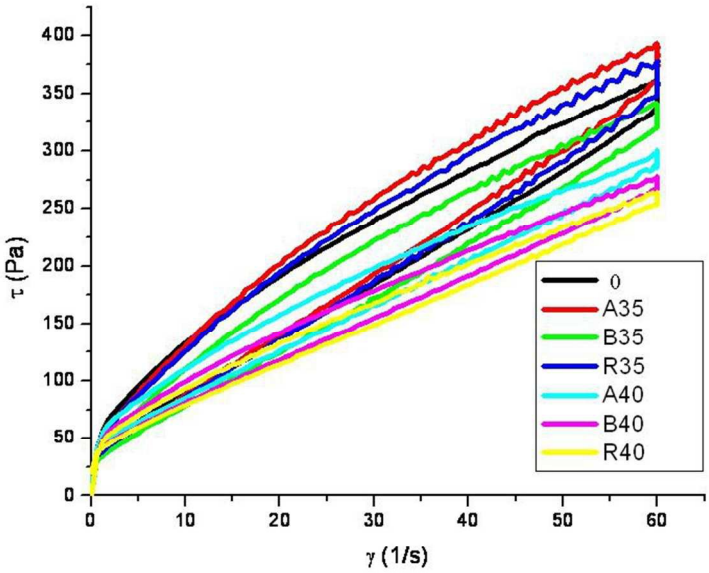

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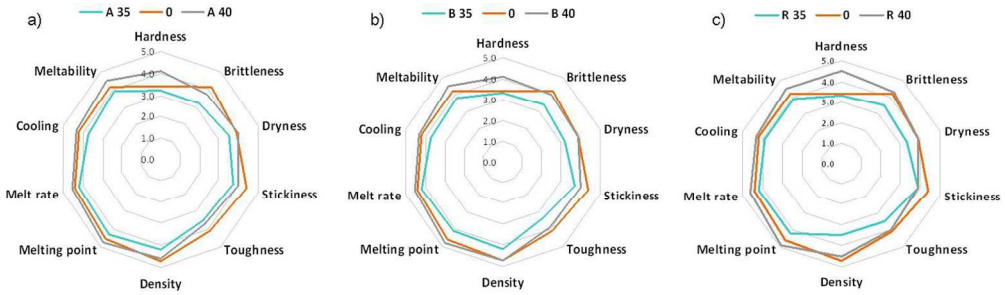

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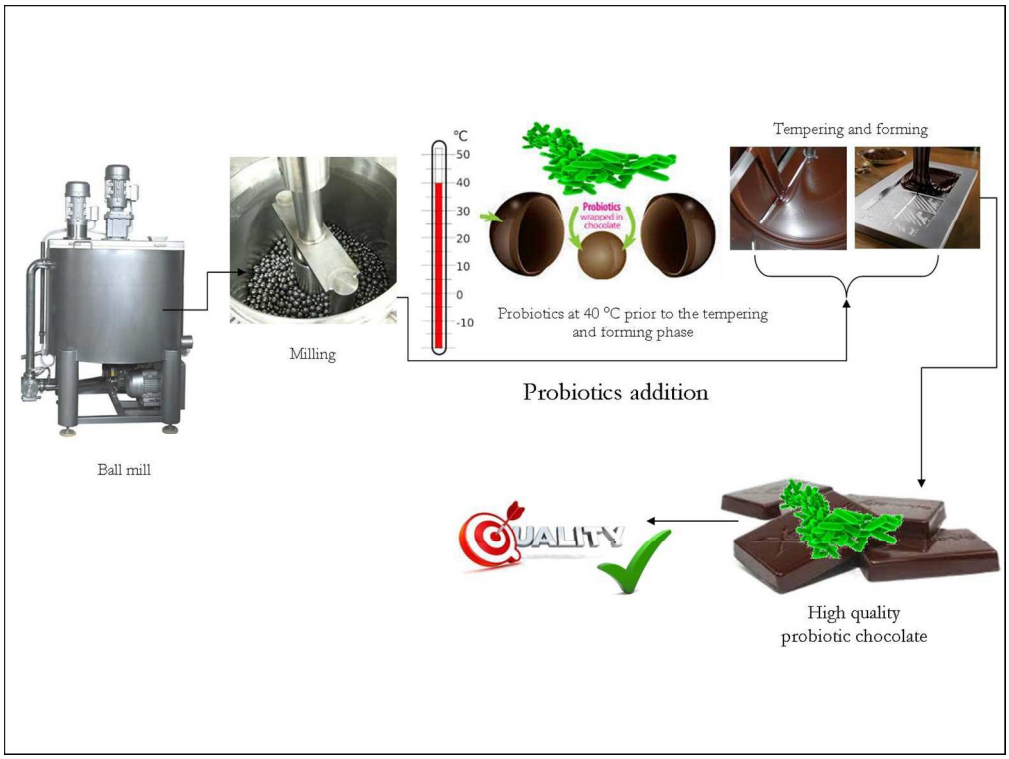

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