

## Effect of Milk Chocolate Supplementation with Lyophilised *Lactobacillus* Cells on its Attributes

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### Abstract

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Manufacturing of novel foodstuffs supplemented with live probiotic bacteria has recently been intensively investigated. The supplementation of confectionery with probiotics is troublesome since some unit technological processes are conducted at high temperatures and the products are usually stored at ambient temperature. Our group has developed a method of the production of milk chocolate, sweetened with either sucrose or isomalt and aspartame, containing 32, 36, or 40 g/100 g fat, and supplemented with live cells of probiotic bacterial strains: *Lactobacillus casei* and *paracasei*. This new milk chocolate displayed the same sensory properties as the reference, probiotic-free chocolate. The number of live bacterial cells was maintained at the functional level of  $10^6 \div 10^8$  CFU/g after keeping for 12 months irrespective of the temperature. The highest number of live probiotic bacteria survived in the chocolate kept at 4°C. Thus the product can be regarded as functional food.

**Keywords:** milk chocolate; properties of chocolate; lyophilisate; LAB

The consumption of products supplemented with live cells of lactic acid bacteria (LAB), in particular with their probiotic strains, is believed to benefit consumers' health due to their well documented positive impact on the function of gastro-intestinal tract and immune system, reduction of blood cholesterol, and apparent anticancer activity (YOON *et al.* 2006). However, the health-promoting effect of LAB depends on the respective strain and the frequency of the consumption of a sufficient dose of their live cells. The functional level of live LAB at the end of the product shelf life is thought to be close to  $10^6$ – $10^7$  CFU (colony forming units) in 1 ml or

1 g of the product (STANTON *et al.* 2005; ARA-GON-ALEGRO *et al.* 2007).

LAB have been used for centuries for the production of various fermented foods like vegetable preserves and fermented dairy products: yoghurts, kefir and acidophilic milk, cheese, some sausages (e.g. salami, chorizo), and also as starters in the bread making (YOON *et al.* 2006). An increasing demand of consumers for foodstuffs supplemented with live LAB, preferentially probiotic ones, gave rise to studies on the enrichment of some other foods with these microorganisms.

For instance, some researchers tried to supplement ice-cream with live cells of probiotic LAB

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such as: *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, and *Lactobacillus johnsonii*. These products had acceptable sensory properties, slightly higher acidity, a lower freezing point and a higher melting rate than the traditional ice-cream but the survival of the probiotic strains examined was different and depended on the experimental conditions (ALAMPRESE *et al.* 2002; SALEM *et al.* 2005).

Also, some sorts of cheese were manufactured by using probiotic or potentially probiotic bacteria such as: *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium animalis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (applied either as a single strain or a mixture of several strains), apart from LAB naturally occurring in starters. The probiotic strains were either added to milk together with the starter cultures or added to cheese (either cottage cheese or hard pressed cheese) during further steps of its processing (e.g. during cheese milling). The viability of LAB was determined during the cheese manufacturing process and cheese keeping for several to several dozen weeks either under refrigerating conditions or at ambient temperature (between 10°C and 20°C). BLANCHETTE *et al.* (1996) who kept batches of cottage cheese under refrigerating conditions for 15 days observed a decrease in the population of *Bifidobacterium infantis*. They explained this phenomenon by adverse environmental conditions such as too low pH and high titratable acidity (BLANCHETTE *et al.* 1996). GOBETTI *et al.* (1998) observed a good viability of *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis* in Crescenza cheese. Bifidobacteria were characterised by an excellent viability in Cheddar cheese during the storage for more than 20 weeks under refrigerating conditions. Even after this time the count of live *Bifidobacterium* cells remained at a high, functional level. The probiotic Cheddar cheese was characterised by an acceptable texture, physicochemical parameters, and sensory properties, which were not statistically different from those of traditional (not supplemented with probiotics) cheese of this sort. *Bifidobacterium infantis* cells display a high activity of  $\alpha$ -galactosidase which catalyses hydrolysis of lactose to galactose and glucose. This enzyme can completely degrade lactose contained in cheese even in one day. Thus, the application of

*Bifidobacterium infantis* enables the production of lactose-free Cheddar-type cheese which can be consumed by humans suffering from lactose intolerance (DINAKAR & MISTRY 1994; DAIGLE *et al.* 1999). The ripening temperature of Cheddar cheese affects neither the viability of probiotic bacteria nor the concentrations of salt, fat, and protein, but it strongly affects the humidity and pH of cheese, and first of all the population of the starter cultures. The ripening time and ripening temperature of cheese and the concentrations of lactic and acetic acids were found to be strain-dependent. The selection of appropriate probiotic strains to be used in manufacturing Cheddar-type cheese is very important because these strains should not affect the ripening temperature and ripening time of cheese (ONG & SHAH 2009).

The presence of prebiotics such as inulin or oligofructose stimulates the growth of bifidobacteria and lactobacilli in cheese (GOMES DA CRUZ *et al.* 2009). The supplementation of soft cheese with inulin can enhance the viability of probiotic or potentially probiotic bacteria like e.g. *Lactobacillus plantarum* 14 strain and improve the cheese sensory properties (MODZELEWSKA-KAPITULA *et al.* 2007).

Cheese can thus be a valuable probiotic alternative to fermented milk and yoghurt and its dense matrix (especially hard cheese matrix) and the relatively high fat content can additionally protect probiotic bacteria in the human gastro-intestinal tract, in particular in stomach (GOMES DA CRUZ *et al.* 2009).

Strains of *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum* were used to produce non-fermented and fermented fruit juices (e.g. from apples), vegetable juices (from carrots, red beetroots, white cabbage, celery), mixed vegetable or fruit-and-vegetable juices (e.g. supplemented with fermented sake lees). The survival of these bacteria was found to depend on the protecting substances added to the juices (sucrose, dietary fiber, polydextrose, dextrans), the form of bacterial culture (fresh cells or lyophilisate) as well as the duration and conditions of their storage (SAKAMOTO KARYO 2004; SAARELA *et al.* 2006; SUGIYAMA *et al.* 2006; YOON *et al.* 2006).

Another new product supplemented with probiotics was oat flakes coated with a mixture of chocolate, lyophilised cells of *Lactobacillus rhamnosus*,

and a protectant (sucrose, and a mixture of oat flour with 20%  $\beta$ -glucan, polydextrose, or wheat dextrin). This foodstuff was also characterised by an appreciable survival of LAB for 7 months with the exception of the variant containing 20%  $\beta$ -glucan (SAARELA *et al.* 2006).

Other examples of a successful incorporation of LAB strains into novel carriers are as follows. A strain of *Lactobacillus paracasei* subsp. *paracasei* LBC 82 survived in the chocolate mousse and inulin (prebiotic)-containing chocolate mousse that were stored at 5°C (ARAGON-ALEGRO *et al.* 2007). The number of cells of *Lactobacillus* and *Bifidobacterium* strains added to a frozen vegetarian soybean dessert was maintained at the functional level for 6 months (HEENAN *et al.* 2004). Other examples described in literature comprise fermented soybean products supplemented with selected strains of *Lactobacillus acidophilus* and *Lactobacillus paracasei* (LEBLANC *et al.* 2004) and additives to foods and feeds for animals, fish, and shellfish (Dokuritsu Gyosei Hojini Suisan Sogo Kenty & Yakult Honsha 2004; RANGANATHAN 2004; RYUKYU BIORESOURCE KAIHATSU 2004).

Confectionery products provide for consumers calories and sweetness (organoleptic properties) while usually having no added value. The development of new technologies facilitating the supplementation of confectionery with LAB can yield novel products, enriched with health-promoting ingredients that are capable of preventing civilisation disorders. Because confectionery products are consumed by children and teenagers, their supplementation with live LAB is advisable. The basic criterion of quality evaluation of this sort of products should be the maintenance of LAB cells at a functional level during technological processes and throughout the storage at ambient temperature. The acceptance of sensory attributes by consumers is also of a great importance and therefore these products should have the same sensorial characteristics as the traditional LAB-free ones.

The authors of this work supplemented milk and dark chocolates, either sweetened with sucrose or sucrose-free. The preparation of LAB was executed by spread drying of yoghurt bearing two LAB strains: *Streptococcus thermophilus* MK-10 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 151. The LAB preparation had the characteristic sour taste of yoghurt, however, despite an excellent survival of LAB its usefulness in chocolate manu-

facturing was found to be limited (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006).

This work reports on the effect of the lyophilisate of probiotic *Lactobacillus* strains (*Lactobacillus casei* ŁOCK 0900 B/00019, *Lactobacillus casei* ŁOCK 0908 B/00020 and *Lactobacillus paracasei* ŁOCK 0919 B/00021) on the physicochemical and sensory attributes of milk chocolate either sweetened with sucrose or sucrose-free. The survival of these 3 strains was determined after keeping the chocolate for 12 months at 18°C (the recommended temperature), 4°C (in refrigerator), and 30°C (above the recommended temperature of keeping).

## MATERIALS AND METHODS

**Materials.** The milk chocolate masses examined and bars of milk chocolate obtained from these masses contained the lyophilisate of live cells of probiotic *Lactobacillus* strains that partially replaced skimmed powdered milk. The chocolate was sweetened either with sucrose or with isomalt. Because isomalt is less sweet than sucrose, the sucrose-free chocolate masses were additionally sweetened with aspartame. The concentration of aspartame was calculated using the following formula:

$$0.6 \times \text{isomalt content (\%, w/w)} + 200 \times \text{aspartame content (\%, w/w)} = 1 \times 100 \text{ (\%, w/w)}$$

Coefficients 0.6, 200, and 1 reflect the relative sweetness of isomalt, aspartame and sucrose (standard), respectively.

The chocolate masses and chocolates examined contained 32, 36, or 40 g/100 g fat while the cocoa liquor content was maintained at 40 g/100 g. Cocoa butter was manufactured by Barry Callebaut and cocoa liquor was purchased from the Factory of Confectionery Industry Goplana S.A. (Nestle, Poznań, Poland). The cocoa liquor contained  $1.8 \pm 0.1$  g/100 g water and  $52.3 \pm 0.3$  g/100 g fat. The average diameter of its particles was  $58.0 \pm 0.5$   $\mu\text{m}$  (it was measured by a micrometric screw NSK Digimark Electronic Micrometer with electronic readout of the results – Japan Micrometer MFG Co., Ltd., Osaka, Japan). Skimmed powdered milk was purchased from Fleur Ltd. (Łódź, Poland). It contained  $3.4 \pm 0.2$  g/100 g water and the average diameter of its particles was  $73.9 \pm 0.5$   $\mu\text{m}$ . Sucrose and Isomalt PF were manufactured by

sugar factory Ostrowy S.A. (Ostrowy, Poland) and Palatinit Süßungsmittel GmbH (Mannheim, Germany), respectively. Isomalt contained  $3.5 \pm 0.2$  g/100 g water and the average diameter of its particles was  $61.1 \pm 0.4$   $\mu\text{m}$ . It completely replaced sucrose in sucrose-free chocolate. The concentration of the standard liquid soybean lecithin with phospholipid content of 55 g/100 g (derived from the Factory of Fat Industry in Kruszwica, Poland) in the chocolate masses was 0.3 g/100 g.

The reference milk chocolate masses and milk chocolate (without *Lactobacillus* cells) were produced for the comparison reasons. All variants of both types of chocolate masses and chocolates were produced in triplicates.

**Strains of LAB.** The lyophilised preparation of LAB contained 3 strains:

- *Lactobacillus casei* strain No. ŁOCK 0900 B/00019,
- *Lactobacillus casei* strain No. ŁOCK 0908 B/00020,
- *Lactobacillus paracasei* strain No. ŁOCK 0919 B/00021.

All these strains were obtained from the Collection of Pure Industrial Microbial Cultures at the Technical University of Lodz ŁOCK 105. These strains were deposited in the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław.

**Properties of the LAB strains.** The strains were selected on the basis of the results of *in vitro* studies. They were resistant to the acidity of gastric juice and to bile, adhered to epithelial cells, and displayed an antimicrobial activity. The studies were carried out according to FAO/WHO recommendations (Report FAO/WHO2001, 2002). On the basis of the sequence of the gene encoding 16S rRNA, the examined bacterial strains were classified as *Lactobacillus casei* and *Lactobacillus paracasei* (97 ÷ 99% similarity). Both these species rank among the typical microflora of human intestines and can be safely used for the production of fermented milk products and probiotics preparation. The strains examined were tolerant to pH 3.5. Almost all cells survived 3 h incubation at pH 3.5 and at neutral pH (6.5) while 80 ÷ 100% cells survived at pH 2.5 (depending on the strain) while in the presence of 4% bile salts only 60% cells survived. All the examined LAB strains exerted inhibitory effects on pathogenic bacteria, both Gram-negative and Gram-positive. The *in vivo* studies employing 2-month old immunocompetent mice Balb/c

revealed no translocation of these bacteria to the blood and other internal organs. Minor amounts of these bacteria in mesenteric lymph nodes could be evidence for immune system activation. The safety of the application of these strains was also proved through *in vivo* studies employing children suffering from atopic skin inflammation (CUKROWSKA *et al.* 2007).

**Production of *Lactobacillus casei* and *paracasei* lyophilisates.** Pure strains of *Lactobacillus casei* and *paracasei* were cultured in fermentors in MRS culture medium (purchased from BTL Ltd., Warszawa, Poland). The inoculum (1 ml) was added to the culture medium (100 ml) and the culture was conducted for 24 h at 37°C. The cells were harvested by centrifuging (Multifuge 3L/3L-R, at 2750 g for 30 min), washed twice with sterile distilled water, mixed with the same amount of skimmed powdered milk, frozen at –20°C, and lyophilised for 24 h in a tray lyophiliser Delta 1-24LSC (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) under the following conditions: the temperature of heating plates –15°C, pressure 50 MPa, temperature of condenser –45°C; ultimate temperature of drying +20°C.

The lyophilisate of *Lactobacillus* cells contained  $5.0 \pm 0.2$  g/100 g water.

**Production of chocolate masses and chocolate.** The milk chocolate masses were produced in a laboratory ball mill. They were ground at 45°C and 75 rpm until the average diameter of solid particles of approximately 25  $\mu\text{m}$  was reached. The soybean lecithin was added to the masses half an hour before the completion of grinding. The lyophilised bacteria were added at 40°C in the proportion of 3.33 g/100 g that provided the functional level of LAB (at least  $10^6$ – $10^7$  CFU/g).

On completion of the grinding in the ball mill, the chocolate masses were tempered at 30°C for 10 min in a laboratory temperer, poured into forms, cooled, and then removed from the forms. The chocolate bars produced were wrapped in aluminum foil (manually) and subjected to physicochemical analyses. The survival of LAB cells was determined after keeping the bars for 12 months at 4°C, 18°C, and 30°C.

## Analytical methods

**Solid substance content.** Solid substance content was assayed by thermogravimetric method. The samples of chocolate (4 g) were mixed with sand

(20 g) and dried at  $102 \div 105^\circ\text{C}$  until constant weight.

**Total acidity.** Total acidity of each sort of chocolate was determined by potentiometric titration using CG 843 pH-meter (SCHOTT VERTE GmbH, Mainz, Germany). The chocolate samples (5 g) were added to 100 g of boiling distilled water, the mixture was boiled under the lid for 10 min and cooled to  $20^\circ\text{C}$  (water evaporated during boiling was replenished). The suspension of chocolate was filtered through filter paper and 50 ml of the filtrate was titrated with 0.1 mol/l NaOH until pH 8.2. The results were expressed in ml of 1 mol/l NaOH per 100 g of chocolate.

**Volatile acidity.** Volatile acidity was assayed by steam distillation (10 g chocolate, 0.1 g tartaric acid, 50 ml distilled water; distillation was terminated when the volume of distillate was 400 ml but only if the pH of the distillate flowing from the cooler was neutral). The distillate was titrated with 0.1 mol/l NaOH using phenolphthalein as pH marker. The results were expressed as g of acetic acid in 100 g of chocolate. The results of this assay were calculated according to the equation:

$$\text{Total acidity} = (0.6 \times a)/m$$

where:

0.6 – mass of acetic acid (expressed in g) in 100 g of chocolate, equivalent to 1 ml of 0.1 mol/l NaOH used to neutralise the distillate

$a$  – volume (expressed in ml) of 0.1 mol/l NaOH used to titrate the distillate

$m$  – mass of the chocolate sample used in the assay (g)

**Casson viscosity and yield value.** The Casson viscosity and yield value were determined using HADVIII+ rheoviscometer with a coaxial rotor (SC4-27) and stator (Brookfield Engineering Laboratories, Inc., Middleboro, USA). The latter was equipped with a pan for small samples. Prior to these measurements, each chocolate mass was melted for about 75 min at  $50^\circ\text{C}$  (in the laboratory oven), transferred to the stator and mixed for 10 min at  $40^\circ\text{C}$  at  $5 \text{ s}^{-1}$  according to SOKMEN & GUNES (2006). The shear stress was measured at  $40^\circ\text{C}$  and at shearing rates ranging between  $5 \text{ s}^{-1}$  and  $60 \text{ s}^{-1}$ . The computer program Rheocalc V2.0 (Brookfield Engineering Laboratories, Inc., Middleboro, USA) was used to calculate the results of these measurements with Casson-Steiner model (shown below). The coefficient  $m$  in Casson-Steiner's equation was 0.5 (STEINER 1958; CHEVALLEY 1991).

$$(1 + a) \times \tau^{0.5} = 2 \times \tau_{\text{CA}}^{0.5} + (1 + a) \times \eta_{\text{CA}}^{0.5} \times D^{0.5}$$

where:

$a$  – the ratio of rotor radius to stator radius = 2.139

$\tau$  – shear stress (Pa)

$\tau_{\text{CA}}$  – Casson yield value, i.e. the shear stress at the shear rate decreasing to zero (Pa)

$\eta_{\text{CA}}$  – Casson viscosity (Pa·s)

$D$  – shear rate ( $\text{s}^{-1}$ )

**Shape of particles.** The shape of solid particles in chocolate masses and raw materials was determined by using a Jenamed 2 optical microscope (Carl Zeiss, Jena, Germany).

**Texture.** The texture (hardness) of chocolate was assayed at  $20^\circ\text{C}$  by using INSTRON 5544 apparatus (Instron Corporation, Norwood, USA), equipped with a penetrator and governed by Merlin software. The squeezing test was undertaken to determine the destructive tension and force. It was repeated 5 times with each chocolate variant.

**Calorie value.** The calorie value of each sort of chocolate was calculated on the basis of its formulation. The calorie values of the components were as follows: fat 37.6 kJ/g, sucrose 16.5 kJ/g, isomalt 10.0 kJ/g, protein (casein) 24.5 kJ/g, and lactose 16.5 kJ/g. The average contents of protein and lactose in the powdered milk were 25.1 and 37.7 g/100 g, respectively.

**Sensorial analysis.** Sensorial analysis of each variant of chocolate was carried out according to Polish Standard PN-A-88032: 1998. The evaluation of the sensorial attributes included the following determinants of quality: the appearance in a packaging, shape, colour, texture (hardness, smoothness), conchoidal fracture, aroma, taste, and surface (upper, bottom). Each of these features was evaluated on 5-point scale ( $1 \div 5$ ), in which 5 and 1 corresponded to the highest or worst quality, respectively. Next, each score ( $1 \div 5$ ) was multiplied by the relevant coefficient of importance, recommended by the Polish Standard. Because our chocolate bars were manually wrapped in aluminum foil, the routine evaluation of their appearance in the packaging was impossible. For this reason, the score of the latter parameter was arbitrarily assigned 5, for all the chocolate bars examined.

The sum of multiplication products constituted the overall point score of each variant of chocolate. The results of the sensorial analysis were expressed in the following scale: 5 – excellent quality; 4 – acceptable quality; 3 – less acceptable (tolerable)

quality; 2 – unacceptable quality; 1 – defective piece. The evaluation of the sensorial properties of each variant of chocolate was carried out in triplicates by 5 qualified panelists.

**Water activity.** Water activity was determined in triplicates in each variant of chocolate by using HYGROPALM AW1 meter (Rotronic, Helvetia, Switzerland) equipped with a digital probe AW-DIO. Freshly disintegrated samples of chocolate (approximately 12 g) were weighed into WP-40 vials that were closed and left for 30 min at  $23 \pm 1^\circ\text{C}$  to approach the state of humidity equilibrium. This incubation was followed by the measurement of water activity at the same temperature. For this purpose, each vial was opened and placed on a plate. The digital probe was put on the sample and the hygrometer was switched on. Water activity and temperature were shown after 2 min on the display.

**Survival of *Lactobacillus* strains.** Live bacterial cells were enumerated as colony forming units (CFU). The chocolate was suspended in a physiological saline solution (8.5 g NaCl/1000 ml) and the series of decimal dilutions were spread in triplicates on agar MRS medium (purchased from BTL Ltd., Łódź, Poland). The plates were incubated at  $37^\circ\text{C}$  for 48 h in the atmosphere of  $\text{CO}_2$  (10 ml/100 ml) and the number of live cells was expressed as logarithm of CFU per 1 g of chocolate (Figure 2). The degree of survival of LAB: *Lactobacillus casei* and *paracasei* during keeping the chocolate was calculated using the formula:

$$\text{Degree of survival (\%)} = \frac{N}{N_0} \times 100$$

where:

$N$  – log CFU/g in the chocolate after the definite time of storage

$N_0$  – log CFU/g in the newly produced chocolate

**Statistical analysis.** All the assays were carried out in triplicates on each variant of chocolate and the results were averaged. One-way ANOVA was carried out to find if the differences between the results were statistically significant.

## RESULTS AND DISCUSSION

Manufacturing technology and physicochemical, sensorial and microbiological characteristics of chocolate supplemented with live LAB cells have been hitherto reported only by our group. Our earlier papers addressed the supplementation of chocolate with traditional yoghurt bacteria (*Streptococcus thermophilus* MK-10 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 151) which had been embedded in spray-dried yoghurt (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006). This publication reports on the properties and survival of LAB in chocolate supplemented with lyophilised probiotic bacteria (*Lactobacillus casei* and *paracasei*).

### Solid substance content

The sucrose-free milk chocolates contained less solid substance than their counterparts sweetened with sucrose (Table 1). The main reason was the higher water content in isomalt (3.5 g/100 g) than in sucrose (0.02 g/100 g). Irrespective of the sweetener, the chocolate supplemented with lyophilised *Lactobacillus* cells contained less solid substance than LAB-free chocolate since the lyophilisate contained more water (5.0 g/100 g) than the other raw materials. Nebesny and co-worker characterised both the chocolates sweetened with sucrose and those sucrose-free that were supplemented with bacteria *Streptococcus thermophilus* MK-10 and

Table 1. Solid substance content (g/100 g) of milk chocolates (means  $\pm$  S.D.)

Type of chocolate	Total fat content per 100 g		
	32 g	36 g	40 g
With isomalt, aspartame and LAB	97.31 <sup>a</sup> $\pm$ 0.01	97.59 <sup>a</sup> $\pm$ 0.02	97.70 <sup>a</sup> $\pm$ 0.04
With sucrose and LAB	98.71 <sup>b</sup> $\pm$ 0.04	98.67 <sup>b</sup> $\pm$ 0.05	98.47 <sup>b</sup> $\pm$ 0.04
With isomalt and aspartame	97.80 <sup>c</sup> $\pm$ 0.05	97.85 <sup>c</sup> $\pm$ 0.04	98.17 <sup>c</sup> $\pm$ 0.04
With sucrose	98.90 <sup>d</sup> $\pm$ 0.04	98.71 <sup>d</sup> $\pm$ 0.02	98.72 <sup>d</sup> $\pm$ 0.03

<sup>a, b, c, d</sup>there were no statistically significant differences between the values in lines  $\alpha$  of 0.05

*Lactobacillus delbrueckii* subsp. *bulgaricus* 151 (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006). Solid substance content in yoghurt, sucrose-free chocolates was below 97 g/100 g (NEBESNY & ŻYŻELEWICZ 2006) while their counterparts containing lyophilised LAB contained as much as 97.31–97.70 g/100 g solid substance (Table 1). The latter value was more advantageous in terms of the product quality. Chocolates sweetened with sucrose and supplemented with either lyophilised LAB or powdered yoghurt (NEBESNY *et al.* 2005) had almost the same solid substance content which was only slightly higher in yoghurt chocolates.

**Total and volatile acidity.** The supplementation of milk chocolate with lyophilised *Lactobacillus* cells changed neither the total nor the volatile acidity irrespective of the sweetener (sucrose or isomalt and aspartame – Table 2). The total acidity of sucrose-containing milk chocolates was higher than that of their sucrose-free counterparts which resulted from the characteristics of these sweeteners. A similar relationship had been observed with yoghurt chocolates (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006). Besides, total acidity of yoghurt chocolates was higher by 0.3–0.4 ml 1 mol/l NaOH per 100 g compared to chocolates supplemented with lyophilised LAB, and this was reflected by a difference in their taste. The slightly acidic taste of yoghurt chocolates was a natural consequence of the content of powdered yoghurt (presence of lactic acid) (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006).

Volatile acidity of chocolates sweetened with isomalt and aspartame (both supplemented with LAB and LAB-free) was higher by 26–51% than that of analogous chocolates sweetened with sucrose. This difference was caused by the properties of the sweeteners. A similar relationship was observed with the chocolates containing powdered yoghurt. The values of volatile acidity of chocolates supplemented with either lyophilised probiotic bacteria or powdered yoghurt were not statistically different (NEBESNY & ŻYŻELEWICZ unpublished data).

**Viscosity and Casson yield value.** 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 the resistance during unit processes like mixing or pumping. This in turn enables to reduce the expensive cocoa butter content in the chocolate mass, the decreasing the quantities of chocolate mass that are necessary for effective coating and forming, faster and more uniform filling of chocolate forms, and an easier removal of air bubbles entrapped in the chocolate mass within the step of tapping. Very viscous chocolate masses contribute to worse sensorial attributes of chocolate during consumption (it sticks to teeth and palate). Therefore the determination of rheological parameters and their disclosure in the product specification are necessary. Industrial recipients of chocolate masses often specify their

Table 2. Volatile and total acidity (g acetic acid/100 g) of milk chocolates (means  $\pm$  SD)

Type of chocolate	Total fat content:		
	32 g/100 g	36 g/100 g	40 g/100 g
<b>Volatile acidity</b> (g acetic acid/100 g)			
With isomalt, aspartame and LAB	0.106 <sup>a</sup> $\pm$ 0.007	0.109 <sup>a</sup> $\pm$ 0.008	0.111 <sup>a</sup> $\pm$ 0.008
With sucrose and LAB	0.070 <sup>b</sup> $\pm$ 0.008	0.078 <sup>b</sup> $\pm$ 0.009	0.086 <sup>b</sup> $\pm$ 0.008
With isomalt and aspartame	0.107 <sup>c</sup> $\pm$ 0.006	0.110 <sup>c</sup> $\pm$ 0.007	0.112 <sup>c</sup> $\pm$ 0.006
With sucrose	0.073 <sup>d</sup> $\pm$ 0.006	0.080 <sup>d</sup> $\pm$ 0.008	0.089 <sup>d</sup> $\pm$ 0.011
<b>Total acidity</b> (ml 1 mol/l NaOH in 100 g)			
With isomalt, aspartame and LAB	4.3 <sup>e</sup> $\pm$ 0.2	4.2 <sup>e</sup> $\pm$ 0.3	4.2 <sup>e</sup> $\pm$ 0.4
With sucrose and LAB	4.8 <sup>f</sup> $\pm$ 0.4	4.8 <sup>f</sup> $\pm$ 0.4	4.9 <sup>f</sup> $\pm$ 0.4
With isomalt and aspartame	4.3 <sup>g</sup> $\pm$ 0.3	4.3 <sup>g</sup> $\pm$ 0.2	4.3 <sup>g</sup> $\pm$ 0.2
With sucrose	4.6 <sup>h</sup> $\pm$ 0.4	4.8 <sup>h</sup> $\pm$ 0.4	4.6 <sup>h</sup> $\pm$ 0.5

a, b, c, d, e, f, g, h there were no statistically significant differences between the values in lines  $\alpha$  of 0.05

rheological parameters that must be adequate for their purpose. The viscosity of dark chocolate masses destined for manufacturing of chocolate bars should be of 2 Pa·s while milk chocolate masses are more viscous because of the properties of powdered milk. Each alteration in the composition of chocolate mass changes its rheological parameters. Usually, the replacement of sucrose with other sweeteners increases Casson viscosity or/and Casson yield value of the chocolate mass. Its enrichment with dairy products (with an exception of fat) also increases these parameters. All raw materials used for chocolate manufacturing should be characterised by very low humidity because water considerably increases both the viscosity and Casson yield value of chocolate masses. Water contained in these masses can disturb the technological process or even damage machines. Chocolate masses and couvertures produced by the industry have various rheological parameters that depend on their purpose. Chocolate masses destined for the production of bars have different rheological attributes than those used in the manufacturing of chocolate figurines or coating cores. Therefore, it is difficult to define rheological characteristics of an ideal chocolate mass. However, each manufacturer of chocolate masses should be aware of the consequences related to the changes in their formulations. This knowledge enables to modify the technological process in order to reduce the problems caused by variations in chocolate mass composition. Rheological properties of chocolate masses can be regulated, but it is not simple. The decrease in their viscosity or/and improvement of fluidity can be achieved by the following methods: reduction of water content (e.g. by roasting at higher temperature, milling in multi-roller mills, long conching at elevated temperature, mixing or conching under vacuum), increasing cocoa butter content (it is not always possible because of the changes in taste and higher costs), conducting processes at elevated temperature (the drawback consists in the high likelihood of chocolate mass burning, particularly probable for milk chocolate masses), addition of lecithin in a concentration of up to 0.5% (optimally up to 0.3%), addition of other emulsifiers, e.g. PGPR which reduces first of all Casson yield value. Each of these methods aimed at reducing rheological parameters should be used skilfully. Investigations on the effect of emulsifiers and fat on rheological properties of

chocolate masses were carried out by Finke (FINKE 1965; WOODS 1976; SCHMITT 1994; NEBESNY *et al.* 1998; NEBESNY *et al.* 2002; NEBESNY & ŻYŻELEWICZ 2005). Casson viscosity and yield values of chocolate masses supplemented with live LAB cells that were obtained within the scope of this work were higher compared to the rheological parameters of analogous masses which did not contain lyophilised bacteria. However, they neither disqualified the product nor disturbed the technological process. Besides, if necessary, the values of these parameters can be decreased to the level typical of LAB-free chocolate masses by using any of the aforementioned methods.

The addition of lyophilised LAB to milk chocolate masses increased their rheological parameters such as Casson viscosity and yield value (Figure 1). It was caused mainly by the relatively high humidity of the lyophilisate (5.0 g/100 g). The sucrose-free chocolate masses (supplemented with LAB and LAB-free) contained more water than their counterparts sweetened with sucrose, and therefore they were characterised by higher values of Casson viscosity. In the presence of water, isomalt crystals can partially dissolve which leads to the formation of a thin layer of syrup on their surface. This in turn can increase the internal friction between isomalt particles manifested in a higher viscosity compared to sucrose-containing chocolate masses. The density of isomalt is slightly lower (1.5 g/cm<sup>3</sup>) than that of sucrose (1.6 g/cm<sup>3</sup>). Because the percentage contents of these substances (% w/w) in chocolate masses were the same, the chocolate sweetened with isomalt contained more solid particles with a larger surface area. Therefore, the latter masses were characterised by higher Casson viscosity than the analogous chocolate masses sweetened with sucrose.

It was observed that Casson yield values of milk chocolate masses sweetened with isomalt and aspartame (either supplemented with a lyophilisate of *Lactobacillus* cells or not) were lower than those of analogous sucrose-containing masses (Figure 1). This difference can be explained by different shapes of particles of the sweeteners on completion of the grinding process. Microscopic observations revealed that isomalt particles were regular and with round edges whereas sucrose particles were less regular and had sharp edges. This three dimensional structure of sucrose particles makes the adjacent layers of liquid (chocolate mass) overlap and this in turn worsens the flow characteristics

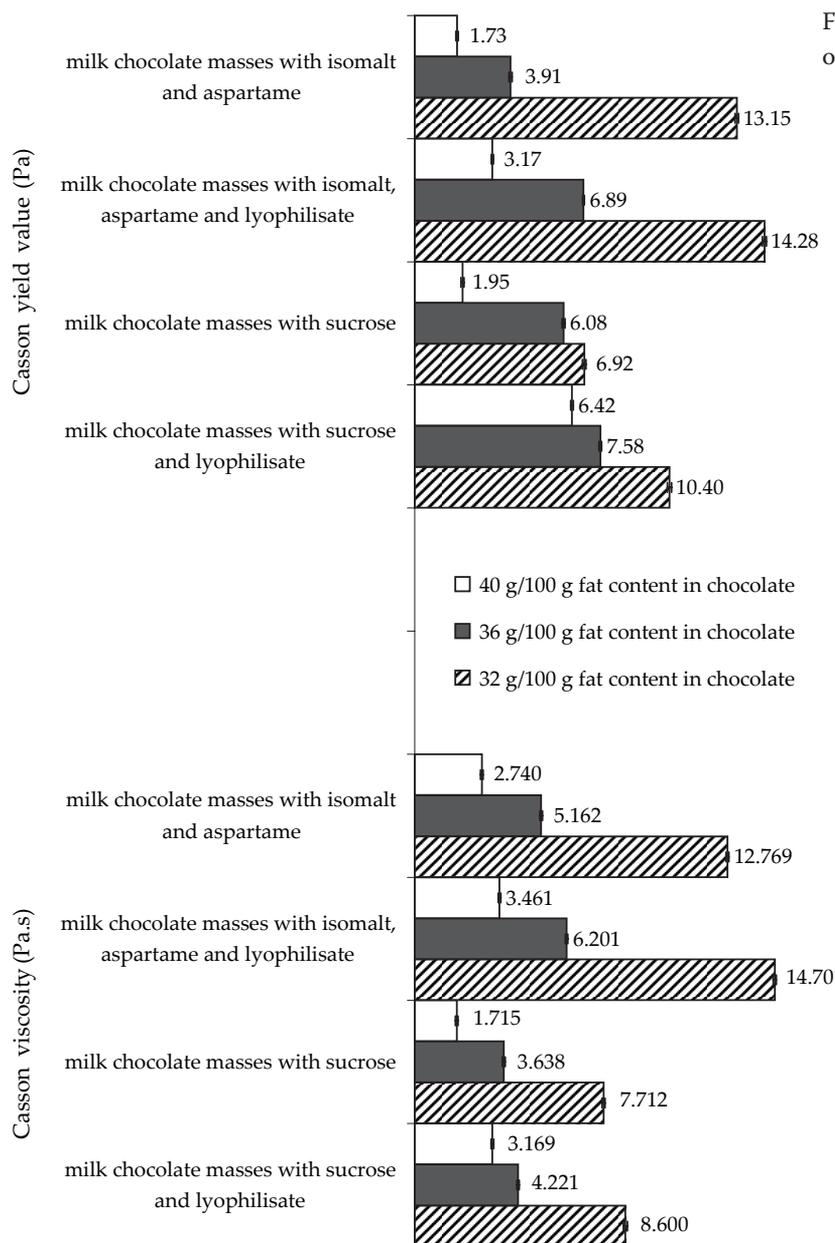


Figure 1. Casson yield value and viscosity of milk chocolate variants

of the system. An exception in this respect was the milk chocolate mass (sweetened with isomalt and aspartame) that contained 32 g/100 g fat because it was characterised by a higher Casson yield value than the respective sucrose-containing masses. Probably this one mass did not contain enough fat to coat all solid particles and to counteract the deleterious impact of water contained in isomalt on the rheological characteristics. The comparison of these results and those of our earlier study on yoghurt chocolates (NEBESNY *et al.* 2005; NEBESNY & ŽYŽELEWICZ 2006) indicates that like with Casson yield value and in contrast to Casson viscosity of chocolate masses supplemented with lyophilised LAB, both Casson viscosity and yield values of

milk yoghurt chocolate masses were lower than the same parameters of milk chocolate masses that did not contain powdered yoghurt. This phenomenon was caused not only by the shape of the powdered yoghurt particles (oval or spherical) but also by its composition. Dried yoghurt was a replacement of part of powdered milk in milk chocolate masses and therefore part of milk proteins was replaced by proteins with lower molecular masses and free amino acids contained in yoghurt. Probiotic bacteria were suspended in skimmed dried milk and therefore the partial replacement of the milk with their lyophilisate caused no changes in the protein profile of the supplemented product. Chocolate masses supplemented with powdered yoghurt were

characterised by lower rheological parameters, in particular Casson viscosity, compared to the masses supplemented with lyophilised LAB, this difference being higher for sucrose-free masses. For instance, Casson viscosity of milk chocolate masses containing 36 g/100 g fat and supplemented with lyophilised probiotic bacteria was 6.201 Pa·s (Figure 1) while with chocolate masses containing powdered yoghurt it was only 4.543 Pa·s (NEBESNY *et al.* 2005). However, if necessary, these two rheological parameters can be regulated by means of the methods described above.

### Texture (hardness)

There was no need to change the tempering conditions due to the supplementation of milk chocolate masses with lyophilised LAB. The hardness of milk chocolates that did not contain lyophilised *Lactobacillus* cells was slightly higher (Table 3). Of all the chocolate variants (irrespective of the sweetener and supplementation with LAB) those with the lowest fat content (32 g/100 g) were the hardest because of the highest content of solid particles. The mean values of the destructive tension and destructive force were at similar levels as with the chocolates supplemented with powdered yoghurt that had been characterised earlier by our group (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006). Because the content of milk fat (contained in skimmed milk) in these chocolates was low, the softness of chocolate and bi-component mixtures: fat with high melting point – milk fat was not increased.

### Calorie value

The replacement of sucrose with isomalt and aspartame and to a lesser extent also the supplementation of milk chocolate with lyophilised LAB cells changed the calorie value of the product (Table 4). The replacement of sucrose with isomalt decreased the calorie value of the chocolate supplemented with LAB and containing 32, 36, and 40 g/100 g fat by 8.5, 7.0, and 5.4%, respectively, whereas for the analogous LAB-free chocolates this decrease was 9.6, 8.4, and 6.8%, respectively. The addition of LAB lyophilisate to the sucrose-free chocolates only slightly increased (by 1.1–1.2%) their calorie values. By contrast, the calorie value of milk chocolates sweetened with sucrose was slightly decreased (by 0.1–0.4%) due to the supplementation with LAB. The comparison of the data for yoghurt chocolates (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006) and sucrose-free chocolates supplemented with lyophilised LAB revealed that the calorie value of the latter was slightly higher, i.e. by 6 kJ. The calorie value of chocolate with sucrose enriched with probiotic LAB preparation was on average lower by 24 kJ as compared to yoghurt chocolates sweetened with sucrose. Thus, the enrichment of chocolate with either lyophilised LAB or powdered yoghurt has a little effect on the calorie value. A stronger impact is observed when sucrose is replaced by isomalt which causes a decrease in the calorie value. However, the addition of lyophilised LAB to chocolate masses sweetened with isomalt negatively affects their rheological parameters (Figure 1), solid substance content (Table 1), and total acidity (Table 2).

Table 3. The hardness of milk chocolates (total fat content in g/100 g)

Type of chocolate	Destructive tension $\sigma_{\text{cmax}}$ (MPa)			Destructive force $F_{\text{max}}$ (N)		
	32	36	40	32	36	40
With isomalt, aspartame and LAB	7.51 <sup>a</sup> ± 0.76	6.52 <sup>a</sup> ± 0.84	5.84 <sup>a</sup> ± 0.85	72.25 <sup>e</sup> ± 4.95	62.78 <sup>e</sup> ± 5.09	60.06 <sup>c, e</sup> ± 5.57
With sucrose and LAB	7.55 <sup>a</sup> ± 0.70	6.40 <sup>a</sup> ± 0.57	5.99 <sup>a</sup> ± 0.30	72.64 <sup>e</sup> ± 4.73	61.64 <sup>e</sup> ± 4.48	57.64 <sup>c</sup> ± 2.88
With isomalt and aspartame	8.90 <sup>b</sup> ± 0.53	7.87 <sup>a, b</sup> ± 0.19	7.23 <sup>a</sup> ± 0.84	85.68 <sup>d</sup> ± 4.74	75.72 <sup>d, e</sup> ± 4.53	69.54 <sup>e</sup> ± 4.03
With sucrose	7.91 <sup>a, b</sup> ± 0.59	7.19 <sup>a</sup> ± 0.78	5.96 <sup>a</sup> ± 0.17	76.09 <sup>d, e</sup> ± 4.68	69.20 <sup>e</sup> ± 4.08	57.36 <sup>c</sup> ± 1.64

mean ± standard deviation; <sup>a, b</sup>no statistically significant differences between the values of destructive tension  $\alpha$  of 0.05; <sup>c, d, e</sup>no statistically significant differences between the values of destructive force  $\alpha$  of 0.05

Table 4. Calorie value (kJ) of milk chocolate variants (100 g)

Type of chocolate	Total fat content (g/100 g)		
	32	36	40
With isomalt, aspartame and LAB	1773	1883	1994
With sucrose and LAB	1937	2024	2108
With isomalt and aspartame	1752	1862	1972
With sucrose	1939	2032	2116

### Sensory analysis

The addition of the lyophilised preparation of *Lactobacillus* cells did not change the sensorial attributes of chocolate. The sensorial attributes of all the examined variants of milk chocolate received  $4.83 \div 4.86$  points on the 5 point scale and there were no statistically significant differences at the confidence level ( $\alpha$  of 0.05) between these scores. The number of points was almost the same as that for yoghurt chocolates (sucrose-free or sweetened with sucrose, milk or dark) given by (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006).

### Microbiological analysis

The number of live LAB cells contained in the product manufactured by our technology was maintained at the functional level ( $10^6 \div 10^8$  CFU/g) after keeping for 12 months irrespective of the temperature (Figure 2). The only exception was the sucrose-free milk chocolate that contained 32 g/100 g fat and was kept at 30°C. The survival of *Lactobacillus casei* and *paracasei* in milk chocolate was high. It implies that the milk contained in chocolate protects LAB cells. There was no difference in the survival of *Lactobacillus* cells between different sweeteners and different fat contents, and it was only slightly higher with the isomalt and aspartame containing chocolates that were kept at 4°C (relative to their analogues sweetened with sucrose).

As much as 85.2–88.3% of *Lactobacillus casei* and *paracasei* cells survived the incubation of the examined variants of chocolate for 12 months at 18°C, the ultimate number of live cells ranging between  $2.0 \times 10^7$  and  $3.5 \times 10^7$  CFU/g (Figure 2).

When the milk chocolates were stored at 4°C for 12 months, the survival of *Lactobacillus* cells was relatively higher (as compared to 18°C) and

varied between 91.9% and 95.3%. The number of live cells ranged between  $7.1 \times 10^7$  CFU/g and  $9.3 \times 10^7$  CFU/g in the chocolates sweetened with sucrose, and between  $1.1 \times 10^8$  CFU/g and  $1.4 \times 10^8$  CFU/g in those sweetened with isomalt and aspartame (Figure 2). Thus a decrement in live LAB cells was slowed down through chocolate refrigeration.

After 12 months of the chocolates incubation at 30°C, the survival of *Lactobacillus casei* and *paracasei* was only 72.3–76%, with the exception of sucrose-free milk chocolates containing 32 g/100 g fat, which were characterised by a significantly lower survival (59.4%). It is to note that, after 12-month storage at 30°C, the number of LAB cells in the other variants of milk chocolate was maintained at the lowest limit of the functional level ( $1.7 \times 10^6$ – $3.1 \times 10^6$  CFU/g). The steepest decrement in the cell number was observed until the 7<sup>th</sup> month of storage with the exception of 40 g/100 g fat-containing milk chocolates, with which a rapid decrease in live cell number was observed until the 5<sup>th</sup> month of storage (Figure 2).

The survival of LAB cells in the examined milk chocolates kept at 4 and 18°C was relatively high while at 30°C fewer cells of *Lactobacillus casei* and *paracasei* survived. However, also at the latter temperature their number remained at the functional level after 1 year storage. According to MATTILA-SANDHOLM *et al.* (2002), the survival of LAB in confectionery is mainly affected by the water activity, osmotic tension, and temperature. The persistence of *Lactobacillus casei* and *paracasei* in the examined milk chocolates can result from a few reasons. Firstly, these bacteria were added to the chocolate masses in the lyophilised form, thus the cells were in the state of anabiosis. Grinding the chocolate mass in a ball mill results in a complete mixing of all its components and the formation of an emulsion in which the solid particles (derived from cocoa beans, sweetener and

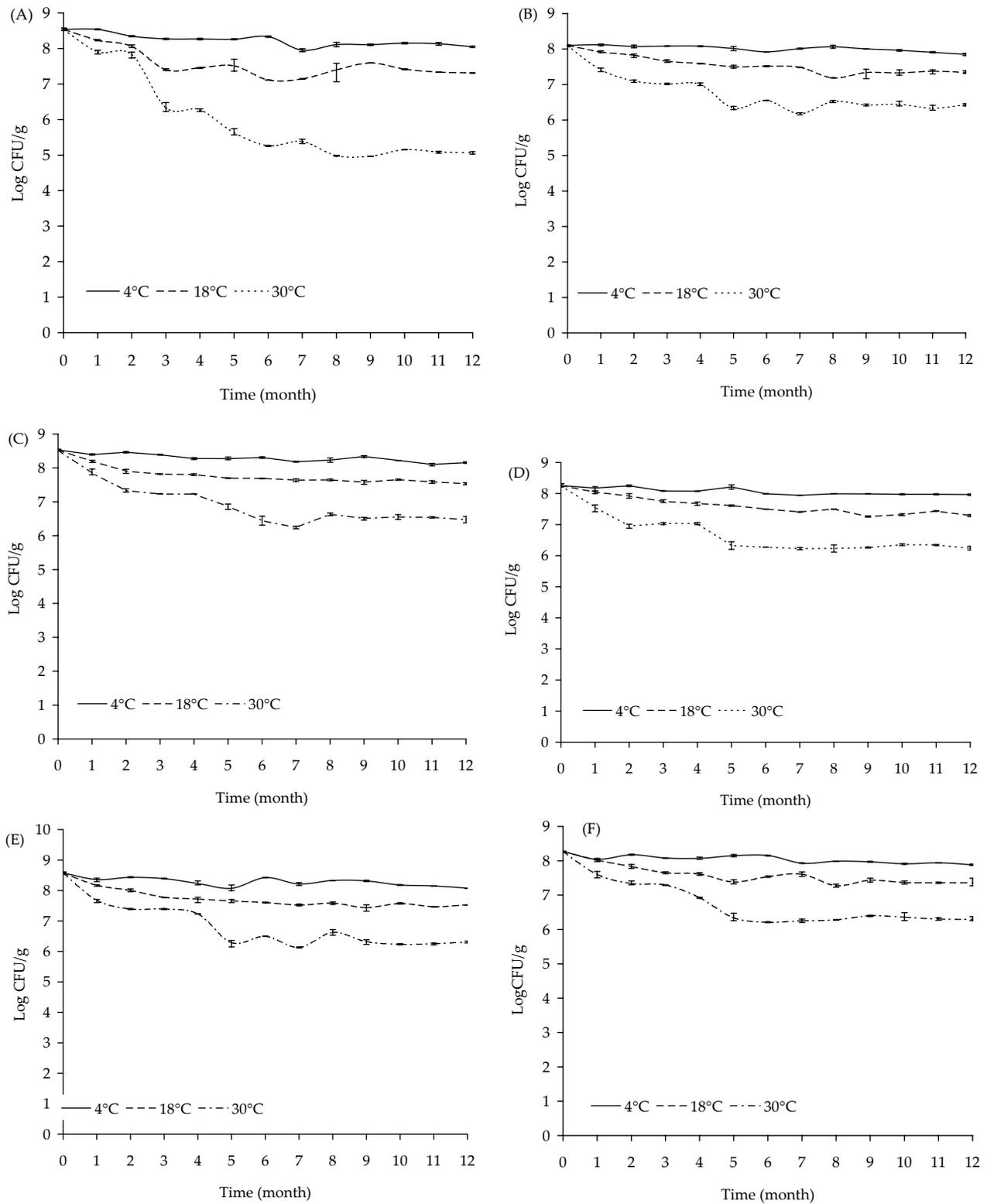


Figure 2. Survival of *Lactobacillus casei* and *L. paracasei* in milk chocolates stored at 4°C, 18°C, and 30°C: (A) milk chocolates with isomalt and 32 g/100 g fat content; (B) milk chocolates with sucrose and 32 g/100 g fat content; (C) milk chocolates with isomalt and 36 g/100 g fat content; (D) milk chocolates with sucrose and 36 g/100 g fat content; (E) milk chocolates with isomalt and 40 g/100 g fat content; (F) milk chocolates with sucrose and 40 g/100 g fat content

powdered milk) form a discontinuous phase and fat (from cocoa butter and milk) forms a continuous phase. Also LAB cells that are suspended in powdered milk are discontinuous phase components. After the transition of the chocolate mass to a chocolate bar, a majority of bacterial cells become entrapped inside the solid product in which gas diffusion is impeded (unlike in aerated chocolates). Only those LAB cells that are located close to the surface of the chocolate bar can have access to oxygen which is harmful to them. To minimise the contact of LAB cells with oxygen and to protect the chocolate bars from humidity and damage during keeping, they were tightly wrapped in aluminum foil (manually). However, oxygen concentration was not measured either inside the chocolate bars or between their surface and aluminum foil. Furthermore, water content in this environment was very low whereas the concentrations of fat and sweeteners were very high. Milk contained in the chocolate (20 g/100 g) protected *Lactobacillus* cells. Another, and probably the most important factor was the very low water activity of chocolate. The minimum water activity, at which gram-positive bacteria, including LAB, can grow, is 0.91 (McFARLANE 1994). The majority of chemical and enzymatic reactions occurring in the food products require water activity above that value, which is equivalent to the monolayer of water molecules. Water activity of all the examined variants of milk chocolate varied between  $0.118 \pm 0.005$  and  $0.200 \pm 0.006$ , being very low. Low water activity and high concentrations of sugars in the chocolate practically eliminate the growth of fungi and bacteria, even those of osmophilic yeast and xerophilic filamentous fungi. Only spores of bacteria and filamentous fungi can persist in chocolate. These spores can be formed by autochthonic microflora of cocoa beans and species acquired during their processing (e.g. during fermentation of cocoa beans). However, the spores cannot be activated and attack the chocolate because of the adverse conditions in this environment (MAZIGH 1994). The gradual disappearance of part of live *Lactobacillus casei* and *paracasei* cells that was observed during 12-month incubation of the chocolates supplemented with their lyophilisate could be the result of sublethal damage of these microorganisms either during the lyophilisation or during the chocolate manufacture.

After 12 months of keeping at 4°C and 18°C, the number of live LAB cells in chocolates con-

taining either the lyophilised probiotic bacteria or powdered yoghurt ranged between  $10^6$  CFU/g and  $10^8$  CFU/g (Figure 2) while the number of live lactic acid bacilli in yoghurt chocolates stored at 4 and 18°C decreased to  $10^3$  CFU/g after 2 to 4 months of keeping (NEBESNY *et al.* 2005; NEBESNY & ŻYŻELEWICZ 2006). After 6 months of keeping yoghurt chocolates at these temperatures, the number of live LAB cells remained at the functional level but almost all of them were cocci.

## CONCLUSIONS

The trend to enrich new foodstuffs with live *Lactobacillus* cells is a novel and promising approach to the application of LAB in the food production. The supplementation of chocolate with live LAB cells is one of these new applications. Our study proved that milk chocolate was a good carrier for *Lactobacillus casei* and *paracasei* cells. Their number was kept at a relatively stable level for 12 months at 18°C (the survival above 85%). Consequently, these chocolates can be stored at ambient temperature together with other confectionery. However, more LAB cells survived in refrigerated chocolate.

The changes in the technology of chocolate manufacturing resulting from its enrichment with live cells of *Lactobacillus casei* and *paracasei* entail neither the purchase nor the construction or application of additional equipment. Therefore, the results of this research project can be easily applied in the food industry. Chocolate is willingly consumed by children and teenagers. The supplementation of this product with live probiotic cells can enrich their diet. Sucrose-free chocolate supplemented with LAB can be consumed also by diabetics.

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