Cell Viability of *Bifidobacterium lactis* Strain in Long-Term Storage Butter Assessed with the Plate Count and Fluorescence Techniques

Magdalena OLSZEWSKA[1], Bogusław STANIEWSKI[2] and Łucja ŁANIEWSKA-TROKENHEIM[1]

[1]Chair of Industrial and Food Microbiology and [2]Chair of Dairy Science and Quality Management, Faculty of Food Science, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

Abstract

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Great interest in functional products containing bacterial strains displaying health-promoting properties is expressed worldwide and is as a result connected with a demand for developing new probiotic-based products, especially those containing bifidobacteria. The *Bifidobacterium* strains play a key role in gastrointestinal homeostasis, providing many health-related attributes, but as fastidious microorganisms require specific conditions (e.g. anaerobic environment, neutral pH) to survive in the long-term at the needed level above 10^6 CFU/g. In consequence, not every food product guarantees optimal maintenance of *Bifidobacterium* viability. From this point of view, the objective of the study was to examine the survival of *Bifidobacterium lactis* strain in butter during long-term refrigerated storage. Two enumeration techniques: microscopic LIVE/DEAD® and plating were compared by monitoring bifidobacterial counts for 4 weeks. The plate method was characterised by underestimation of the cell counts in relation to the results evaluated microscopically. However, the good survival exhibited by *B. lactis* was found with both techniques. Moreover, the microscopic LIVE/DEAD® method permitted to trace delicate changes in the viable/non-viable bifidobacterial population at the single-cell level.

Keywords: *Bifidobacterium* sp.; survival; food products; LIVE/DEAD® method; plate count method

Bacteria of the *Bifidobacterium* genus were discovered and described for the first time by Tissier in 1899 as microflora of breast-fed newborns. A natural environment for *Bifidobacterium* sp. is the human and animal gastrointestinal tracts. The population size of these bacteria in the gastrointestinal tract is high, amounting to 10^8–10^9 CFU/g, but it is subject to changes, depending mainly on the diet and the age of the organism (Soomro *et al.* 2002). Many health-related properties are attributed to these bacteria, including the regulation of intestine microbial homeostasis, inhibition of pathogenic bacteria, modulation of immunological response, anticancerogenic effects, production of bacteriocins or bioconversion of the diet components into the so-called bioactive compounds (Soomro *et al.* 2002; Hernandez-Mendoza *et al.* 2007).

In recent years, the production and consumption of products with the addition of strains displaying health benefits has been dynamically increasing (Khurana & Kanawjia 2007). The growing interest of consumers in such products is related to the demand for convenient and functional food. A

response to such demand is broadening and improving the range of products of therapeutic and prophylactic properties (Antunes *et al.* 2007). The concentration of probiotic bacteria required to gain health-promoting benefits is often valuated as > 10^6 CFU/ml in the small intestine and > 10^8 CFU/ml in the large intestine (Sanders 2003; Bertazzoni Minelli & Benini 2008). In the light of this, one should consume an adequate number of live and active cells, but there is simply not yet enough evidence to recommend the most appropriate dosage that is valid to all probiotic bacteria. The dose required differs significantly depending on the strain and product. Some products have proved to be efficient at lower levels, while some necessitate considerably higher ones, in the order of 10-fold or 100-fold in terms of CFU. The common dosage in humans is referred to as 10^7–10^9 CFU/ml (mg) per day (Bertazzoni Minelli & Benini 2008). The role of the matrix in bringing functional effects also has to be taken into account – the viability of the strain in a particular product that will remain up to the end of the shelf-life (Grand *et al.* 2003; Suvarna & Boby 2005; Hernandez-Mendoza *et al.* 2007). With this aim in view, monitoring the viability of intestine-derived bacteria in different types of products is required. Epifluorescence microscopy can be an advantage in this type of research. This method, after an appropriate sample preparation, makes it possible to determine relatively quickly the number of microorganisms in food products (Warmińska-Radyko *et al.* 2010). The plate count method, widely used in determining bacteria count, is based on the cell ability to proliferate and form colonies on a solid medium. Epifluorescence microscopy, depending on the staining technique applied, allows the users to specify the number of live cells on the basis of cytoplasmic membranes integrity, for example. One such method is LIVE/DEAD® staining. This assay includes a set of two fluorochromes: SYTO9, which penetrates the cell through the integrated, as well as damaged membrane, and propidium iodide (PI), which can penetrate only a damaged membrane (Breeuwand & Abee 2000; Warmińska-Radyko *et al.* 2010). In effect, live cells can be distinguished as visibly fluorescing green, and dead – as fluorescing red. The monitoring of subtle changes in the dynamics of the proliferation/decline of the population at the level of single cells is the basic advantage of its application in studies on the physiology of microorganisms (Joux & Lebaron 2000; Łaniewska-Trokenheim *et al.* 2010).

Therefore, a study has been undertaken into determining the viability of intestine-derivative *Bifidobacterium lactis* strain in butter during a four-week refrigerated storage. After microbiological analysis with the application of the fluorescence and plate methods, the size of the *Bifidobacterium* population assessed by those two techniques was compared.

MATERIAL AND METHODS

Preparation of butter product. *Bifidobacterium lactis* B100.6 strain (CSK Food Enrichment, Toruń, Poland) was studied. The model butters were prepared in seven repetitions by inoculating churned, pasteurised cream (30% (v/v) fat) with > 10^6 CFU/ml of the given culture. The prepared butters were stored at 6°C for 4 weeks. Sampling was performed at selected time intervals 0, 1, 2, 3, and 4 weeks.

Enumeration procedure. To estimate the live/dead bifidobacterial counts, the samples were examined by Direct Epifluorescent Filter Technique using LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, USA). The LIVE/DEAD® BacLight™ was performed according to the protocol provided by Molecular Probes. The stained cells suspensions prepared were filtered by means of a filter tower and a vacuum pump (both Millipore, Schwalbach, Germany) with the use of black polycarbonate filters (13 mm diameter, 0.22 μm pore size; Millipore, Schwalbach, Germany). Air-dried filters were mounted in nonfluorescent immersion oil (Molecular Probes, Eugene, USA) on glass slides underneath cover slips. Microscopic counts were performed with an image analysis software (Cell F) linked to a Olympus BX51 microscope (both Olympus Co., Hamburg, Germany) which was equipped with U-MNG2/U-MNB2 filters. The mean values of twenty fields per assay were obtained. As 'live cells' with integrated membranes fluoresce green, and 'dead cells' or injured ones with destabilised membranes fluoresce red, it was assumed that all green cells were viable and the red ones were dead. The optimisation of the staining procedure was followed by the manufacturer's instruction. At the same time, the plate count method was applied to evaluate the number of bifidobacteria. The samples were plated on Garshe agar (asparagin 10.0 g/l;

peptone (casitone) 20.0 g/l; cystin 0.4 g/l; lactose 20.0 g/l; $CH_3COONa \cdot 6H_2O$ 10.0 g/l; $MgSO_4 \cdot 7\ H_2O$ 1 g/l; $Na_2HPO_4 \cdot 12\ H_2O$ 4.66 g/l; KH_2PO_4 1.8 g/l; *p*-aminobenzoic acid 2.0 g/l; calcium pantothenate 2.0 g/l; biotin 20 µg; agar 1.8%; plus supplement g/100 cm^3 ddH_2O: lithium chloride 6.0; nalidixic acid 0.03; neomycin sulphate 0.2; paramycin sulphate 0.25) and incubated anaerobically (Anaerocult A; Merck, Darmstadt. Germany) at 37°C/72 hours.

Statistical analysis. After verifying the test assumptions, the significance of differences of the results obtained was based on the following statistical analysis. Analysis of variance (ANOVA) with repeated measurements was used to verify that the population size of *B. lactis* evaluated by microscopic counts vary significantly over time. Anova Friedman's Rank Test was applied to resolve whether the bifidobacterial counts determined by the plate method change statistically during storage. Mann-Whitney U Test was used to compare the results of the number of bifidobacteria determined by two methods of quantitative analysis, and for this purpose the comparison of the results in each measurement point ($P_{0,1,2,3,4}$) was made. The results of this test resolve whether the numbers of cells determined using those methods differed significantly. The significance of differences was analysed with respect to $P < 0.05$. The calculations were performed using Statistica StatSoft® Vers. 9 (StatSoft Inc., Tulsa, USA).

RESULTS AND DISCUSSION

At each stage of butter storage, significant differences were found between the counts of *B. lactis* assessed by the fluorescence method and by the plate method ($P_{0,1,2,3}$: $P = 0.002$; P_4: $P = 0.04$). The cell count determined by the plate method at each measurement point ($P_{0,1,2,3,4}$) was underestimated in relation to the count of live cells determined by the microscopic SYTO9 method (Figure 1). The value of the so-called underestimation of the plate method depended on the storage stage, and it was the highest in the samples of butter at zero determination and after one week – the difference in the numbers of cells between the plate and the microscopic SYTO9 methods was 2 logarithmic units. The results of determination by the plate method were also characterised by a larger range in comparison to the results obtained by the microscopic SYTO9 method, except for the last measurement, in which minimum-maximum values determined by the SYTO9 method affected the proximity of exceeding the criterion of the lack of differences between the determinations by both methods ($P = 0.04$).

The variation analysis showed that the count of *B. lactis* assessed by the plate method did not significantly change during the butter storage ($P = 0.14$), maintaining a high level of about 7 logarithmic units of CFU/g (Figure 1). Discrepancies in the results concerning the cell count determined by the plate method were the reason for introducing additional statistical calculations, comparing the numbers of bacteria in arrangements: $P_{0\leftrightarrow1;\ 1\leftrightarrow2;\ 2\leftrightarrow3;\ 3\leftrightarrow4}$ separately, and also here, no differences were found towards the established significance level. According to the determinations by the microscopic method with SYTO9, the count of live cells at the initial stage of storage was found at the level of 9 logarithmic units (Figure 2a). Afterwards, along with the butter storage time, it was observed that the number of viable cells revealed a tendency to decrease by more than one logarithmic cycle in the last week. A decreasing trend was confirmed by the results of statistical tests, indicating a significant influence of time on the size of the *B. lactis* population in butter ($P = 0.002$) (Figure 2a). The microscopic LIVE/DEAD BacLight® method proved a good viability of live cells of the *B. lactis* strain, preserving the integrity of membranes in the examined butter, and it enabled to monitor the rate at which the cells die out during the storage period. As it was calculated directly after manufacturing, a significant number of dead cells were introduced

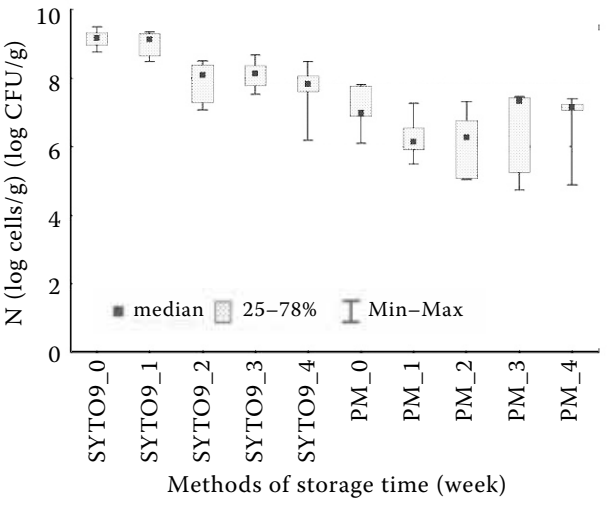
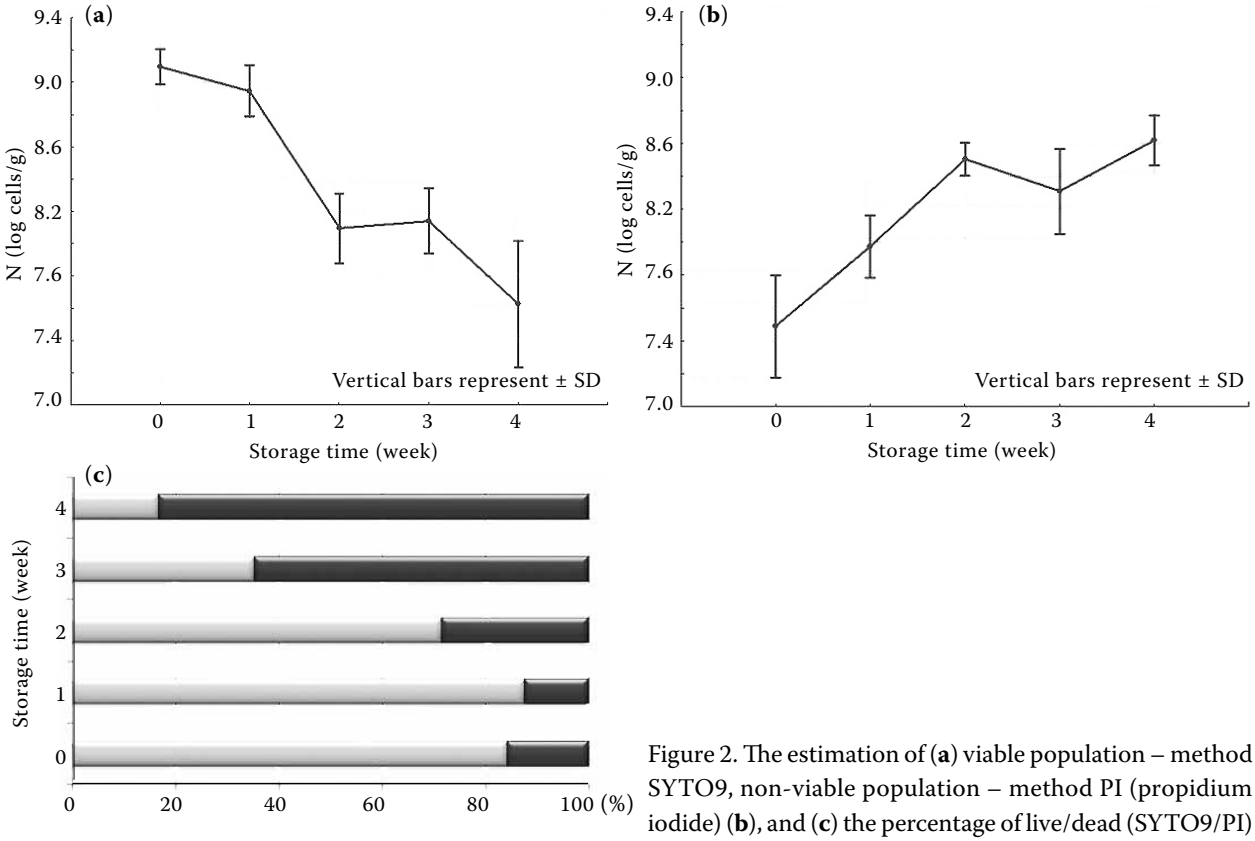


Figure 1. The comparison of *Bifidobacterium lactis* B100.6 counts in long-term storage butter obtained by microscopic method (SYTO9) and plate method (PM)



	0	1	2	3	4
SYTO9	84.25	87.72	71.59	35.31	16.80
PI	15.75	12.28	28.41	64.70	84.20

Figure 2. The estimation of (**a**) viable population – method SYTO9, non-viable population – method PI (propidium iodide) (**b**), and (**c**) the percentage of live/dead (SYTO9/PI) cells – microscopic method (LIVE/DEAD®) of *B. lactis* B100.6 in long-term storage butter assessed with microscopic method (SYTO9)

together with live cells, accounting for about 16% of the population (Figure 2c). In subsequent weeks, dying-out of the cells was observed and the balance between the numbers of live and dead cells was established between weeks 2 and 3. The cell dying process was slow – by week 4, the number of dead cells had increased by about one logarithmic unit, and according to the results of the statistical tests, it significantly increased during the storage period (P = 0.001) (Figure 2b). The LIVE/DEAD BacLight® method revealed that the population of *B. lactis* was characterised by stability under the conditions assumed by the experiment, displaying subtle differences in the counts of live and dead cells. The count of *B. lactis* strain determined in butter, as regards both live and dead populations, significantly changed during the four-week storage of the product, which could not be demonstrated while applying the plate method. The proportions of live and dead cells of *B. lactis* calculated on the basis of the microscope data (LIVE/DEAD BacLight® method) graphically display both tendencies revealed on the basis of statistics, namely, a reduction in the live population and an increase in the dead population during the refrigerated storage of butter (Figure 2c).

On the basis of the results obtained, it was found that the plate method was characterised by underestimation of the cell counts in relation to the values determined microscopically. The underestimation could result from the possibility of *Bifidobacterium* rods occurring in clusters and, consequently, from the doubtful origin of an individual colony from an individual cell (Moreno *et al.* 2006; Warmińska-Radyko *et al.* 2010). Another cause for inaccuracies in determining their number by the plate method could be the choice of the culture medium and incubation conditions, resulting from high nutritive and environmental requirements of *Bifidobacterium* and, consequently, from the uncertainty of their optimisation for the species and even the strain (Lahtinen *et al.* 2006). In addition, other things to consider should include the occurrence in the population of cells incapable of growing on the culture media, known as VBNC (viable but non-culturable) (Colwell 2000; Joux & Lebaron 2000; Oliver 2005; Olszewska & Łaniewska-Trokenheim 2011). This concerns the bacteria

cells in the products stored in the long-term as well as in starter cultures (Breeuwand & Abee 2000; Bunthof & Abee 2002; Lahtinen *et al.* 2005; Gatti *et al.* 2006; Lahtinen *et al.* 2006; Rault *et al.* 2007; Warmińska-Radyko *et al.* 2010). The cell population in the dormant state is identified as a result of various unfavourable conditions, such as e.g. temperature shock, starvation caused by the lack or exhaustion of nutrients, the presence of various bacteriostatic agents (Barer & Harwood 1999; Joux & Lebaron 2000; Bunthof *et al.* 2001; Guchte *et al.* 2002; Ben Amor *et al.* 2007). Lahtinen *et al.* (2006), while examining the viability of two strains of *Bifidobacterium* in an oat beverage stored in refrigerated conditions for 30 days, demonstrated a lower ability of the *B. longum* strain to stay alive in comparison to *B. lactis*, as assessed by the plate method and the LIVE/DEAD BacLight® method. However, they also demonstrated a statistically significant reduction in the number of *B. longum* during the product storage, from the level of 7.8 to 0.4 logarithmic units when determining their number by the plate method, and to 7.2 by the LIVE/DEAD BacLight® method. The count of the *B. lactis* assessed with the plate method changed within a month from the level of 7.2 to 6.3. LIVE/DEAD BacLight® results did not reveal this pattern, where the level of 7.2 (at the beginning) and 7.0 (at the end) was established – only in this case it was not a significant change of the count with time. The researchers, on the basis of those discrepancies in the determinations between the methods applied, suggest that those strains lost their ability to proliferate and part of the population (particularly large in the case of *B. longum*) entered the dormant state. Auty *et al.* (2001) also published results proving higher counts of *Lactobacillus paracasei* NFBC 338, *Bifidobacterium* sp. UCC 35612, and *Bifidobacterium* sp. UCC 401 in milk and in fermented milk as determined by the direct cell count method based on LIVE/DEAD BacLight® staining, in relation to the plate method – and the differences presented depended on the strain and the product in which they were quantitatively examined. Kramer *et al.* (2009), studying the viability of e.g. *B. lactis* BB-12 strain in a freeze-dried product during a three-month storage period, found a lack of significant changes in the number of those cells over the entire period of research, and the usefulness of the LIVE/DEAD BacLight® staining method in the determination of their count, being a supplement to the plate technique, which is characterised by the limitation of determining only the culture part of the population. Other researchers (Collado *et al.* 2005) took up the task of determining the viability of 10 strains of *Bifidobacterium* isolated from commercially available products, in *in vitro* model of the gastrointestinal conditions, quantitatively determined, as above. The individual strains differed in their resistance to stress factors. Nevertheless, it is worth emphasizing that microbiological analysis with the use of the two techniques revealed differences of about 1 logarithm, with the highest count always determined by the LIVE/DEAD method, leading to conclusions about the identification of the VBNC status. Ben Amor *et al.* (2002) undertook the assessment of viability of *B. lactis* and *B. adolescentis* cells in the presence of bile salt. The first strain proved to be more resistant, which in the environment of the bile salt concentration of about 0.1% demonstrated viability on a level between about 50% (plate method) to about 70% (fluorescence method), while the other strain revealed viability between close to zero (plate method) to about 40% (fluorescence method). Again, the determination results obtained by both techniques were not equal, which confirms the complexity of the induced physiological changes in those cells under the influence of stress factors.

The presence of VBNC cells, or ABNC (active but non-culturable) cells prevents their determination by the plate methods using proliferation as a determinant of microbe detection (Hermida *et al.* 2000; Joux & Lebaron 2000; Łaniewska-Tokenheim *et al.* 2010). Assuming that underestimation of the plate method resulting from its assumptions and from culture difficulties is constant and at the same level, the microscope and plate methods applied together in the research model can constitute a tool for differentiating populations into fully-active cells and cells with VBNC status (Decker 2001; Olszewska & Łaniewska-Trokenheim 2011). The estimated percentage of the dormant population (on the basis of the ratio of the number of live cells determined by the SYTO9 method to 'culturable cells' determined by the plate method) presents its proportion at each of the butter storage stages in Figure 3. The mean proportion of the dormant cell population was about 20%, but the differences observed during the four-week storage period were at the level of between 30% directly after manufacturing and 10% in the last week, with a continued decreasing trend. A higher proportion

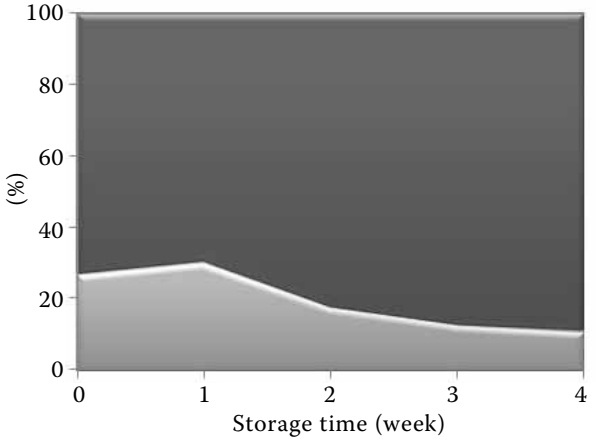


Figure 3. The percentage of the dormant population estimated on proportion between ■ 'viable cells' (determined by the SYTO9 method) and ■ 'culturable cells' (determined by the plate method) at each of the butter storage stages

of the dormant and/or damaged cells determined at the first stage resulted from a reduced activity of bacterial inoculum cells due to the form of a deeply frozen concentrate used for direct inoculation during butter working phase of churning. The percentage of cells in the physiological status characterised by non-proliferation, preserving the membrane integrity, is maintained during the first week of storage, and structure stabilisation is then gradually lost by the not fully active cells, the proportion of the dormant population being reduced. *Bifidobacterium* ssp. require an anaerobic environment and neutral pH to survive and maintain levels greater than 6 log CFU/g or ml (Boylston *et al.* 2004). Therefore, not all foodstuffs provide the most favourable conditions for the growth and survival of the *Bifidobacterium* genus. Scientific research demonstrates that the *Bifidobacterium* genus is characterised by a lower viability during storage in acidic foods such as yoghurt and cultured milk (pH of 3.7–4.3) (Barreto *et al.* 2003; Gueimonde *et al.* 2004; Antunes *et al.* 2005) which, in such cases, does not apply to butter made of sweet cream with a pH close to neutral (pH of 6.6). Moreover, Boylston *et al.* (2004) presented evidence that cheese (pH level between 4.8–5.6) provides an appropriate environment for a long-term survival of *Bifidobacterium*, proposing the effective incorporation of cells into cheese as the main criterion for the maintenance of bifidobacteria viability. The data indicated that, in spite of the fastidious characteristics of the *Bifidobacterium* genus, the count of the examined strain in butter for the entire period of storage was maintained at a high level of > 7 log units which could be determined by either of the methods applied. The plate method demonstrated a lack of significant changes in the cell count, the population of which was maintained at a stable level. The microscopic method also demonstrated a good viability of the *B. lactis* strain cells maintaining the integrity of membranes in the examined butter but, at the same time, it provided the possibility of monitoring the changes in the counts of live and dead cells during storage. This method provides an interesting alternative to the conventional method by basing the cell detection criterion on other viability determinants than proliferation. However, a combination of techniques based on various approaches to the assessment of the physiological status of cells can become an advantage while analysing the multidimensionality of a population and enriching knowledge of the physiology of microorganisms.

The obtained results of *B. lactis* viability in butter encourage the continuation of model research for such a matrice, in order to expand the range of products of health-related properties. Unless the maintenance of the *B. lactis* viability for a whole examined period was observed, other factors like the evaluation of health claims of this specific strain should be also taken into account. While considering therapeutic aspects of intestinal microflora, according to FAO/WHO and WGO the term 'probiotic' should be used cautiously, since they involve the requirement to carry out clinical trials confirming their effects. What is more, EFSA highlights the relation between the health claim and the number of bacteria consumed with the food dose. It was seen that *B. infantis* was considered effective in lowering the symptoms of Irritable Bowel Syndrome at min 8 log CFU/day. As a result, the daily dose of probiotics ranges from 8 to 9 log CFU with daily amount of 100 g infant formula. From the functional point of view butter should be considered as an additional source of probiotics and contribute to the diversity on the probiotic markets.

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Corresponding author:

Magdalena Olszewska, University of Warmia and Mazury in Olsztyn, Faculty of Food Science, Chair of Industrial and Food Microbiology, Pl. Cieszyński 1, 10-726 Olsztyn, Poland
tel. + 48 89 523 37 29, e-mail: magdalena.olszewska@uwm.edu.pl