

## Bifidogenic properties of cell-free extracts derived from probiotic strains of *Bifidobacterium bifidum* and *Lactobacillus reuteri*

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Comprehensive study of the biological activity of structural components and metabolites of “beneficial” microorganisms opens the prospects of efficient and rational use of their biotechnological potential in the correction of microecological and related disorders. The study tested proliferative activity and biofilm formation by *Bifidobacterium bifidum* probiotic strain under the influence of cell-free extracts containing structural components and metabolites of the probiotic strains of *B. bifidum* and *Lactobacillus reuteri*. Cell-free extracts were obtained by disintegrating suspensions of probiotic cells by cyclic freezing-thawing, cultivating probiotic microorganisms in their own disintegrates and subsequent filtration of the obtained disintegrates and cultures. The proliferative activity and biofilm formation of the probiotic test culture were studied by spectrophotometric microtiter plate method with 10%vol, 30%vol and 50%vol content of cell-free extracts in the cultivation medium. All investigated extracts showed a significant concentration-dependent stimulatory effect on the proliferative activity of *B. bifidum*. According to the degree of stimulatory effect on the *B. bifidum* proliferation, cell-free extracts arranged in ascending order: MLG (filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate supplemented with 0.8 M glycerol and 0.4 M glucose) < MB (filtrate of *B. bifidum* culture, grown in *B. bifidum* disintegrate) < B (filtrate of *B. bifidum* disintegrate) < ML (filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate) < L (filtrate of *L. reuteri* disintegrate). With the same content in the culture medium, filtrates of disintegrates had a more pronounced stimulatory effect than filtrates of cultures grown in their own disintegrates. Cell-free extracts from *L. reuteri* (L and ML) exerted a more pronounced stimulatory effect than cell-free extracts from *B. bifidum*. Not all studied cell-free extracts stimulated the biofilm formation by *B. bifidum*. The effect of cell-free extracts on this process depended on their type and concentration. Extract L had a predominantly inhibitory effect on biofilm formation by *B. bifidum*. The most pronounced stimulatory effect on biofilm formation by *B. bifidum* came from extract MLG. ML, B and MB extracts stimulated this process approximately equally. The detection of significant bifidogenic effect of the studied cell-free extracts may contribute to their pharmaceutical applications. Cell-free extracts can be used as metabolites or prebiotics for increasing the survival of the injected probiotic, facilitating its inoculation in the gastrointestinal tract when used together. The obtained data encourage further careful study of the biochemical composition of cell-free extracts and efforts to clarify the mechanism of their action.

**Keywords:** probiotic bacteria; biofilm formation; biotechnological potential; proliferative activity; cell-free extract.

### Introduction

The gut microbiota is involved in a wide variety of physiological processes: degradation of non-digestible food compounds, absorption of nutrients, metabolism of bile acids and xenobiotics, production of essential vitamins, bioactive metabolites and signaling molecules. It has a strong influence on the intestinal epithelium and immune system development, host behaviour and metabolism. Recent studies have revealed a correlation between the composition of the microbiota and diseases such as obesity, metabolic syndrome, diabetes, cardiovascular and cerebrovascular diseases: hypertension and atherosclerosis, liver disease, malnutrition, inflammatory bowel disease, rheumatoid arthritis, cancer, autism, and Parkinson's disease (Landman & Quevrain, 2016; Wang et al., 2017; Feng et al., 2018).

The gut microbiota of a healthy person is characterized by the dominance of obligate anaerobic members of the phyla Firmicutes, Bacteroidetes and Actinobacteria (Kho & Lal, 2018). An increase in the number of facultative anaerobic Enterobacteriaceae (phylum Proteobacteria) is a marker of gut dysbiosis (Litvak et al., 2017). Members of the genus *Bifidobacterium* play a central role in maintaining and restoring gut homeostasis (O'Callaghan & van Sinderen, 2016; Rivière et al., 2016; Sar-

kar & Mandal, 2016). *Bifidobacterium bifidum*, *B. longum* and *B. breve* are the predominant microbes that colonize the intestine in the first days after birth. They constitute 40–80% of the gut microbiota of newborns. In infants from 6 months of age, the total diversity of bacteria increases, but the proportion of bifidobacteria decreases to 30–40%. In childhood and adolescence, the number of bifidobacteria continues gradually to decline. In adults, their content varies from 0 to 25%. The elderly phase of life is characterized by a further decrease in the number of bifidobacteria. High content of bifidobacteria in the gut microbiota is associated with a ‘healthy’ state and longevity. The level of bifidobacteria is a biomarker of gut flora health and can be influenced by nutrition, use of antibiotics and other drugs (Valdes et al., 2018). The loss of bifidobacteria is harmful for the health of the host: it adversely affects the remaining microorganisms, increases the risk of invasion and colonization of the gut epithelium by pathogenic and opportunistic microorganisms (Sarkar & Mandal, 2016; Rivière et al., 2016; O'Neill et al., 2017).

*B. bifidum* along with other bifidobacteria provides mucosal colonization resistance. Antagonistic activity against many opportunistic and obligatory pathogenic microorganisms is realized through the synthesis of antimicrobial substances: bacteriocins, exopolysaccharides, organic acids. *B. bifidum* can secrete molecules that inhibit the expression of virulence

genes responsible for colonization and systemic dissemination of intestinal pathogens. These beneficial microorganisms not only enhance the gut barrier, but also reduce the risk of occurrence of upper respiratory infections. *B. bifidum* strains play a key role in the evolution, maturation of the immune system and modulation of the host immune response. *Bifidobacterium*-mediated immune modulation is considered as one of the new methods of treatment (Ku et al., 2016; Quigley, 2017; O'Neill et al., 2017).

Recently there has been a growing interest in compounds that positively influence the composition and activity of gut microbiota. The most common way to obtain a bifidogenic effect is to consume probiotics and prebiotics (Rivière et al., 2016). Bifidobacterial growth-promoting factors of several groups are known: 1) breast and cow milk components ( $\kappa$ -casein, caseinomacropeptide, enzymatically digested  $\kappa$ -casein), 2) oligosaccharides (for example, raffinose, stachyose, lactulose), 3) polysaccharides (for example, inulin), 4) alcohol saccharides (for example, lactitol), 5) quinones (for example, 2-amino-3-carboxy-1,4-naphthoquinone) (Cicvarek et al., 2011). High expectations are pinned on microbial products as a means to renovate balance of intestinal microbial populations (Hamet et al., 2016; Richards et al., 2016; Gagliardi et al., 2018). Use of the biological activity of the probiotics' derivatives is considered as an alternative approach to prevention of infections by enriching colonization with protective commensal species and enhancing the protective properties of the immune system. This approach can lead to interesting therapeutic results (Landman & Quevrain, 2016; Turroni et al., 2017; Keith & Pamer, 2018). Therapy with preparations based on probiotics' structural components and metabolites can replace insufficiently effective intact cell probiotics therapy in the future (Shenderov, 2013; Sarkar & Mandal, 2016; Singh et al., 2018). In addition, the determination and use of substances that have a stimulatory effect on probiotic cultures has a commercial advantage, since it will increase the overall productivity of cellular biomass. This circumstance is of particular importance in conditions of lower productivity and higher cost of *Bifidobacterium* spp. production as compared with other aerobic or facultative anaerobic microorganisms (Ku et al., 2016).

Considering that the beneficial effect of probiotic bacteria is due to the biological activity of their structural components and metabolites, our efforts were aimed at obtaining them. We have developed a method for the obtaining of cell-free extracts, containing biologically active probiotics' derivatives (Knysh et al., 2018). The applied method of slow disintegration provides access to the extracellular space of the structural components and metabolites of the probiotic cells, which are subjected to stress and damaging factors during the freeze-thaw stages. The resulting disintegrate, in addition to viable cells that survived repeated freeze-thaw cycles, contains substances suitable for bacterial nutrition. This allows the use of the disintegrate as a nutrient medium for the cultivation to receive metabolites of actively proliferating probiotic bacteria.

The aim of the research was to investigate the influence of cell-free extracts, contained derivatives of *L. reuteri* and *B. bifidum* on proliferative activity and biofilm formation by *B. bifidum in vitro*.

## Materials and methods

The effect of five cell-free extracts containing probiotic derivatives on the proliferation and biofilm formation by *B. bifidum* was investigated. These were:

- L – filtrate of *L. reuteri* disintegrate;
- ML – filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate;
- MLG – filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate supplemented with 0.8 M (73.7 g/l) glycerol and 0.4 M (72.1 g/l) glucose;
- B – filtrate of *B. bifidum* disintegrate;
- MB – filtrate of *B. bifidum* culture, grown in *B. bifidum* disintegrate.

Disintegrates and cultures of probiotics were obtained using the method developed by the authors (Knysh et al., 2018). Cell-free extracts contained structural components and metabolites of the commercial strains *B. bifidum* 1 (from medical product “Bifidumbacterin-Biopharma”, JSC Biopharma, Ukraine) and *L. reuteri* DSM 17938 (from medical product “BioGaia”, BioGaia Production AB, Sweden). The commercial strain

*B. bifidum* 1 was used as a proliferating and biofilm-forming test culture. The lyophilized microbial mass was rehydrated and recovered by cultivation in tryptone soya broth (TSB; HiMedia, India) anaerobically for 24 hours at 37 °C. After checking the purity of the culture, the cells were harvested by centrifugation for 10 min at 3000 g and the pellet was washed twice with sterile isotonic saline solution to remove nutrient components. Finally, the pellet was resuspended in physiological saline solution (0.9% NaCl) and an inoculum was prepared from it. The inoculum was a microbial suspension in a physiological saline solution with an optical density of 10 units on the McFarland scale ( $\sim 10^8$  CFU/ml). The turbidity of the suspension was measured using the Densi-La-Meter II device (PLIVA-Lachema Diagnostika, Czech Republic).

The study of the effect of cell-free extracts on the *B. bifidum* proliferation was performed in sterile 96-well polystyrene microtiter plates (JSC “Eximcargotrade”, Ukraine). TSB supplemented with 1% glucose, cell-free extracts and inoculums were added into the test wells in the ratio of 8 : 1 : 1; 6 : 3 : 1 and 4 : 5 : 1. Thus, the final concentration of each extract in the cultivation medium was 10%vol, 30%vol or 50%vol. TSB supplemented with 1% glucose, physiological saline (PhS) and inoculums were added into the positive control wells (PC) in the same ratio: PC<sub>10</sub> (10%vol of the PhS in the cultivation medium); PC<sub>30</sub> (30%vol of the PhS in the cultivation medium); PC<sub>50</sub> (50%vol of the PhS in the cultivation medium). The final concentration of microbial cells in the test and positive control wells was  $\sim 10^7$  CFU/ml. The negative control wells (NC) contained only TSB. The plates were covered with lids and incubated anaerobically for 24 hours at 35–37 °C in static conditions. Optical density (OD) of the wells was measured at 578 nm using a microtiter-plate reader “LisaScanEM” (“ErbaLachemas.r.o.”, Czech Republic). The inhibition (or stimulation) indices were calculated by the formula: II (SI) =  $(\Delta OD - \Delta OD_{PC}) \div \Delta OD_{PC} \times 100\%$ , where  $\Delta OD$  and  $\Delta OD_{PC}$  are changes in the optical density of the test and control samples within 24 hours, respectively.

The study of the effect of cell-free extracts on the biofilm formation by *B. bifidum*. After measuring the optical density of the wells, the plates continued to be incubated anaerobically in static conditions for 24 hours at 35–37 °C. Then the contents of wells were decanted. Each well was washed three times with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.2). Attached biofilms were fixed by exposing them to hot air at 60 °C for 60 min; they were stained by 1% crystal violet for 15 min. After that, the wells were washed ten times with distilled water. Ethanol as an eluent was gently added for resolubilization of the dye and after that microtiter plate covered with the lid. It was left at room temperature for 30 min. Optical density (OD) of the eluate in the test and control was measured at 630 nm by using a microtiter-plate reader “Erba Lisa Scan™ EM” (Germany). Based on the OD values obtained for NC and test samples, biofilm formation was defined as:

- weak ( $OD_{NC} < OD \leq 2 \times OD_{NC}$ );
- moderate ( $2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$ );
- strong ( $4 \times OD_{NC} < OD$ );
- no biofilm formation ( $OD \leq OD_{NC}$ ), according to the description

Lee et al. (2017).

The inhibition (or stimulation) indices were calculated by the formula: II (SI) =  $(OD - OD_{PC}) \div OD_{PC} \times 100\%$ , where OD and  $OD_{PC}$  are optical density of the test and control samples, respectively.

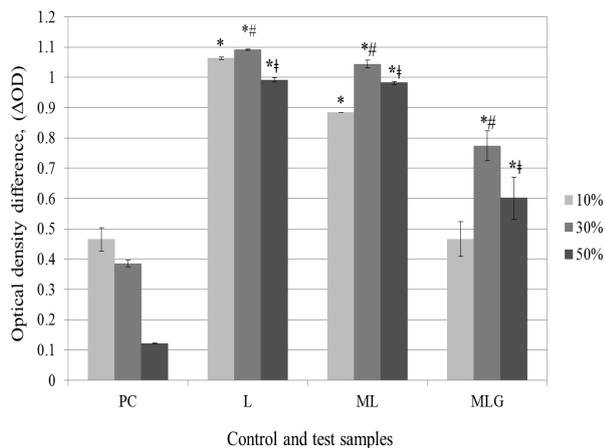
All experiments were performed three times. Each sample was tested in triplicate. The obtained data were statistically processed with Excel 2010 software (Microsoft, USA). Average values of obtained indices (x) with standard deviations (SD) were determined.

## Results

First of all, it should be noted that an increase in the volume of the PhS replacing the nutrient medium in the control sample from 10% to 30% and from 10% to 50%, is accompanied by a decrease in the optical density gain of the culture by an average of 17% and 73.6%, respectively (Fig. 1). This is a dilution effect due to a decrease in the amount of nutrients in the cultivation medium. Addition to the cultivation medium of L and ML cell-free extracts at a concentration of 10% leads to a significant increase in the optical density gain of the culture compared to PC<sub>10</sub> (SI

are 128.3% and 90.0%, respectively). MLG extract at a concentration of 10% does not have a significant effect on the change in the optical density of the culture. The increase of L, ML and MLG extracts' content in the cultivation medium for up to 30% is accompanied by a significant increase in the optical density gain of the culture both in comparison with PC<sub>30</sub> (SI are 183%, 170.7% and 100.8%, respectively) and in comparison with PC<sub>10</sub> (SI are 134.3%, 124.2% and 66.2%, respectively). Addition to the cultivation medium of L, ML and MLG extracts at a concentration of 50% results in a significant increase of the optical density gain of the culture both in comparison with PC<sub>30</sub> (SI are 714.3%, 706.8% and 394.3%, respectively), and in comparison with PC<sub>10</sub> (SI are 112.8%, 110.9% and 29.2%, respectively).

Thus, L, LM and MLG extracts have a significant stimulatory effect on the proliferative activity of *B. bifidum*. It is noteworthy that at the same concentration in the cultivation medium L and LM extracts have more pronounced stimulatory effect on the optical density gain of the *B. bifidum* culture than MLG extract.



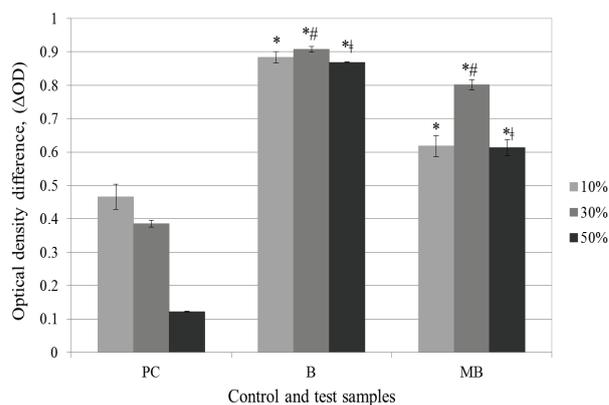
**Fig. 1.** The effect of cell-free extracts derived from *L. reuteri* on the proliferation of *B. bifidum* ( $x \pm SD$  at 578 nm,  $n = 3$ ): PC – positive control (PC<sub>10</sub>, PC<sub>30</sub>, PC<sub>50</sub>); L – filtrate of *L. reuteri* disintegrate; ML – filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate; MLG – filtrate of *L. reuteri* culture, grown in *L. reuteri* disintegrate supplemented with glycerol and glucose; \* – the differences are significant compared to the PC<sub>10</sub>; # – the differences are significant compared to the PC<sub>30</sub>; † – the differences are significant compared to the PC<sub>50</sub>,  $P < 0.05$

As shown in Figure 2, B and MB cell-free extracts in the composition of the cultivation medium at a concentration of 10% cause a significant increase in the optical density gain of the culture compared to PC<sub>10</sub> (SI are 89.8% and 32.6%, respectively). An increase of B and MB extracts' concentration in the cultivation medium of up to 30% leads to even more significant increase in the optical density gain of the culture compared to PC<sub>30</sub> (SI are 135.4% and 107.7%, respectively) and compared to PC<sub>10</sub> (SI are 95% and 72%, respectively). If cell-free B and MB extracts make up 50% of the cultivation medium, then a significant increase in the optical density gain of the culture is observed both in comparison with PC<sub>50</sub> (SI are 613.8% and 403.5%, respectively) and in comparison with PC<sub>10</sub> (SI are 86.6% and 31.6%, respectively).

The obtained data allow us to conclude that B and MB extracts have a significant stimulatory effect on the *B. bifidum* proliferation. It should be noted that with the same concentration in the culture medium B extract has more pronounced stimulatory effect on the proliferation of the *B. bifidum* culture than MB extract.

Optical density of the eluate enables us to indirectly judge the biofilm formation by bacteria. An increase in the volume of the PhS replacing the nutrient medium in the control sample from 10% to 30% and from 10% to 50% is accompanied by a decrease in the optical density of the eluate by an average of 27% and 62.9%, respectively (Fig. 3). The biofilm formation by culture *B. bifidum* at 10% and 30% content of the PhS in the cultivation medium is defined as moderate. With an increase in the PhS content in the cultivation medium up to 50% the biofilm formation by *B. bifidum* is defined as weak. The addition to the cultiva-

tion medium of extract L at a concentration of 10% does not significantly affect the biofilm formation by bifidobacteria. The inclusion of this cell-free extract in the composition of the cultivation medium at a concentration of 30% is accompanied by a decrease in the optical density of the eluate compared with PC<sub>30</sub> (II is 47.3%, the biofilm formation by *B. bifidum* is defined as weak). Increasing the concentration of extract L to 50% led to a decrease in the optical density of the eluate compared to PC<sub>50</sub> (II is 21.4%). Optical density of such eluate did not significantly differ from the optical density of the NC (data not shown). ML cell-free extract, when added to cultivation medium at the 10% concentration, caused a significant increase in the optical density of the eluate compared to PC<sub>10</sub> (SI is 28%, the biofilm formation by *B. bifidum* is defined as strong). 30% content of the ML extract leads to increase in the optical density of the eluate compared with the PC<sub>30</sub> (SI is 49.3%). However, there is a tendency to decrease in optical density compared with eluate of samples containing this extract at a concentration of 10%. The biofilm formation by *B. bifidum* culture at 30% content of the ML extract in the cultivation medium is defined as moderate. Optical density of the sample eluate, which contains 50% of the ML extract, is not significantly different from PC<sub>50</sub> (biofilm formation by the *B. bifidum* culture is also defined as weak). Adding MLG extract to the culture medium at concentrations of 10%, 30% and 50% results in the most significant increase in the optical density of the eluates compared with the corresponding control values (PC<sub>10</sub>, PC<sub>30</sub>, PC<sub>50</sub>). SI are 39.6%, 142.6% and 73.7%, respectively. When the content in the cultivation medium of the MLG extract is 10% and 30%, the culture goes into the discharge with strong biofilm formation.



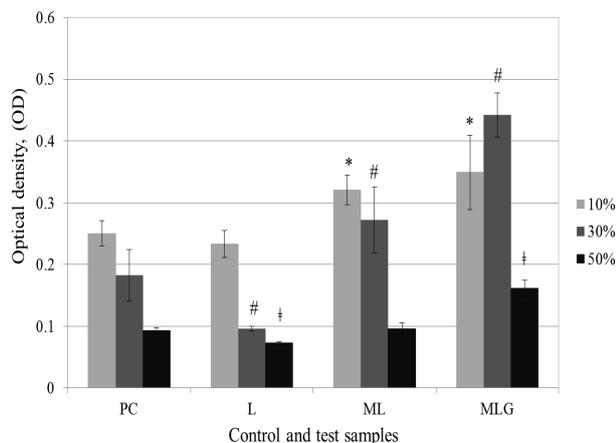
**Fig. 2.** The effect of cell-free extracts derived from *B. bifidum* on the proliferation of *B. bifidum* ( $x \pm SD$  at 578 nm,  $n = 3$ ): PC – positive control (PC<sub>10</sub>, PC<sub>30</sub>, PC<sub>50</sub>); B – filtrate of *B. bifidum* disintegrate; MB – filtrate of *B. bifidum* culture, grown in *B. bifidum* disintegrate; \* – the differences are significant compared to the PC<sub>10</sub>; # – the differences are significant compared to the PC<sub>30</sub>; † – the differences are significant compared to the PC<sub>50</sub>,  $P < 0.05$

From the obtained results it follows that the L extract has an inhibitory effect, while LM and MLG extracts have a stimulatory effect on the biofilm formation by *B. bifidum*. MLG extract has a more pronounced stimulatory effect on the biofilm formation by *B. bifidum* than ML extract.

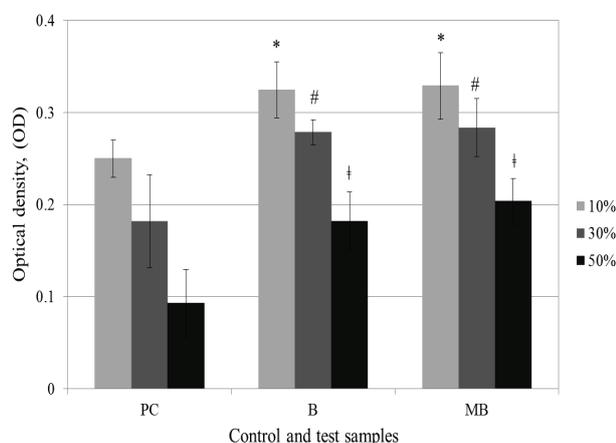
The data presented in Figure 4 suggest that the addition of B and MB cell-free extracts to the cultivation medium at a concentration of 10% leads to a significant increase in the optical density of the eluates compared to PC<sub>10</sub> (SI are 29.8% and 31.5%). An increase in the content of B and MB extracts in the cultivation medium up to 30% is accompanied by the increase in the optical density of the eluates compared to PC<sub>30</sub> (SI are 53.0% and 55.8%) and at the same time a significant (B) or insignificant (MB) decrease in the optical density of the eluates compared to samples containing the studied extracts at a concentration 10%. Optical density of the eluates of samples containing B and MB extracts at a concentration of 50% is significantly higher compared to PC<sub>50</sub> (SI are 96.3% and 119.7%), but much lower compared to samples containing the studied extracts at a concentration 10% and 30%.

The biofilm formation by *B. bifidum* culture at 10% and 30% content of B and MB extracts in the cultivation medium is defined as strong.

At 50% content of B and MB extracts in the cultivation medium the biofilm formation by *B. bifidum* culture is defined as moderate. Thus, B and MB extracts stimulate biofilm formation by *B. bifidum*. It should be noted that there was no significant difference in the intensity of the stimulatory effect of B and MB extracts on the biofilm formation.



**Fig. 3.** The effect of cell-free extracts derived from *L. reuteri* on the biofilm formation by *B. bifidum* ( $x \pm SD$  at 630 nm,  $n = 3$ ): see Fig. 1



**Fig. 4.** The effect of cell-free extracts derived from *B. bifidum* on the biofilm formation by *B. bifidum* ( $x \pm SD$  at 630 nm,  $n = 3$ ): see Fig. 2

## Discussion

Summarizing the obtained data, it should be noted that all the studied cell-free extracts stimulate the proliferative activity of *B. bifidum*. According to the degree of stimulatory effect on the *B. bifidum* proliferation, cell-free extracts arranged in ascending order:  $MLG < MB < B < ML < L$ . The stimulation abilities of cell-free extracts are manifested in a concentration dependent way. With the same content in the culture medium, filtrates of disintegrates have a more pronounced stimulatory effect than filtrates of the cultures grown in their own disintegrates. Cell-free extracts from *L. reuteri* (L and ML) have a more pronounced stimulatory effect than cell-free extracts from *B. bifidum*. The least pronounced stimulatory effect on *B. bifidum* proliferation is observed under the influence of MLG extract. It is known that *L. reuteri* can convert glycerol into the antimicrobial compound reuterin (Spinler et al., 2017). Perhaps the least stimulating effect of the MLG extract is related to the sensitivity of the test culture to reuterin.

Similar results indicating the bifidogenic effect of probiotic derivatives were obtained by other authors. They show that some strains of propionic acid bacteria have ability to produce metabolites, which stimulate the growth of *Bifidobacterium* strains (Warminska-Radyko et al., 2002). "Bifidogenic effects are widely known, yet the underlying mechanism is poorly understood" (Sarkar & Mandal, 2016). Currently, we can only assume which components of the complex extracts play the role of a bifidogenic factor.

Possibly, the explanation of the mechanism of action of the obtained extracts should be sought in the method of obtaining the extracts, which determines their composition. To obtain disintegrates, a slow disintegration method was used, in which bacterial cells were subjected to powerful stress and damaging effects, receiving sublethal and lethal damage. During the freeze-thaw stages microorganisms are exposed to thermal, osmotic, thermomechanical, dehydration and rehydration shock. Some cells die. Structural components and metabolites leave the destroyed cells and enter the environment. In the remaining living cells, metabolism is altered. It is known that in response to cold shock, a cascade of cellular reactions, accompanied by a high level of temporary expression of cold-induced proteins (CIPs) occurs. CIPs perform pleiotropic functions, such as the regulation of transcription, translation, and splicing. They have the ability to orchestrate multiple cellular processes, including proliferation and differentiation (Lindquist & Mertens, 2018). Thus, disintegrate obtained by repeated freezing-thawing of a bacterial suspension contains the structural components of bacterial cells (MAMPs, microbe-associated molecular patterns), bacterial cold shock proteins (DAMPs, damage-associated molecular patterns), which possess powerful bioregulatory potential. Some substances can be catabolized by bifidobacteria and serve as signaling molecules involved in the regulation of gene expression and in the quorum sensing mechanism implementation.

The results of studies by many authors demonstrate that some exopolysaccharides (EPSs) produced by *Lactobacillus* or *Bifidobacterium* are able to exert a bifidogenic effect *in vitro* (Patten & Laws, 2015; Ryan et al., 2015). Subsequent *in vivo* studies confirmed the effectiveness of EPSs as a growth factor for bifidobacteria. For example, kefiran, a branched hydrosoluble glucogalactan, heteropolysaccharide produced by *L. kefiranoferiens*, is able to change intestinal and faecal microbiota of BALB/c mice, by increasing the number of bifidobacteria populations (Hamet et al., 2016). One of the possible mechanisms of the beneficial action of kefiran is its ability to induce the expression of gene related to carbohydrates transport and metabolism of *B. bifidum* PRL2010 (Serafini et al., 2014). It has been shown that lyophilized EPSs produced by lactobacilli have the ability to cause a bifidogenic effect comparable to the action of prebiotic inulin (Sarıkaya et al., 2017).

The biosynthesis of these bioactive molecules is different in different genera (Patel et al., 2010). Probiotic EPSs have different chemical structure, degree of branching, sugar composition, molecular weight and glycosidic linkages of each polymer of EPSs (Hamet et al., 2016). The presence and severity of bifidogenic effect depends on physical and chemical properties of EPSs (Polak-Berecka et al., 2013; Hamet et al., 2016). EPSs with a different type of monosaccharide composition stimulate the growth of bifidobacteria at different rates (Sarıkaya et al., 2017). The yield, composition and structure of EPSs produced by the same species of microorganism depend on the growth conditions and the composition of the cultivation medium, specifically on the type of the carbohydrate source in the growth medium (Patel et al., 2010; Polak-Berecka et al., 2013). Various probiotic strains synthesize EPSs mixtures of various structures under the same cultivation conditions. The monosaccharide units of homopolysaccharides produced by some strains of *L. reuteri* were determined: levan, reuteran, dextran, mutan. EPSs-producing bacteria are generally not able to destroy their own EPSs. Unrelated microbes are capable to catabolize polysaccharides of other bacteria (Patel et al., 2010). Several studies have demonstrated the resistance of EPSs to biodegradation under conditions of simulating gastric and intestinal digestion (Patten & Laws, 2015). Therefore, EPSs with a bifidogenic effect can be considered as an alternative to traditional probiotic preparations, which show a low survival level in the gastrointestinal tract. Besides EPSs, the bifidogenic effect is attributed to such structural components and probiotic metabolites as: lipoteichoic acids, short chain fatty acids and bifidocins (Sarkar & Mandal, 2016; Valdes et al., 2018). Which biologically active components of the studied cell-free extracts have a stimulating effect on the growth of *B. bifidum* remains to be studied.

Not all studied cell-free extracts stimulate the biofilm formation by *B. bifidum*. The effect of cell-free extracts on this process depends on their type and concentration. Extract L has a predominantly inhibitory effect on biofilm formation by *B. bifidum*. The most pronounced stimu-

latory effect on biofilm formation by *B. bifidum* is from an extract MLG. ML, B and MB extracts stimulate this process approximately equally. Currently, it is not possible to accurately determine the mechanism of this stimulating influence. However, we can make theoretical assumptions about the possible mechanisms of the effect of cell-free extracts on this complex biological process. It is thought that EPSs play a key role in bacterial biofilms: they are a major constituent in these matrices, stabilize the 3-D structure of biofilms and minimize intercellular repulsions between bacteria by shielding the electrostatic charges on their surfaces. It is known that excessive EPSs production by one strain of bacteria can significantly enhance the stability of biofilms of other, non-EPS-producing bacteria (Patten & Laws, 2015). But from these positions it is difficult to explain the mechanism of the inhibitory effect of L extract on biofilm formation by *B. bifidum*, especially taking into account its most pronounced stimulatory effect on the proliferation of a probiotic test culture. The obtained results indicate the existence of a mechanism for inhibiting the biofilm formation that is not associated with inhibition of proliferation.

According to recent studies, bacterial exposure to subinhibitory antimicrobials of many chemically different classes increases biofilm formation. This phenomenon can be explained by relying on the fundamental biomedical paradigm of hormesis, according to which small doses stimulate, and large inhibit biological parameters. This hormetic response can be considered as a quick and nonspecific way to protect the population from chemical threats (Ranieri et al., 2018). We assume that the most pronounced stimulatory effect of MLG extract on biofilm formation by *B. bifidum* is related precisely to the content in the extract of reuterin, which has antimicrobial activity.

Thus, the mechanisms of the stimulatory effect on proliferation and of the different effects on the *B. bifidum* biofilm formation of cell-free extracts containing probiotic derivatives are not entirely clear and need to be thoroughly investigated.

## Conclusion

The present study shows that cell-free extracts have the ability to stimulate the growth and differently affect biofilm formation by *B. bifidum*. The detection of significant bifidogenic effect of the studied cell-free extracts may contribute to their pharmaceutical applications. Cell-free extracts can be used as metabiotics or prebiotics for increasing the survival of the injected probiotic, facilitating its inoculation in the gastrointestinal tract when used together. This study also encourages further careful study of the biochemical composition of cell-free extracts and efforts to clarify the mechanism of their action.

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