Acta Alimentaria, Vol. 46 (4), pp. 439–448 (2017)
DOI: 10.1556/066.2017.46.4.6

PROBIOTIC BIOFILM ON CARRIER SURFACE: A NOVEL PROMISING APPLICATION FOR FOOD INDUSTRY

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(Received: 25 November 2016; accepted: 13 March 2017)

In this essay work, the ability of probiotic biofilm formation on carrier surface was demonstrated. Probiotic biofilms exhibit the same properties as pathogen microbial biofilms but with higher resistance to low pH values and bile salts. The ability of different probiotic strains (*Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum*) to interact with pre-selected carriers divided into 3 categories (polymers, complex food matrices, and inorganic compounds) was tested. *Lactobacillus acidophilus* and *Bifidobacterium longum* combined with inorganic silica carrier exhibited the interaction leading to biofilm formation only. Prepared biofilm (*Lactobacillus acidophilus*) was then subjected to comparative study with planktonic bacterial culture. The ability to survive in the presence of low pH value (pH 1–3) and bile salts (0.3% solution) was evaluated. Low pH value (pH 1) had a harsh effect on free cell culture causing decreased cell viability (71.9±3.2% of viable cells). Biofilm culture exhibited higher resistance to low pH value, the viability exceeded 90%. The exposure of free cell probiotic culture to porcine bile resulted in an almost constant decrease in viability during the study period (68.2±1.1% of viable cells, after 240 min incubation). Viability of biofilm after the exposition to bile was almost constant with a slight decrease of no more than 5% during the study.

Keywords: probiotics, biofilm, carrier, acid tolerance, bile tolerance

The first mention of probiotics appeared at the beginning of the 20th century in the writings of Elie Metchnikoff. He suggested that the longevity and healthy life of Bulgarian people is hidden in their consumption of fermented milk products (Tripathi & Giri, 2014). In 2001, probiotics as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001) was defined.

During the last 15–20 years, it was recognized that the developmental process of biofilm formation is the natural way of life for microorganisms, without differences between harmful and commensal microorganisms. Therefore, on one hand, biofilm formation is an important clinical pathogenic mechanism, a menace to the aging population, immunocompromised and poly-traumatic patients, on the other hand, a modern medical instrumental intervention (Römling et al., 2014). In the common line, biofilm cells have a higher resistance to antimicrobial agents and agents harmful to microbes in biofilm than planktonic bacteria. The formation of a barrier or biofilm prohibits the direct contact with harmful agents (Srey et al., 2013).

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Probiotics, including *Lactobacillus* and *Bifidobacterium* species, have been found also to naturally exist in the gut in the complex biological conglomerate called biofilm, which tightly adheres to the gut lining (Caggianiello et al., 2016). Biofilm is a sessile high-density community of bacterial cells, which is shielded by a self-secreted protective layer formed by extracellular polymeric substance (EPS) (Cheow & Hadinoto, 2013). Although the exact mechanism of acting of EPS is still unknown, cells involved in biofilm exhibit a specific rate of resistance presumably (Caggianiello et al., 2016) due to (i) the specific dormant metabolic state of biofilm cells and their specific communication called quorum sensing and/or (ii) the protective function of the EPS (Ventolini, 2015). Moreover, the protection of bacterial cells against harsh biotic and abiotic conditions including temperature, pH, and osmotic stress during passage through intestinal tract, EPS can also be involved in adhesion to surfaces and biofilm formation and in cell adhesion/recognition mechanisms (Donot et al., 2012; Salazar et al., 2016).

The protective function of biofilm on cells starts considerations on the application of this knowledge in pharmaceutical and food industry, where probiotic cells are stressed by handling, storage, and digestion and, therefore, they lose their potential healthy activity.

The aims of the present study were to evaluate the abilities of probiotic strains to form biofilm on a wide range of carriers, and to compare biofilm formation on the selected surfaces. The viability of biofilm at low pH and in the presence of bile was evaluated under model conditions. Thus, harsh conditions of the gastrointestinal tract were induced to assess differences in the survival of planktonic and biofilm forms of probiotic bacteria.

1. Materials and methods

1.1. Materials

The following materials were used in the present work as carriers: poly-(vinyl-pyrrolidone-*co*-vinyl acetate), nano-cotton, potatoes fibre, sodium alginate, carrageenan, oat fibre, calcium phosphate, zinc oxide, silica, and finely milled complex food matrices (i.e. flour, groats of oats, pea, lentils, poppy, and buckwheat). Complex food matrices were bought from local markets and milled in the laboratory. Silica was provided by Pharmaceutical Biotechnology (CZ). All other ingredients used in the cultivation step were of pharmaceutical quality and could be purchased as standard pharmaceutical ingredients for food supplements production or drug production.

1.2. Microorganisms and cultivation media

The adherence abilities of probiotic strains to selected carriers were evaluated for three different bacteria: *Lactobacillus acidophilus* (CCM 4833), *Bifidobacterium longum* (CCM 4990), and *Bifidobacterium breve* (CCM 3763). All tested bacteria in this study were provided by the Czech Collection of Microorganisms in Brno. Probiotic bacterial cultures were grown in commercial deMan, Rogosa and Sharpe (MRS) broth (*Lactobacillus acidophilu*s) and *Bifidobacterium* broth with and without carriers. Both media were delivered by Himedia. The media were supplemented with 1.5% w/v carries. All strains were cultivated at 37 °C for 16–24 h.

1.3. Determination of biofilm formation

The creation of biofilm was performed by static fermentation. The biofilm formulation was assessed by Gram-staining and observed under optical microscope (Nikon Eclipse E 400). The biofilm formation was confirmed by electron microscope (Mira3 Tescan).

1.4. Determination of viability in low pH environment

A solution simulating the stomach environment with a composition of NaCl (2.05 g l^{-1}), KH_2PO_4 (0.60 g l^{-1}), $CaCl_2$ (0.11 g l^{-1}), and KCl (0.37 g l^{-1}) was prepared according to Corcoran and co-workers (2005). The pH of the solution was adjusted to values 1, 2, and 3 by 1 M HCl. Fresh biofilm culture after cultivation, where the high degree of adhesion was confirmed by electron microscope, and planktonic form culture were incubated in prepared acidic solutions in a shaker at 37 °C for 120 min. At 30, 60, and 120 min, the samples were taken and neutralized by 1 M $NaHCO_3$. The number of CFU in the prepared sample was determined according to a modified Miles-Misra method (Miles, 1979; Hedges, 2002).

Each experiment was performed in three replications. The viability of the biofilm and planktonic form was expressed as a percentage of viable cells at the sampling time to the viable cell at the start of the experiment.

1.5. Determination of probiotic cells viability in bile

The bile tolerance of *Lactobacillus acidophilus* CCM 4833 strain in planktonic and biofilm form was evaluated. Samples of probiotic cells' fresh cultures grown in the presence of silica carrier and without carrier were incubated in the 0.3% solution of bile salts (Sigma) for 4 h at 37 °C (Ruas-Madiedo & De Los Reyes-Gavilan, 2005). In the period of 1 hour the samples were taken and the CFU number was determined according to Miles-Misra methods (Miles, 1979; Hedges, 2002). Each experiment was performed in three replications.

1.6. Statistical analysis

Two-sample Student's unpaired *t*-test for the evaluation has been used. This test can determine whether the two normal distributions with the same variance, coming from two independent samples, have the same mean. The conformity of variances has been verified by *F*-test. If the resulting P-value>0.05, the variances of both data can be treated as equal, *t*-test can be used.

2. Results and discussion

2.1. Probiotic biofilm formation on carrier surface

Materials investigated in this work were chosen according to the following criteria: (i) carrier has to be commonly used in the pharmaceutical industry, (ii) according to the legislation, carrier has to be used in food supplements, and (iii) carrier has to be insoluble in boiling water. The screening of the selected materials studied for biofilm formation for three different probiotic strains is given in Table 1. Surprisingly, the best biofilm formation was observed in the case of *Lactobacillus acidophilus* and *Bifidobacterium longum* on silica. On the other hand, silica was not an appropriate carrier for *Bifidobacterium breve.* Figure 1 shows the rate of adhesion of *Lactobacillus acidophilus* and *Bifidobacterium longum* on the silica carrier.

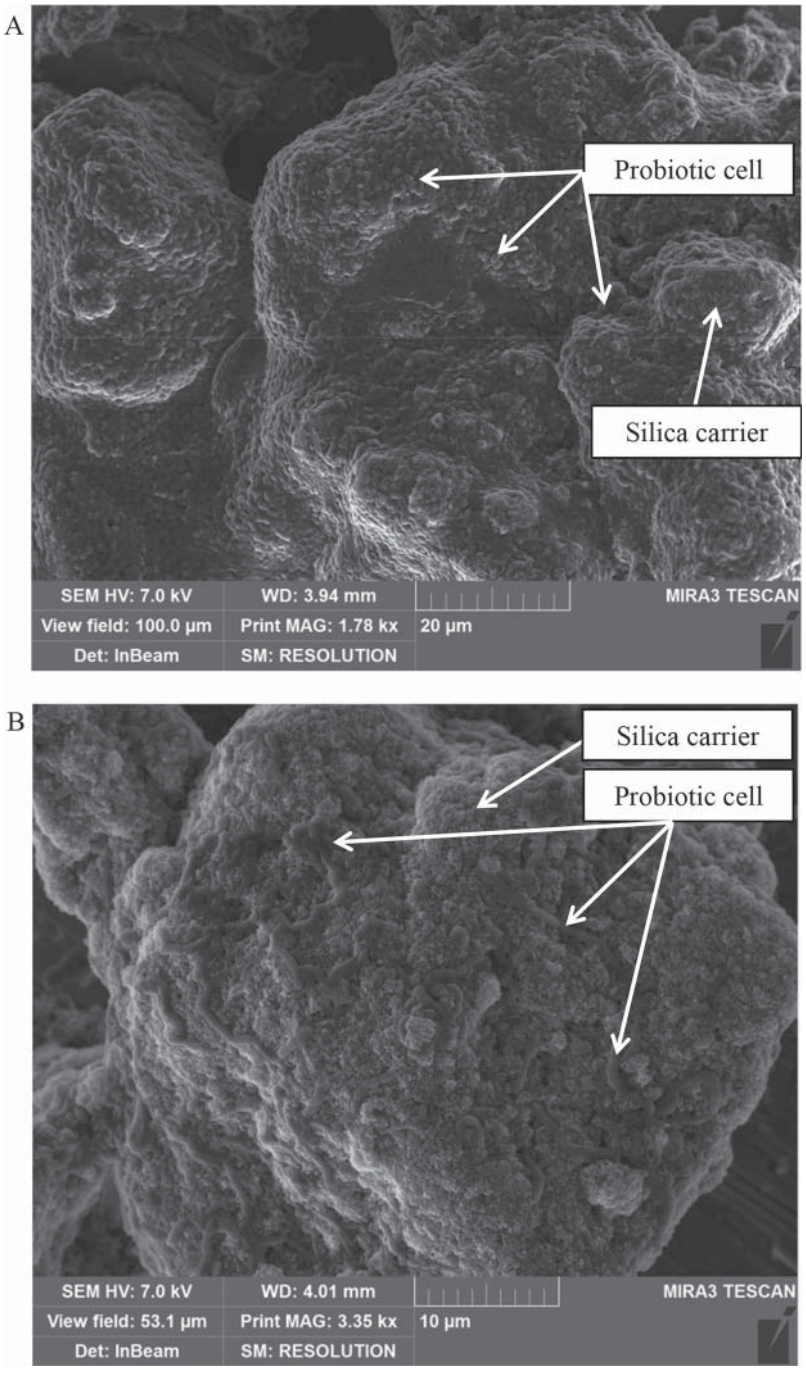

Fig. 1. Probiotic biofilm on silica carrier (A): *Lactobacillus acidophilus*; (B): *Bifidobacterium longum*; under electron microscope Mira3 Tescan

Sodium alginate, potatoes fibre, carrageenan, oat fibre, pea, and buckwheat showed partial adhesion of *Lactobacillus* strain only, which was later on rejected. Thanks to a complex and polymeric structure of studied materials, it was later found that probiotic cells are detained in the complex structure of carriers and biofilm formation was not confirmed. Sodium alginate, potatoes fibre, oat fibre, and buckwheat show the same properties in the presence of *Bifidobacterium longum*; and sodium alginate, oat fibre, pea, and buckwheat in the presence of *Bifidobacterium breve*. Nano-cotton, poly(vinylpyrrolidone-*co*-vinyl acetate), corn starch, calcium phosphate, kaolin, flour, groats of oats, lentils, and poppy were not suitable materials for adhesion of *Lactobacillus* and *Bifidobacterium* strains. In Figure 2 biofilm formation in the presence of silica observed by optical microscopy is introduced. Furthermore, *Bifidobacterium longum* did not exhibit the ability to form biofilm on the surface of carrageenan and finely milled pea, and *Bifidobacterium breve* did not form biofilm in the presence of potatoes fibre and carrageenan.

Table 1. Formation of probiotic biofilm (*Lactobacillus acidophilus, Bifidobacterium longum,* and *Bifidobacterium breve*) on selected carriers

Selected carrier	*Lactobacillus acidophilus* (CCM 4833)	*Bifidobacterium longum* (CCM 4990)	*Bifidobacterium breve* (CCM 3763)
Polymers			
Poly (vinylpyrrolidone-*co*-vinyl acetate)	–	–	–
Sodium alginate	+	+	+
Nanocotton	–	–	–
Potato fibre	+	+	–
Carrageenan	+	–	–
Oat fibre	+	+	+
Corn starch	–	–	–
Mineral compounds			
Calcium phosphate	–	–	–
Kaolin	–	–	–
Silica	++	++	–
Complex food matrices			
Flour (finely milled)	–	–	–
Groat of oats (finely milled)	–	–	–
Pea (finely milled)	+	–	+
Lentils (finely milled)	–	–	–
Poppy (finely milled)	–	–	–
Buckwheat (finely milled)	+	+	+

Data are evaluated as a rate of adhesion to the carrier, where –: no adhesion, + : probiotic cells are detained into the complex structure of carriers; ++ : adhesion of probiotic cells on the carrier.

The data presented in Table 1 show that probiotic strains are able to adhere to some carrier surface and form biofilm. Furthermore, the present results suggest that the adherence is driven by properties of both selected carrier and probiotic strain.

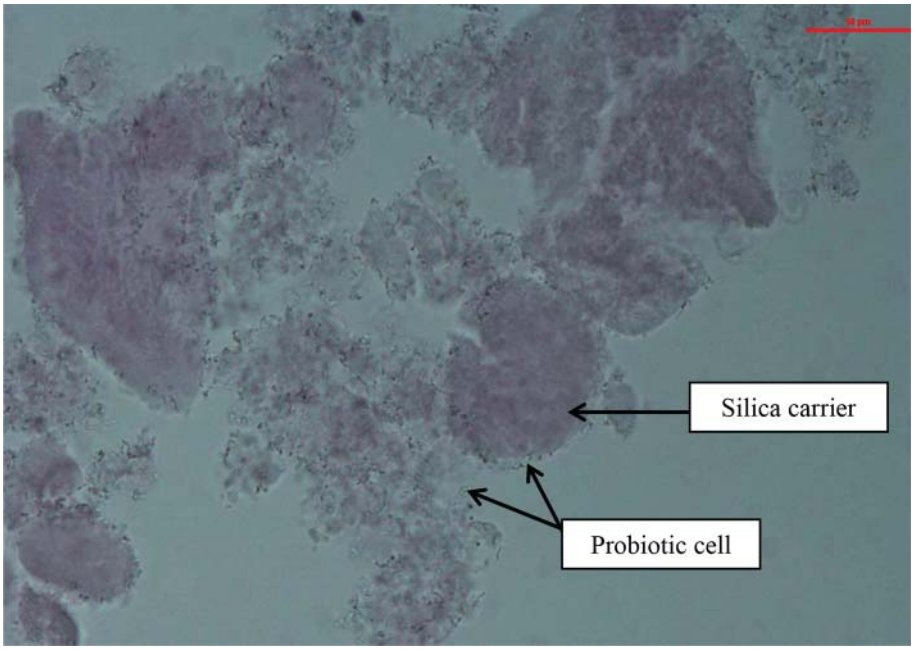

Fig. 2. Lactobacillus acidophilus CCM 4833 in the presence of silica under optical microscope NIKON Eclipse E 400 (figure size: 50 mm)

The properties of surfaces, carrier top layer and probiotic cell, are responsible for adhesion, and thus, this ability is ensured by the sum of specific (mediated by different pili-like structures managed by genetic assembly system (Longo et al., 2014)) and nonspecific (driven by electrostatic and hydrophobic forces, steric hindrance, van der Waals forces, temperature, pH, and hydrodynamic forces (Dunne, 2002)) interactions between these two surfaces. Although biofilm formation on different carriers is well known in the case of pathogenic or commensal microorganisms, probiotic strains have not yet been subjected to study for biofilm formation, especially on free carriers. There are some studies focusing on biofilm formation on polystyrene microplates (Aoudia et al., 2016), but silica and other here presented materials have not been tested yet.

The tests performed have not confirmed adherence of probiotic strain to more than one carrier. Elementary/single carrier particles are proposed as the preferable material suitable for adhesion of probiotic strains. On the other hand, food matrices contain a wide range of components and they may not offer a coherent surface for probiotic adhesion.

The properties of cell surface are connected to the type of microorganism. This study confirmed that the ability of adhesion and subsequent biofilm formation on the carrier is dependent on the type of microorganism. Similarities among studied strains and carrier suitable for all studied probiotic strains were not found. Therefore, we expect that strain dependent properties play unmistakable roles also in biofilm formation. This concept was proposed also by other authors who studied biofilm formation on polystyrene microplate (Aoudia et al., 2016).

2.2. The viability of probiotic cells at low pH

To apply their effect in the intestine and to confer any health benefits to the host, probiotic bacteria should be capable of surviving the passage through the gastrointestinal tract, which presents low pH in the stomach and bile in the small intestine tract (TULUMOGLU et al., 2013). Therefore, high acid level tolerance was evaluated as a critical parameter of a good source of probiotics in the pharmaceutical industry.

In the present study, the viability of probiotic cells in biofilm and in planktonic state subjected to low pH values was studied. The viability of cells was expressed as CFU units and calculated also as a percentage of viable cells in the sample to viable cells in the control culture, which was prepared under standard cultivation conditions of *Lactobacillus acidophilus* CCM4833 (Table 2). Biofilm bacterial cells, as it could be expected, are more resistant than planktonic cells at low pH (pH 1) at all times studied. The drop of viability was observed during the whole experiment, and the highest rate of decrease was observed after 120 min. While planktonic culture showed just 71.9±3.2%, biofilm sample exhibited 90.5±0.1% of viability.

Table 2. Average change in number of surviving bacteria under low pH

pH	Incubation time (min)	CFU value without carrier (expressed as percentage of control %)	CFU value without carrier (cell number)	CFU value with carrier (expressed as percentage of control %)	CFU value with carrier (cell number)
Control	0	100.0±0.1	$7.30•10^8±7•10^5$	100.0±0.1	$5.40•10^8±5•10^5$
	30	$(75.5±1.8)^a$	$5.51•10^8±1•10^7$	$(95.7±0.4)^a$	$5.17•10^8±2•10^6$
1	60	$(71.9±3.2)^b$	$5.25•10^8±1•10^7$	$(93.2±0.2)^b$	$5.03•10^8±1•10^6$
	120	$(71.9±3.2)^c$	$5.25•10^8±1•10^7$	$(90.5±0.1)^c$	$4.89•10^8±5•10^5$
	30	$(85.5±3.2)^d$	$6.24•10^8±2•10^7$	$(95.6±2.6)^d$	$5.16•10^8±1•10^7$
2	60	$(86.5±1.5)^e$	$6.31•10^8±1•10^7$	$(91.8±0.3)^e$	$4.96•10^8±2•10^6$
	120	$(75.5±2.3)^f$	$5.51•10^8±2•10^7$	$(90.7±0.4)^f$	$4.90•10^8±2•10^6$
	30	$(84.7±2.3)^g$	$6.18•10^8±2•10^7$	$(94.8±0.3)^g$	$5.12•10^8±2•10^6$
3	60	$(88.4±1.3)^h$	$6.45•10^8±9•10^6$	$(94.8±0.4)^h$	$5.12•10^8±2•10^6$
	120	$(75.5±1.8)^i$	$5.51•10^8±1•10^7$	$(93.8±0.7)^i$	$5.07•10^8±4•10^6$

Data are evaluated as average value and standard deviation. Mean values are the average of at least three replicates ± standard deviation. Values with the same letters in the each line were evaluated ($P>0.05$). Statistical difference was not confirmed between values marked by letter d and h. Values with letters d and h in the same line are not statistically different ($P>0.05$).

Furthermore, a higher pH value (pH 2) indicates a partially suitable environment for probiotic viability. After 30 min, the free cell culture exhibited some kind of adaptation. At other monitored time points differences between viabilities were found. The greatest difference was measured after 120 min of incubation, where biofilm culture exhibited 90.7±0.4% and free cells sample achieved 75.5±2.3% of viable cells.

At the last tested pH value (pH 3) bacteria also showed a partial adaptation to the environment. Especially, after 60 min incubation, increased resistance to low pH of free cells was observed. A sample without carrier exhibited 88.4±1.3% of viability and biofilm sample achieved 94.8±0.4% of cell viability.

The hypothesis that bacterial cells in biofilm on the carrier are more stable at low pH was confirmed. The difference between viability of biofilm and free cells exceeded 15% at all studied pH values after 120 min cultivation. Furthermore, the viabilities of free cells oscillated around 72% and viabilities of biofilm cultures exceeded 90%.

Although most sources indicate a high decrease in the viability of free cells upon contact with the environment of the stomach compared to encapsulated or immobilized probiotics (Gebara et al., 2013; Fijałkowski et al., 2016), there is still a lack of comprehensive data in the case of biofilm formed on free carrier. Some kinds of probiotic biofilm forms were studied, but these works focused on resistance to stresses of self-forming biofilm encapsulated in natural polymers. Such biofilms exhibited a higher resistance to processing and digestion stresses than encapsulated planktonic cells (Cheow et al., 2014), but the resistance of biofilm on free carriers was not documented yet.

The obtained results are in agreement with the published data of Doleyres and Lacroix (2005). According to these authors, the immobilized population of bifidobacteria with sufficient high cell density could induce a quorum-sensing response, leading to the improvement in physical and technological characteristics of lactic acid bacteria, especially adaptation to changing environmental conditions (Doleyres & Lacroix, 2005). On the other hand, the viability of both free cell culture and biofilm is strictly dependent on the pH value, but linear dependence of decrease in cell viability as the function of pH was not confirmed. Furthermore, the results show that free cell cultures can also exhibit a weak resistance to low pH value.

2.3. The viability of probiotic cells in the presence of bile salts

The comparison of resistance of planktonic form and biofilm form cultures is given in Table 3. The viability of planktonic culture was greatly influenced by bile salts, and at study points (60, 120, 180, and 240 min) viability decreased to 78.9±1.8%, 77.3±0.7%, 74.2±1.4%, and 68.2±1.1%, respectively. While the percentage of viable cells of planktonic culture dropped to 68.2±1.1%, the percentage viability of the biofilm cells remained 98.2±1.0% even after 4-h treatment. Whereas free cell culture exhibited a decreasing linear trend, biofilm culture subjected to 0.3% bile salts solution showed an almost constant value during the whole studied period. *Lactobacillus acidophilus* CCM 4833 biofilm on the appropriate carrier was more resistant to bile than free cell culture at all studied time points.

Table 3. Percentage of surviving bacteria in 0.3% bile salts

Time (hour)	Average of change in CFU without carrier (%)	CFU value without carrier (cell number)	Average of change in CFU with carrier (%)	CFU value without carrier (cell number)
0	100.0±1.6	$6.80 \cdot 10^8 \pm 1 \cdot 10^7$	100.0±0.7	$4.50 \cdot 10^8 \pm 3 \cdot 10^6$
60	78.9±1.8	$5.37 \cdot 10^8 \pm 1 \cdot 10^7$	97.5±1.0	$4.39 \cdot 10^8 \pm 5 \cdot 10^6$
120	77.3±0.7	$5.26 \cdot 10^8 \pm 5 \cdot 10^6$	95.6±1.9	$4.30 \cdot 10^8 \pm 9 \cdot 10^6$
180	74.2±1.4	$5.05 \cdot 10^8 \pm 1 \cdot 10^7$	97.0±1.2	$4.37 \cdot 10^8 \pm 5 \cdot 10^6$
240	68.2±1.1	$4.64 \cdot 10^8 \pm 7 \cdot 10^6$	98.2±1.0	$4.42 \cdot 10^8 \pm 5 \cdot 10^6$

Data are evaluated as average value and standard deviation. Mean values are the average of at least three replicates ± standard deviation. All values in the same line are statistically different ($P>0.05$).

The previously presented results indicate that probiotic cells form biofilm as pathogenic or commensal organisms. Furthermore, probiotic biofilms gain also other features, such as bile tolerance, studied here. Although bile tolerance of probiotic biofilm on free carrier was not previously studied, bile tolerance of probiotic cells incorporated in biofilm on the free carrier can be expected.

Bile tolerance of probiotic cells greatly varies from strain to strain (Ruiz et al., 2013) and may be dependent on the type of source from which the potential organism has been isolated. It has been reported that probiotic strains of human origin have a higher resistance to bile than of different origins (Panicker & Behre, 2014). Furthermore, some environmental factors, such as pH and temperature, may increase bile tolerance or enhance the survival rate of probiotic cells (Li, 2012).

3. Conclusions

In this study the biofilm formation of probiotic microorganisms on the surface of different carriers was tested. The prepared biofilm (*Lactobacillus acidophilus* CCM 4833) was subjected to low pH (pH 1, 2, and 3) and 0.3% solution of porcine bile salts. From the wide range of materials, insoluble in hot water and authorized for food supplements in the European Union, only silica was found as possible carrier for biofilm formation. The biofilm formation on a carrier provides protection to probiotics against low pH for a certain exposure time. Although the planktonic form of studied strain exhibited a weak adaptation at some points, the formation of biofilm showed better survival of probiotic cells. Bile tolerance of biofilm was also confirmed in comparison to free cell culture.

Silica as a carrier of probiotic cultures represents a new direction to increase stability and ensure higher efficiency of probiotics administered in food supplements or in food products.

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The work was supported by the project „Materials Research Centre – Sustainability and Development" Nr. LO1211 of the Ministry of Education, Youth and Sports of the Czech Republic.

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