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Influence of co-encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with inulin and ascorbic acid on its viability

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Abstract: Eight types of capsules containing *Bifidobacterium animalis* subsp. *lactis* Bb12 with addition of inulin and/or ascorbic acid were prepared by emulsion method with milk protein matrix or by extrusion method with alginate matrix. The size of protein and alginate capsules containing only Bb12 was 204 ± 18 μm and 1.7 ± 0.1 mm, respectively. Addition of both inulin (1% w/w) and ascorbic acid (0.5% w/w) increased the size of alginate capsules. Both methods of encapsulation prevented efficiently the manifestation of Bb12 cell metabolic activity. All types of encapsulation provided higher resistance of Bb12 cells to the conditions of a model gastrointestinal tract (GIT) compared to free cells. The influence of co-encapsulation with inulin (1% w/w) and ascorbic acid (0.5% w/w) on viability in model GIT was not demonstrable in alginate capsules but it was significant in protein capsules. The most efficient was co-encapsulation in a protein matrix with 1% w/w inulin and 0.5% w/w ascorbic acid.

Keywords: alginate; emulsion encapsulation; extrusion encapsulation; gastrointestinal tract; milk protein

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" (FAO/WHO 2002). The role of *Bifidobacterium* spp., which represents one of the widely used probiotics, in the human microbiota was summarized by O'callaghan & Van Sinderen (2016). *Bifidobacterium* spp. are Gram-positive, strictly anaerobic, non-sporeforming and non-motile rod-shaped bacteria that belong to the phylum *Actinobacteria*. They mainly occur in the gastrointestinal tract of mammals, birds and insects, and they are also present in sewage or human breast milk. Bifidobacteria are saccharolytic microorganisms, the fermentation via the typical fructose-6-phosphate shunt results in the production of acetic and lactic acid (Lee & O´Sullivan 2010). *Bifidobacterium animalis* subsp. *lactis* has been isolated from different sources (Bunesova et al. 2017) and is commonly found in the gut of healthy humans (Turroni et al. 2017). The specific strain Bb12, selected by Chr. Hansen, is the world's most documented probiotic with widespread commercial use (Jungersen et al. 2014).

When probiotic bifidobacteria are applied into food, it is necessary to ensure their stability during processing and storage of the final product, and protection when passing through GIT. Encapsulation of cells may be an effective protection option. It is a physicochemical and mechanical process by which bacteria are coated with or entrapped within different materials through the use of emulsions, extrusions or through drying technologies. Commonly used materials for the encapsulation of bacteria include polysaccharides (starch, carrageenan), polysaccharide derivatives (gum Arabic, xanthan gum, alginate) and some proteins (gelatine, milk proteins) (Martín et al. 2015).

Co-encapsulation is an encapsulation process which uses a combination of two or more bioactive substances which can positively influence each other (Cham-

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pagne & Fustier 2007). Co-encapsulation of probiotics with prebiotics has been tested in order to protect their viability during the passage through GIT (Atia et al. 2017). As the viability of probiotics is negatively influenced by a detrimental effect of oxygen, which damages the membrane lipids, antioxidants (ascorbic acid, l-cysteine and tocopherol) were also tested to prevent oxidative stress when added into an encapsulation matrix with probiotics (Chen et al. 2017).

The aim of this study was to prepare microcapsules containing *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) with the addition of inulin and/or ascorbic acid both by emulsion method using milk proteins and by extrusion method using an alginate matrix, and further to compare the viability of the free and encapsulated bifidobacteria during storage and in conditions simulating those in GIT.

MATERIALS AND METHODS

Cultivation and determination of bifidobacteria count. The freeze-dried commercial probiotic strain of *Bifidobacterium animalis* subsp. *lactis* Bb12 was obtained from Christian Hansen (Hørsholm, Denmark). The strain was cultivated in 200 mL of MRS broth (Oxoid Ltd., Hampshire, UK; pH 6.5) with 0.05% (w/v) L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany) anaerobically at 37 °C for 18 hours. The cells were harvested by centrifugation at 9 000 rpm for 10 min at 4 °C (Universal 32R; Hettich, Tuttlingen, Germany), washed three times with physiological saline solution (pH 7) and immediately used for encapsulation.

For determination of their counts, after appropriate dilution bifidobacteria were cultivated in an anaerobic jar (Oxoid) at 37 °C for 72 h on L-cysteine-MRS agar, pH 6.5, and counted as colony-forming units (CFU mL^{-1}). Sterile sodium citrate solution [2% (w/v), pH 7.5] instead of saline solution was used for the dilution of samples containing protein or alginate capsules to allow their solubilisation and a release of cells from the matrix.

Encapsulation process. Emulsion encapsulation was done according to Heidebach et al. (2009) and Lisová et al. (2013) with some modifications. In brief, 120 g of 35% (w/w) reconstituted skimmed milk (Laktino skimmed milk powder, Promil, Nový Bydžov, Czech Republic) was mixed with fresh culture from 200 mL cultivation in MRS (de Man, Rogosa and Sharpe medium). After agitation (800 rpm, 5 min), 1.6 mL of Naturen^{TM} Premim 145 rennet (Chr. Hansen, Nienburg, Germany), diluted with distilled water at the ratio of 1 : 4 (w/w), was added. After that, the stirring speed was reduced to 500 rpm for 30 min and subsequently 720 µL of 10% (w/w) sterile $CaCl_2$ was added. Then, the whole mixture was transferred to 600 g of pre-tempered (5 °C) sunflower oil (Vegetol; Setuza, Ústí nad Labem, Czech Republic) with 0.5% (w/w) soya lecithin (Epikuron 100 PIP; Cargill GmbH, Krefeld, Germany) and stirred at 500 rpm. After 5-min agitation the mixture was heated to 40 °C with continued stirring for 15 minutes. The encapsulated cells were separated from the oil by gentle centrifugation (6 000 rpm, 4 °C, 2 min), washed three times in physiological solution and stored at 6 ± 1 °C before the following use.

For extrusion encapsulation, alginate was used as a coating material (Krasaekoopt et al. 2004). Fresh culture (1 g) was added to 10 mL of sterile 1% (w/v) sodium alginate solution (Sigma-Aldrich, St. Louis, USA). The mixture was injected through a sterile disposable 0.5-mm needle into 100 mL of sterile 0.1 mol L^{-1} $CaCl_2$ solution. The alginate beads were kept in the solution for gelification for 30 min; then they were washed twice with saline solution and stored at 6 ± 1 °C.

The addition of 1 % (w/w) inulin (Orafti® HPX, Beneo-Orafti, Oreye, Belgium) and/or 0.5% (w/w) ascorbic acid (Penta, Prague, Czech Rep.) was performed directly into reconstituted skimmed milk or into sodium alginate solution.

Survival of free and encapsulated cells in simulated gastrointestinal conditions. Freeze-dried culture (0.1 g) or microcapsules (1 g) were added into 50 mL of a solution simulating the stomach gastric juice containing distilled water, 0.5% w/v NaCl and 0.3% w/v pepsin (Sigma-Aldrich, St. Louis, USA). The final pH was adjusted to pH 2 with 10% w/v HCl. The samples were incubated at 37 °C in anaerobic atmosphere. After 2 h, pH was adjusted to 6.8 (with sterile 10% w/v NaOH) and ox bile (0.3% w/v; Merck) and pancreatin (0.1% w/v; Sigma-Aldrich, St. Louis, USA) were added to simulate the conditions in the ileum for another 4 hours. At the time of 0, 2, 4 and 6 h the number of viable cells was determined. The results are expressed as the means from three independent measurements (n = 3).

Viability of free and encapsulated bacteria during storage. The storage stability (UHT skim milk, Madeta, Planá, Czech Republic) of free and encapsulated cells in milk was tested for 6 weeks at 6 ± 1 °C. Every 14 days the number of viable cells and pH value (Jenway pH meter; Jenway, Staffordshire, UK) were measured. The results are the means from two independent replications, both parallel samples were analysed twice (n = 4).

Capsule size determination. Laser diffraction, based on the optical properties of dispersion, was used

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to determine the size of microcapsules prepared by the emulsion method. The capsules were re-suspended in demineralised water with refractive index 1.33 and the mean size distribution was measured using a Mastersizer 2 000 particle size analyzer (Malvern, Worcestershire, UK) with the Hydro G dispersion unit (Malvern, Worcestershire, UK) at 25 °C. The refractive index 1.45 and absorption coefficient 0.001 were selected for samples. Ninety percent fractiles ($d_{0.9}$) were calculated (i.e. 90% of the total volume is composed of microcapsules with diameters equal or lower than $d_{0.9}$). The results are the means from two independent encapsulation procedures, both parallel encapsulated cell samples were analysed three times.

The size of alginate capsules was measured with a digital calliper. The results are the means of measuring at least 30 randomly selected capsules, each from two independent encapsulation processes.

Table 1. Type and size of capsules prepared by different methods containing *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of inuline and/or ascorbic acid

Encapsulation method	Matrix	Inulin 1% (w/w)	Ascorbic acid 0.5 % (w/w)	
				v.b. fractiles ($d_{0.9}$) (μm)
Emulsion	milk protein	0	0	204 ± 18[a]
		+	0	192 ± 27[a]
		0	+	87 ± 10[b]
		+	+	227 ± 11[a]
				size (mm)
Extrusion	alginate	0	0	1.7 ± 0.1[a]
		+	0	2.3 ± 0.1[b]
		0	+	2.2 ± 0.1[b]
		+	+	2.3 ± 0.1[b]

Values in the same column followed by different lowercase letters are significantly different ($P < 0.05$); v.b. – volume based

RESULTS AND DISCUSSION

Eight types of capsules with inulin and/or ascorbic acid were prepared by emulsion method with protein matrix or by extrusion method with alginate (Table 1). Ascorbic acid can affect the growth of bifidobacteria (Shu et al. 2013) and it can also have a physiological effect (Garaiova et al. 2015). Inulin is a well-known prebiotic, its effect on bifidobacterial growth and human microbiota has been confirmed in many studies (Meyer & Stasse-Wolthuis 2009). It can be concluded that the addition of ascorbic acid reduced the size of protein microcapsules prepared by the emulsion method. On the contrary, in the case of alginate capsules (extrusion method), the addition of inulin and/or ascorbic acid increased the size of capsules. In the study of Heidebach et al. (2009) protein microcapsules containing probiotics with a volume-based median $d_{0.5}$ = 68 ± 5 μm were prepared. The authors concluded that there was no difference in the size of microcapsules containing bacterial cells or without them but they reported the influence of encapsulation conditions on $d_{0.5}$. Other authors (Vivek 2013) also proved the influence of various factors on the capsule size such as stirring speed, water/oil ratio, emulsifier concentration. In accordance with results of Valero-Cases & Frutos (2015), we also found that the addition of inulin increases the size of capsules. When applying encapsulated material into different types of food, the size of capsules must be taken into account. The application of larger particles obtained by the extrusion method is not suitable for liquid and semi-liquid foods, but they can be used in solid foods (Chen et al. 2017).

In the next part of this work, the influence of encapsulation and co-encapsulation with inulin and/or ascorbic acid on the stability of Bb12 compared to the stability of free Bb12 cells during 6-week storage at 6 ± 1 °C in sterile milk was tested. The results are summarized in Table 2. Milk is known for its protective effects on the viability of microorganisms (Sagheddu et al. 2018). Free Bb12 cells were found quite stable; nevertheless the number of cells at the end of storage was significantly different. It decreased by about 1.5 log cycles while the number of encapsulated cells remained statistically unchanged. The encapsulation had a positive effect on the viability of cells but the addition of inulin and ascorbic acid had no demonstrable effect either in protein or alginate capsules (at $P < 0.05$). This result is different from the conclusion of Valero-Cases & Frutos (2015), who found a positive effect of inulin on the viability of *L. plantarum* in alginate capsules.

Changes in the pH value of milk (considered as metabolic activity) were also monitored. Table 3 shows that free cells were able to produce organic acids and decreased pH from 6.33 ± 0.03 to 5.10 ± 0.02. In contrast, the pH of milk with encapsulated cells remained the same as at the beginning of storage. Both encapsulation methods prevented efficiently the manifestation of Bb12 fermentation activity.

The main objective of encapsulation is to protect cells against adverse conditions. The viability of bacte-

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Table 2. The viability (log CFU mL^{-1}) of free, encapsulated and co-encapsulated (with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid) cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 during 6-week storage in sterile skimmed milk at 6 ± 1 °C

	Week				Week			
	0	2	4	6	0	2	4	6
Free Bb12	8.1 ± 0.2^{a,A}	7.5 ± 0.1	6.8 ± 0.1	6.5 ± 0.1^{b,A}	10.4 ± 0.1^{a,A}	8.8 ± 0.1	8.5 ± 0.1	8.2 ± 0.1^{b,A}
	Emulsion method with milk protein				Extrusion method with alginate			
Encapsulated Bb12	8.8 ± 0.1^{a,A}	8.7 ± 0.1	8.3 ± 0.1	8.2 ± 0.3^{a,B}	10.4 ± 0.1^{a,A}	10.2 ± 0.1	10.2 ± 0.1	9.9 ± 0.3^{a,B}
Co-encapsulated Bb12 + inulin	8.3 ± 0.3^{a,A}	8.2 ± 0.2	8.2 ± 0.1	8.0 ± 0.3^{a,B}	10.3 ± 0.2^{a,A}	10.2 ± 0.1	9.9 ± 0.1	9.8 ± 0.1^{a,B}
Co-encapsulated Bb12 + ascorbic acid	8.2 ± 0.2^{a,A}	8.3 ± 0.1	8.4 ± 0.1	8.1 ± 0.1^{a,B}	10.3 ± 0.1^{a,A}	10.1 ± 0.1	10.2 ± 0.1	9.9 ± 0.2^{a,B}
Co-encapsulated Bb12 + inulin + ascorbic acid	8.2 ± 0.3^{a,A}	8.1 ± 0.2	8.3 ± 0.1	8.1 ± 0.1^{a,B}	10.3 ± 0.1^{a,A}	10.0 ± 0.1	9.9 ± 0.1	9.9 ± 0.1^{a,B}

Values are in mean ± SD; values in the same row followed by different lowercase letters are significantly different ($P < 0.05$); values in the same column followed by different capital letters are significantly different ($P < 0.05$).

ria in the stomach environment and in the presence of bile is one of the main requirements for probiotics (Karimi et al. 2011). Therefore, the stability of free and encapsulated Bb12 cells was checked in conditions simulating those when passing through GIT. The results are presented in Figure 1. All types of encapsulated cells showed a greater resistance to the conditions of the model digestive tract compared to free cells of Bb12. Differences in stability were particularly evident in conditions simulating the ileum. During the experiment a decrease in the number of encapsulated cells was only by 0.9–2.8 log cycles compared to 8 log cycles for free cells. No significant differences in cell survival were found when using protein or alginate capsules in the case of encapsulation into the matrix itself (decrease in cell number by 2.7–2.8 orders). In alginate capsules, the positive effect of inulin and ascorbic acid was not demonstrable, in all cases there was a total cell loss of 2.5–2.8 orders. On the other hand, the enriched protein capsules showed a lower cell loss; only by 0.9–1.8 orders. Our results are in good agreement with previously published data concerning the effect of encapsulation on the stability of bifidobacteria in human GIT. Comparable results were achieved by Madureira et al. (2011) with whey protein encapsulation of Bb12. The positive effect of encapsulation into an alginate matrix on resistance of *B. infantis* against gastric juices was also published by Cook et al. (2012). Fritzen-Freire et al. (2013) proved a positive effect of co-encapsulation of Bb12 with inulin into a protein matrix on the cell resistance to gastric juice and bile.

Table 3. The change in pH of milk with free, encapsulated and co-encapsulated [with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid] cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 after 6-week storage at 6 ± 1 °C

	Week		
	0	6	
Free Bb12	6.33 ± 0.03	5.10 ± 0.02	
	both capsules	emulsion method	extrusion method
Encapsulated Bb12	6.36 ± 0.03	6.15 ± 0.03	6.25 ± 0.04
Co-encapsulated Bb12 + inulin	6.36 ± 0.03	6.30 ± 0.03	6.45 ± 0.03
Co-encapsulated Bb12 + ascorbic acid	6.35 ± 0.04	6.32 ± 0.02	6.14 ± 0.04
Co-encapsulated Bb12 + inulin + ascorbic acid	6.32 ± 0.03	6.40 ± 0.02	6.08 ± 0.04

Values are in mean ± SD

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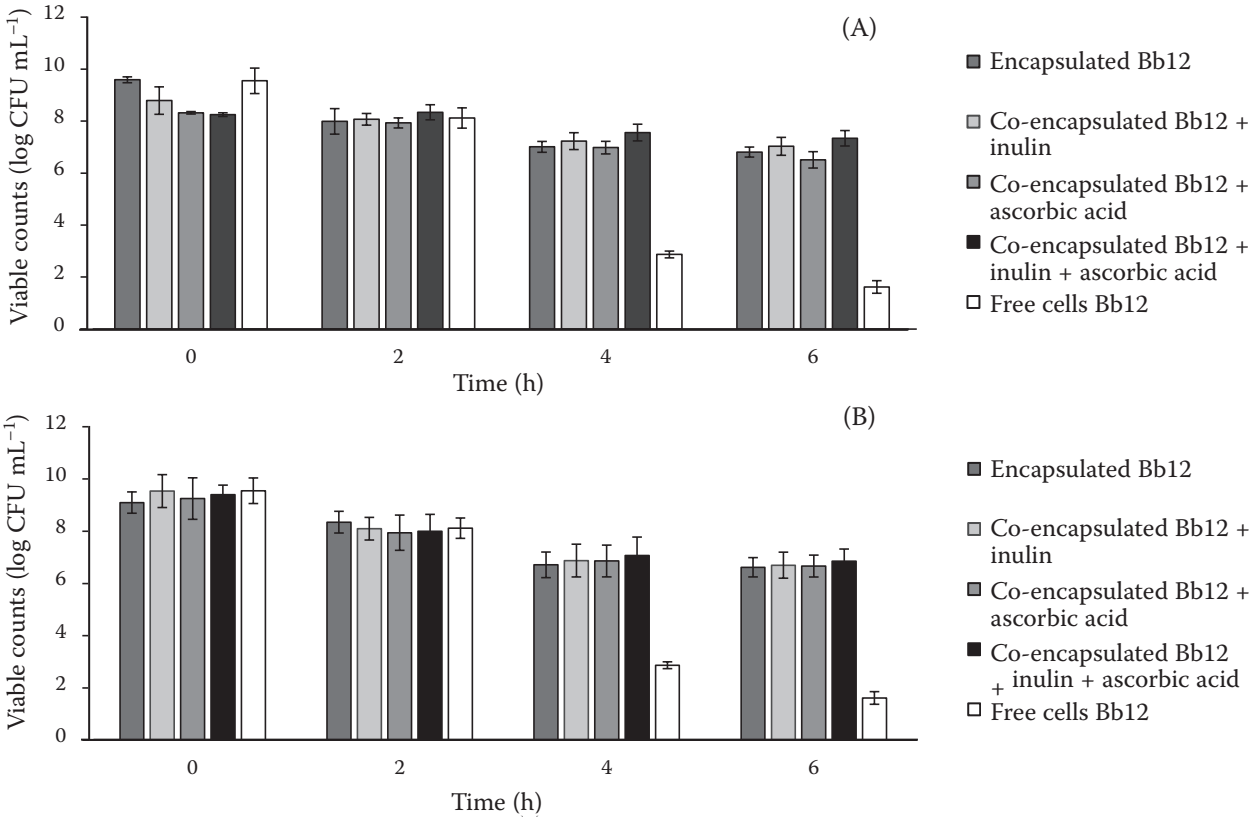

Figure 1. The viability (log CFU mL^{-1}) of free, encapsulated and co-encapsulated [with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid] cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 in conditions simulating those in stomach (0–2 h) and in ileum (2–6 h); (A) – emulsion encapsulation method with milk protein matrix; (B) – extrusion encapsulation method with alginate matrix

CONCLUSION

In this study, protein microcapsules of 204 ± 18 μm in size and alginate capsules of 1.7 ± 0.1 mm in size were obtained by various methods of encapsulation of *B. animalis* subsp. *lactis* Bb12 bacteria. Co-encapsulation with inulin (1% w/w) and/or ascorbic acid (0.5% w/w) affected the size of alginate capsules. Both encapsulation techniques resulted in the protection of Bb12 cells during 6-week storage in milk at a refrigerator temperature. Protein and alginate capsules as well as those with added inulin and/or ascorbic acid greatly enhanced the viability of Bb12 in conditions simulating the gastrointestinal tract. Capsules with probiotics, prebiotic inulin and antioxidant ascorbic acid can be considered as a suitable way of enriching food with biologically active ingredients with a high level of protection of sensitive bacteria. However, for application, the particle size must also be taken into account not to negatively affect the sensory properties of a product.

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