The influence of different polymers on viability of *Bifidobacterium lactis* 300b during encapsulation, freeze-drying and storage

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Revised: 23 May 2014 / Accepted: 3 June 2014 / Published online: 8 July 2014
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Abstract Seven different types of natural polymers namely hydroxypropyl methylcellulose (HPMC), sodium-carboxymethyl cellulose (Na-CMC), microcrystalline cellulose (MCC), starch BR-07, starch BR-08, dextrin and pullulan were used in order to develop the optimal formula for the entrapment of *Bifidobacterium lactis* 300B in Ca-alginate based granules. Laminar flow drip casting with Brace-Encapsulator was used in order to prepare the granules. The results showed that alginate/pullulan and alginate/HPMC formulation provide high protection for the bacterial strain used for encapsulation. These two formulations were further used to obtain freeze dried granules, for which the viability in time and at different temperatures was tested. The final results showed a higher viability than the level of the therapeutic minimum ($>10^7$ CFU/g) after 15 days of storage. Other parameters like entrapment efficiency, production rate, sphericity, flowability were also discussed.

Keywords Bioencapsulation · Biomaterials · Viability · *Bifidobacteria* · Granules Production

A wide range of fields such as foods, pharmaceuticals, environmental protection, and biotechnology, benefits from the utilization of valuable microbial cells. Nevertheless, pure active agents, as probiotics are difficult to handle in their respective form (Brandau 2002) and are likely to degradation when exposed to environmental conditions.

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The growing interest in microorganisms, living cells and other functional molecules encapsulation (Dalmoro et al. 2012b; Dalmoro et al. 2012a) has its origin in the lack of stability and target release of currently used formulations and in the attempt to provide appropriate growth conditions and protection (Rathore et al. 2013).

Numerous studies have been focused on microencapsulation of probiotic cells as a technique capable of cell loss limitation, insurances of mechanical stability, isolation, protection and target/controlled release (Albertini et al. 2010; Betoret et al. 2011; Burgain et al. 2011; Rathore et al. 2013). Major challenges, for the probiotic cells survival, include not only the complex and hazy events that occur during passage through the gastrointestinal tract (Savard et al. 2011), but also the adverse circumstances during processing and storage of the granules that serve as carriers for the active cells. Many food components may interact with the active cells, as polysaturated fatty acids and phytochemicals, or with the protective walls, as acidic food products (Vidhyalakshmi et al. 2009), of the granules. It is therefore, mandatory that the encapsulation technique protects the cells during the whole period of processing, storage and ensures, last but certainly not least, the target release. The efficiency of added probiotics in different functional foods (Stanton et al. 2001; Zhang et al. 2014) depends on their level and viability, which must be maintained during storage (Vidhyalakshmi et al. 2009).

In recent years, medical scientists have developed an increase interest on probiotics, as *Bifidobacterium lactis* (Prasanna et al. 2014), for human consumption due to the benefits they confer to the humoral immune system (Cui et al. 2000), how they affect the intestinal microflora balance (McMaster et al. 2005), and their antimicrobial properties, which serve to inhibit gastrointestinal pathogens (Haros et al. 2007).

In a dripping process, the shape, the structure and the size of the granules is strongly influenced by the matrix used in the

encapsulation process (Chan et al. 2011b). Chan et al. (2011a) related the survival of the encapsulated probiotic cell to the structural integrity of the granules. Alginate based granules are frequently used for the entrapment of probiotics (Anal and Singh 2007; Brinques and Ayub 2011; Burgain et al. 2011), due to its biodegradability and biocompatibility, resistance in acidic conditions, successful release in intestinal media (Sultana et al. 2000) its thermo tolerance and freeze-drying resistance (Cheow and Hadinoto 2013).

Natural polymers with valuable physical and biochemical proprieties, can improve the alginate based granules characteristics. This can be transposed in higher entrapment efficiency, improvement and enhance of the probiotic cells stability in the product until consumption better protection in biological environments.

The aim of this study was to investigate the entrapment of *Bifidobacterium lactis* 300B in Ca-alginate based granules, in order to obtain adequate physical and biochemical properties that sustain the viability of the cells. Seven different encapsulation matrices were used: three types of celluloses, two types of starch, dextrin and pullulan.

Materials and methods

Probiotic strain

In the present study, *Bifidobacterium lactis* 300B was used as probiotic strain. The strain was purchased as lyophilized probiotics powder from Howaru, Germany. The probiotic was used as received from the supplier. A viability test was performed before each trial. All materials and solutions were sterilized by autoclaving at 121 °C for 15 min. prior utilization.

Encapsulation of probiotic cells

Lyophilized probiotic, 75 g/L, were encapsulated using cross linking gelation. The encapsulation formulation consists in 15 g/L alginate (FMC, Norway) and 15 g/L filler material. The Spherisator M, type 2002SP-AE5-D0 was used in the granules formulation process, at Brace GmbH Germany. The slurry and the polymers were pumped from the feed tank to the nozzle head where the vibrating device induces the break-up of the flow into uniform droplets. These are formed into spheres by the surface tension of the feed. The droplets are solidificated during falling into the hardening bath. The obtained granules were hardened for 30 min in calcium chloride 40 g/L (Brenntag, Australia), the hardening bath, and then rinsed with sterile sodium chloride 8.5 g/L (Sigma-Aldrich, Germany). The filler used for encapsulation of the probiotic powder, and each sample codification are shown in Table 1. The entrapment efficiency of the fresh granules was

determined according to (Sandoval-Castilla et al. 2010) with slight change as follows:

$$\text{Entrapment efficiency} = (a \times F/b) \times 100 \text{ (CFU/g)}$$

Where a is CFU/g in the granules, and b is CFU/g in the biopolymer slurry before production, and F is the sphere packing factor (Aste and Weaire 2008). We considered the cubical densest package for all calculations $F=0.70$ (Aste and Weaire 2008; Holleman et al. 1985).

Mixture properties

The density of the alginate-filler mixture was calculated using the mass (m) and volume (V) ratio

$$\rho = m/V \text{ g/cm}^3 \quad (1)$$

The viscosity was measured with an HAAKE viscometer VT-02 (ThermoFisher, Germany) at 23 ± 1 °C, for each sample after sterilization and cooling down. The value for the surface tension of the alginate based solutions at 15 g/L was obtained from Chan et al. (2011a) and considered constant for all seven samples.

Micrometrics properties

Granules size and shape

The size of the granules was determined using the method described by Chan et al. (2011b) with slight change as follows.

Theoretical diameter of detached liquid drop, $D_{l(T)}$ (mm):

$$D_{l(T)} = (6d_T\gamma/\rho g)^{1/3} \quad (2)$$

where d_T is the capillary tip diameter (mm); γ is surface tension (g/s^2); ρ is density (g/mm^3); and g is gravitational acceleration (mm/s^2).

Corrected diameter of detached liquid drop, $D_{l(C)}$ (mm):

$$D_{l(C)} = k_{LF} * D_{l(T)} \quad (3)$$

where k_{LF} is the liquid lost factor, $k_{LF}=0.98-0.04d_T$.

Corrected diameter of Ca-alginate granules after gelation, $D_{b(C)}$ (mm):

$$D_{b(C)} = k_{SF(gelation)} * D_{l(C)} \quad (4)$$

where k_{SF} is the shrinkage factor attributed to the gelation process, which was found to be for Ca- alginate granules

Table 1 *Bifidobacterium Lactis* 300B encapsulation formulation

Granules prepared with sodium alginate 1.5 % (w/v) and	1.5 % (w/v) HPMC (Harke Pharma, Germany)	AHPMC
	1.5 % (w/v) Na CMC (Dow Chemicals, Germany)	ACMC
	1.5 % (w/v) Microcrystalline cellulose (Rettenmayer, Germany)	AMCC
	1.5 % (w/v) Starch BR-07 (Brace, Germany)	AS07
	1.5 % (w/v) Starch BR-08 (Brace, Germany)	AS08
	1.5 % (w/v) Dextrin (Sigma, Germany)	AD
	1.5 % (w/v) Pullulan (Hayashibara, Japan)	AP

$k_{SF(gelation)}=0.88$ (Chan et al. 2011b). The shrinkage factor is given by the ratio of the diameter of the granules before gelation and the diameter of the granules after gelation.

The reduction in granules size after lyophilization was calculated and expressed by a shrinkage factor, as shown below:

$$k_{SF(lyophilization)} = (D_b - D_{b(lyophilized)}) / D_b \quad (5)$$

where $k_{SF(lyophilization)}$ is the shrinkage factor attributed to the lyophilization process; D_b is the diameter of the granules obtained as described above before lyophilization (mm); and $D_{b(lyophilized)}$ is the diameter of the granules obtained after lyophilization (mm).

Determination of granules shape

The granules shape was quantified using the sphericity factor (SF), which is given by the following equation:

$$\text{Sphericity factor (6) (SF)} = (d_{\max} - d_{\min}) / (d_{\max} + d_{\min})$$

where $d_{\max}$ is the largest diameter and $d_{\min}$ is the smallest diameter perpendicular to $d_{\max}$.

Twenty granules were used for each determination of the $d_{\max}$ and $d_{\min}$ and the average was calculated. The $d_{\max}$ and $d_{\min}$ was obtained using a microscope.

Determination of bulk density, tapped density and flowability

The bulk density (ρ_{BD}) of the lyophilized granules was determined by pouring a known mass of granules (mp) into a calibrated cylinder, and it was calculated by dividing the mass (mp) by the bulk volume (v_B), as shown in following equation:

$$\rho_{BD} = m_P / v_B \quad (7)$$

The tapped density (ρ_{TD}) was determined by tapping a calibrated cylinder containing granules until the equilibrium tap volume (v_T) was obtained. The tapping was performed until no volume change was observed. Hausner's ratio of microcapsules was computed according to the following equation:

$$\text{Hausner ratio} = \rho_{TD} / \rho_{BD} \quad (8)$$

The Hausner ratio is a parameter that inversely influence the granule flowability.

Granules freeze-drying

Freeze drying is more suitable than other drying process (e.g. spray drying) for some bacterial strains (Capela et al. 2007; Johnson and Etzel 1995). The fresh obtained granules were shock frozen at -18°C and connected to a VaCo 5 freeze dryer from Zirbus (Germany) at once and freeze dried at -50°C and 0.05 mbar for 24 h. The fresh granules were connected to the freeze drier using sterile bottom flasks, for each sample was weight approximately 30 g. The freeze dried material was collected sterile recipients. Samples were analyzed immediately and after 3, 6, 9 and 15 days at room temperature ($\sim 22^\circ\text{C}$) and refrigeration ($\sim 4^\circ\text{C}$) in order to determine the evolution of cell viability in time and at different temperatures.

Probiotic cells viability

Non-encapsulated and encapsulated *Bifidobacterium lactis* 300B were enumerated immediately after the encapsulation, and freeze drying process respectively, using the plate counting method, on BSM agar (Sigma-Aldrich, Germany). The granules were dissolved completely in sodium citrate (20 g/L) with an adjusted pH=7.3, before enumeration of viable cells. Dilutions steps 1:10 were performed in saline solution (8.5 g/L). From the last three dilutions, one ml of the dilution was introduced in the Petri dish where the nutrient agar medium was added. The operation was repeated three times for each dilution. After 72 h incubation at 37°C in the anaerobic jar (Sigma-Aldrich, Germany) the number of colony-forming units (CFU) was counted. Colonies of bacteria were calculated and converted to $\log_{10}$ CFU.

The survival of bacteria cells in each of the freeze-dried samples tested was determined using the mathematical formula: (9) survival=(n/n₀), where "n₀" is the number of bacteria per gram of wet granules before drying, and "n" is the number of the freeze-dried granules right away after drying (Simpson et al. 2005). The viability of the cells as a function of storage time, at room temperature and at 4°C was obtained by calculating the ratio: (CFU/g of granules after 3, 6, 9 and 15 days storage/CFU/g of granules immediately after freeze-drying).

Statistical analyses

Analysis of variance (ANOVA) was applied to all data for the alginate based granules in all samples in order to determine differences between the $\log_{10}$ CFU/g of *Bifidobacterium lactis* 300B granules of the different treatments: right away after encapsulation, after freeze drying and at the end of the storage time (3, 6, 9 and 15 days). The mean values and the standard error were calculated from the triplicate data. The statistical evaluation was carried out using Graph Prism Version 4.0 (Graph Pad Software Inc., San Diego, CA, USA). For the size determination, all the calculations were performed using Microsoft Excel 2010.

Results and discussion

Microencapsulation yield and granules properties

Throughput and processing conditions were investigated in order to evaluate the possibility of industrial scale production of probiotic entrapment in one of the formulations described above. It was found that the production of granules, in terms of quantity and quality, was influenced by the nature of the seven types of filler material used for the *Bifidobacterium lactis* 300B entrapment. This production was for laboratory purpose. Scaling up the process for small industrial production would be possible with slight modifications to the process, and according to Brandau (2002), it is suitable for large industrial production. The production of 600 g/h, the value obtained in our study, is an easily achievable outcome. Future attempts resulting in the production of higher outputs ranging from 3.6 kg/h to 10 kg/h may be reasonably expected.

As can be observed in Fig. 1, in terms of production per minute, the highest throughput is obtained for the samples AS08, AP and AS07 in this order.

The production for the samples AHPMC- 580 g/h, ACMC- 537,4 g/h and AD-552,4 g/h are similar while the sample AMCC- 441 g/h, shows the lowest production rate.

High entrapment capacity was observed for all formulations, as is represented in Fig. 2. In our study, the entrapment efficiency of *Bifidobacterium lactis* 300B into the granules varied between 57.20 and 69.96 %. In the literature (Jyothi et al. 2010; Reid et al. 2005) the entrapment efficiency data is linked to the viability losses in the granules. The highest entrapment efficiencies were obtained for the samples AHPMC, AP and AS07 in this order. Due to their excellent physicochemical and mechanical properties, HPMC, pullulan and starch enhance the alginate action. The granules filled with microcrystalline cellulose (sample AMCC) and dextrin (sample AD) showed the lowest entrapment efficiency, but not statistically significantly lower than the other samples. The

Fig. 1 Throughput of alginate based granules production. ((●) sample AHPMC, (■) sample ACMC, (▲) sample AMCC, (▼) sample AS07, (◆) sample AS08, (◼) sample AD, (◻) sample AP)

rest of the alginate based granules, respectively the ones using the two types of cellulose and starch BR-08 as fillers showed similar trends. Our results concerning the entrapment efficiencies when using the different cellulose types and starch as fillers agree with those previously found by Nochos et al. (2008) and Sultana et al. (2000).

Micrometric properties

Granules size and shape

The various granule formulations show an average particle size in the range of 1054–1066 μm .

As can be seen in Table 2, similar trends can be observed for all formulations in the dripping and gelation processes

Fig. 2 Entrapment efficiency in alginate based granules for each sample (mean $\pm$ SD, $n=3$, $P>0,05$)

Table 2 Density and viscosity of the mixture, nozzle size and the calculated diameters

Sample	Density (g/cm ³)	Viscosity (mPa·s)	Tip diameter (mm)	Theoretical diameter of detached liquid drop (mm)	Corrected diameter of detached liquid drop (mm)	Corrected diameter of granule after gelation (mm)	Experimental diameter of granule after gelation (mm)
AHPMC	1,037±0.01	190±11.8	0.3	1,2248	1,1907	1,099	1,0668
ACMC	1,040±0.01	490±20.6	0.3	1,2061	1,1765	1,084	1,0539
AMCC	1,039±0.015	260±23.2	0.3	1,2166	1,1827	1,092	1,0596
AS07	1,038±0.018	255±17.61	0.3	1,2169	1,183	1,090	1,0599
AS08	1,027±0.019	250±18.9	0.3	1,2208	1,1868	1,095	1,0633
AD	1,019±0.01	248±16.8	0.3	1,224	1,1899	1,097	1,0661
AP	1,017±0.014	310±12.4	0.3	1,2158	1,1819	1,091	1,0589

Values are mean ($n = 3$) ± standard deviation

regarding the size of the granules. Furthermore, in the Table 2 can be observed that the drop size decreased consistently along the hardening process.

The factors affecting the size of the granules involve the viscosity of the polymer solution, the diameter of the nozzle and the distance between the outlet and the coagulation solution (Anal et al. 2003; Anal and Singh 2007; Anal and Stevens 2005) and the manufacturing methods used (Grabnar and Kristl 2011). In our study for all the samples the same size diameter of the nozzle was used. A correlation between the size of the obtained granules and the viscosity can be observed. The sample AHPMC, the less viscous from the samples, with 190 (mPa·s) viscosity had the biggest diameter, 1.0668 mm. This kind of correlation between the viscosity and the granules size underlined also by other studies (Chan et al. 2011b; Chandramouli et al. 2004). Similar trends were observed for the samples AD, AS07, AS08, ACMC and AP with no significant differences neither when the viscosity is discussed nor the diameter of the obtained granules.

In the vibration dip casting, the drop was formed by a vibration system. When the droplet was extruded by the flow rate, it broke up with the vibration under resonance, the liquid drop detached from the nozzle and immerse into the hardening bath where bound ions and create linkages lead to the gel formation. Granules were smaller than the drop detached from the nozzle, a phenomenon attributed to the syneresis effect happened in the formed gel. The calculated diameters of the granules after gelation were found to give a high approximation to the obtained experimentally as can be observed in Table 2.

Previous reports (Donati et al. 2005) have shown that the shrinkage factor, which is influenced by the M/G (mannuronic/glucuronic acid) ratio of the alginate, can be used to correct the diameter of the granules after gelation. Chan et al. (2011a) have shown that low viscosity of the filler leads to high shrinkage factor. In accordance to what was previously found, this trend is also observed in the present work as can be seen in Fig. 3. In our study, the highest amount

of shrinkage of the lyophilized granules is attributed to AHPMC and AD, the samples that proved the lowest viscosity of the mixture. The least amount of shrinkage can be attributed to the samples ACMC and AP due to the same motive regarding the viscosity (Nienaltowska et al. 2010). A similar trend can be seen with SiO₂ as filler (Brandau 2002).

The shape of the alginate based granules was delineating using the sphericity factor due to its effectiveness in determining shape changes. A perfect sphere is defined by a sphericity factor equal to 0; meanwhile, the elongated objects have values of the sphericity factor approaching to unity. According to Goh et al. (2012) a high concentration of polymer leads to an increased sphericity. In the present study, all the obtained granules have had spherical shape in spite of the type of the filler. The lyophilization process induced a deformation in the structure of the granules caused by the sublimation of the water from the hydrogel matrix. This fact resulted in granules with an unpredictable and irregular shape, occurrence observed in former studies (Chan et al. 2011b; Rassis et al. 2002; Zohar-Perez et al. 2004). Nevertheless, in our study, the deformation of the granules was attenuated by

Fig. 3 Effect of viscosity of the samples on the shrinkage factor of lyophilized Ca-alginate based granules

the different fillers used as can be observed in Table 3, were the sphericity factor of the lyophilized granules is presented.

Bulk, tapped density and flowability

Generally, entrapment efficiency is used as a quality parameter for the dried granules. Nevertheless, other assessed quality control parameters as bulk density, tapped density and the Hausner Ratio provided the powder flowability (Kennedy and Panesar 2006). The results obtained for these quality parameters in this study are presented in Table 4.

The bulk density can be defined as the mass of granules divided by the total volume occupied, which includes the granules volume, the inter-particle void volume and the internal pore volume. In the present study, the results indicate that the bulk density of the samples ranges from 0.18 to 0.28 g/cm³. As it is well-known, a dry product with a high bulk density can be stored in a smaller container than a product with a relatively lower bulk density.

Tapped density refers to the bulk density of the granules after a specified compaction process. The variation of tapped density in our study was from 1.20 to 0.32 g/cm³. In this study, it was found that the tapped density was the highest in samples AMCC, AP, APMC and AS07, and the tapped density was found to be higher than the bulk density. A correlation between the viscosity of the sample and the densities was found. The sample AMCC and AP show the highest values of viscosity, and also the highest values for the densities. The same tendency was observed in all the samples. This behavior was also observed in previous work (Chan et al. 2011b) where the difference of the samples viscosity was due to various concentrations of the filler.

The Hausner ratio of a granular material is defined as a measure of the interparticle friction or cohesiveness of the material (Kennedy and Panesar 2006). The description of granules degree of compaction is defined by this ratio, and it can be defined as the ratio of the tapped density to the bulk density. The presence of high inter-particle friction is indicated by a larger value (Abdullah and Geldart 1999). The friction is affected by the class of material used; the granules size and shape, the surface, the size distribution, the atmospheric conditions (humidity and temperature) and the inter-particle forces (e. g. cohesion and electrostatics).

A higher Hausner ratio means that the material is more cohesive and less able to flow freely. A Hausner ratio of less than 1.5 has been used to indicate good flowability (Thalberg

et al. 2004) since particles at this ratio show little potential for further consolidation. In this study, all lyophilized granules were free flowing as indicated by Hausner ratios ranging from 1.07 to 1.16. In spite of this quality, the highest Hausner ratio was observed for the sample AMCC, which shows an increased inter-particle friction. The sample AHPMC showed the lowest Hausner ratio value followed by the sample AP. However, the Hausner ratio of the granules were similar despite the type of the filler. Chan et al. (2011b) correlate the values of the Hausner ratio with the concentration of the polymer rather than with the type of the filler. In our study, the similarity can be derived from the fact that was not a significant difference in regarding the size of the obtained granules.

Entrapment of Bifidobacterium lactis 300B in fresh granules

The entrapment of cells is influenced by the encapsulation process. The counts of the encapsulated cells were calculated based on the ratio of viable cells after encapsulation over the initial number of viable cells in the slurry.

Entrapped cells survival after the encapsulation process decreased in all the cases. However, the granules matrix composition provided a different degree of protection to the entrapped cells resulting in different survivability values. The encapsulation procedure was the same for all seven samples. It can be observed a greater number of surviving cells, by providing *Bifidobacterium lactis* 300B with a proper covering matrix. It is reported in the literature (Rodriguez-Huezo et al. 2007) that the oxygen protection immediately after gelation has a beneficial effect by reducing the decaying rate of cells consistently. The specific property of pullulan to form strong, oxygen-impermeable films is consistent reported (Leathers 2003; Singh et al. 2008).

This statement supports our results, shown in Fig. 4 where the survival rate of the probiotic cells under the same conditions is observed, indicating that the formulation of sample AP, where the pullulan filler was used, offer the best protection, providing a value of 10¹³ CFU/g immediately after encapsulation. The granules where the HPMC filler was used, reduce, to some extent, the protective effect, but the survivability is still high compared to the rest of the samples: 10¹² CFU/g compared to 10⁹ CFU/g the mean of the rest samples. It is interesting to observe that this granules variation was also positive for the freeze drying process and storage conditions.

Table 3 Sphericity factor (SF) of the lyophilized granules

Sample	AHPMC	ACMC	AMCC	AS07	AS08	AD	AP
(SF)	0.203±0.003	0.149±0.008	0.092±0.009	0.157±0.005	0.154±0.005	0.183±0.003	0.103±0.004

Values are mean (n=3)±standard deviation

Table 4 Bulk density, Tapped density and flowability of the lyophilized granules

Sample	Bulk density (g/cm ³)	Tapped density (g/cm ³)	Flowability (kg/s)
AHPMC	0.18±0.015	0.20±0.013	1.06±0.003
ACMC	0.26±0.014	0.28±0.019	1.07±0.005
AMCC	0.27±0.016	0.32±0.021	1.16±0.008
AS07	0.25±0.019	0.26±0.017	1.09±0.002
AS08	0.20±0.014	0.21±0.016	1.13±0.001
AD	0.19±0.013	0.21±0.015	1.09±0.004
AP	0.28±0.011	0.30±0.019	1.06±0.006

Values are mean ($n=3$)±standard deviation

The number of live cells found in the granules after the encapsulation process influences the number of living cells that will be found in any final product. Chan et al. (2011b) correlate the survival of entrapped cells after the encapsulation process to the physical properties of the granules. Our results fell in between because the samples with HPMC, pullulan and starch showed the best physical properties (in this order), while the best survival rate was observed in the alginate/pullulan formulation. In this specific case, the oxygen protection of pullulan balanced the expense of the physical properties.

Viability after freeze drying process

The application of shock freeze to the fresh granules caused considerably less shrinkage (data not show). For all the samples, the reduction in mass was greater than 92 %. This value is important in the scaling up process.

The freezing rate controls the nucleation and growth of ice crystals that are necessary to initiate the freezing process (Maa and Prestrelski 2000). Slow freezing creates conditions that allow the ice nuclei to grow into larger crystals. Rapid freezing

affects mainly the number of the nuclei and not their size. However, fast freezing creates smaller ice crystals than slow freezing (Maa et al. 1999; Maa and Prestrelski 2000). These findings are associated with changes of protein state, as well as of the cells phospholipid membrane, during the freeze drying process. The deteriorative reactions are: damages created by large crystals to the cell membrane, and freezing induced unfolding of proteins. This process affects the survivability of the entrapped cells as is evident in Fig. 5.

Since the AP and AHPMC samples proved to be the best formulae for entrapment of *Bifidobacterium lactis* 300B we decided to analyse the survival rate in these freeze dried granules. In order to prevent the rupture of the probiotic membrane by the large ice big crystals formed in a slow freezing process, the samples were shock frozen at -18 °C for 30 min.

The number of encapsulated cells in the freeze dried granules were calculated based on the ratio of viable cells after freeze drying over the initial number of viable cells in the fresh made granules is shown in Fig. 5. A higher survival rate of *Bifidobacterium* is observed in both samples. Such behavior can be attributed to the cell wall and membrane composition of *Bifidobacterium* (Carvalho et al. 2004). A 14.16 % and 17.98 % loss of cell viability was registered in the AHPMC respectively AP granules in the freeze drying process. For the freeze drying of the nonencapsulated *Bifidobacterium*, the literature (Capela et al. 2006) reports a mean of 77.78 % survival. This fact demonstrated that the encapsulation applied in this research sustains the viability of the probiotic cells during freeze drying process.

Stability of entrapped cells in freeze dried granules

Survivability of *Bifidobacterium lactis* 300B loaded in AP and AHPMC granules has the tendency to decline during storage. The survival was maintained at 10¹⁰ CFU/g after 15 days of storage at room temperature and 4 °C for alginate/pullulan

Fig. 4 Viability of anaerobic bacteria in the slurry and loaded in the seven types of alginate based granules after encapsulation, expressed as log₁₀ CFU/g. The error bars represents standard deviation of the means

Fig. 5 Survival of *Bifidobacterium lactis* 300B before and after freeze drying in samples AHPMC and AP. The error bars represents standard deviation of the means

based granules and for the alginate/HPMC cellulose based granules at 10^7 CFU/g after 15 days at room temperature and 10^9 CFU/g after 15 days at 4 °C. These results are comparable with the literature (Homayouni et al. 2008) which used encapsulation, in alginate/starch granules, of *Bifidobacterium lactis* for long term protection in lyophilized ice cream.

The influence of the temperature on freeze dried alginate/pullulan and alginate/HPMC cellulose based granules is shown in Fig. 6. A temperature close above 0 °C generally leads to higher survival compared to more elevated storage temperatures (Endo et al. 2014; Heidebach et al. 2009; Picot and Lacroix 2004; Weinbreck et al. 2010), because lower temperatures result in reduced rates of detrimental chemical reactions, such as fatty acid oxidation (Tanghe et al. 2003). In addition, the freeze dried granules kept at refrigeration temperature demonstrated better protection for the entrapped anaerobic bacteria compared to the granules stored at room temperature in our study too (Homayouni et al. 2008) affirms

Fig. 6 Survivability of *Bifidobacterium lactis* 300B in freeze dried granules after 3, 6, 9 and 15 days kept at room temperature and at 4 °C. Symbols: (●) 4°C alginate/pullulan granules, (■) room temperature alginate/pullulan granules, (▲) 4°C alginate/HPMC granules, (▼) room temperature alginate/HPMC granules

that the survival of bacteria against unfriendly conditions is species dependent also. Our study's results show that *Bifidobacterium lactis* 300B after 15 days at room temperature and at refrigeration, is maintained to the level of the therapeutic minimum ($>10^7$ CFU/g) or higher. The HPMC cellulose filling was found to increase the survivability of *Bifidobacterium lactis* 300B during storage at room temperature (Klayraung et al. 2009), nevertheless, the pullulan filler proved even a higher protection at room temperature as it is shown in Fig. 6.

Conclusions

This study compared the effect of seven types of fillers, namely HPMC, Na-CMC, MCC, starch BR-07, starch BR-08, dextrin or pullulan, on the viability of *Bifidobacterium lactis* 300B entrapped in alginate based granules, during encapsulation, freeze drying and storage for different periods of time. Parameters like entrapment efficiency, production rate, sphericity, flowability were also discussed.

Our study has indicated that the survival of immobilized bacteria may be dependent on the properties of the adding filler, which influences the granule's characteristics. Among the seven types of fillers utilized in the encapsulation formula from this research, the pullulan and HPMC provide the highest protection. The viability recorded after encapsulation was 10^{13} CFU/g for the alginate/pullulan granules and 10^{12} CFU/g for the alginate/HPMC granules. The alginate/dextrin type of granules proved to have a lower viability rate after encapsulation, 10^6 CFU/g, while a similar behavior was determined for the two types of starches and cellulose with 10^9 CFU/g. Encapsulation formulas, alginate/pullulan and alginate/HPMC, were effective in keeping the counts of probiotic bacteria higher than the level of the therapeutic minimum ($>10^7$ CFU/g) in the freeze dried form. In conclusion, encapsulation in alginate/pullulan granules offers an effective means of protection in the freeze drying process and storage.

Future work is needed to analyze the resistance of entrapped cells in different products and in the gastrointestinal tract. Cryo- and osmo-protective components can be incorporated into the matrix increasing the survival of cells during processing and storage. Finally, a further surface coating can be applied in order to enhance a specific delivery. From the economic standpoint, the technique described in this paper is suitable for industrial production.

Acknowledgments This work was carried out at Brace GmbH - Karlstein am Main, Germany. The authors would like to thank Dr. Holger Strohm and Mr. Manfred Stöckl for their helpful collaboration during the experimental work.

Declaration of interest The authors declare that they have no conflicts of interest in concerning this article. This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765. The authors are responsible for the content and writing of the article.

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