THE EFFECT OF CO-ENCAPSULATION OF PROBIOTICS WITH PREBIOTICS ON THE VIABILITY OF PROBIOTIC BACTERIA

MATOUŠKOVÁ Petra, VRTNÁ Monika, ŠNADAROVÁ Karolína, BOKROVÁ Jitka, MÁROVÁ Ivana

Brno University of Technology, Faculty of Chemistry, Materials Research Centre, Brno, Czech Republic, EU
xcmatouskovap@fch.vutbr.cz

Abstract

The aim of this work was to evaluate the effect of four natural prebiotics (inulin, psyllium, apple fiber and hemp fiber) on viability of probiotic bacteria. Two probiotic strains, namely *Lactobacillus acidophilus* (CCM 4833) and *Bifidobacterium breve* (CCM 7825T) were co-encapsulated with selected prebiotics into alginate particles. The viability of encapsulated microorganisms during storage in acidic conditions was analysed. Also, the physicochemical evaluation of prepared particles and the viability of cells during simulated gastrointestinal conditions were tested. To analysis of probiotics light/fluorescence microscopy and flow cytometry were used. Results indicated significant improvement in survival of co-encapsulated cells compared to free cells. As the best prebiotic for co-encapsulation the hemp fiber was found. The hemp fiber exhibited the highest increase of cells count and the high viability of encapsulated bacterial cells during long-term storage. The co-encapsulation of probiotics into alginate particles leads to increased tolerance of bacteria to acidic environment. Particles also maintained their integrity during passage through the gastrointestinal tract until they reached their target destination. Therefore, prepared particles could be used in foods or food supplements with targeted transport of probiotic bacteria.

Keywords: Probiotics, prebiotics, co-encapsulation, alginate

1. INTRODUCTION

Probiotics are microorganisms that, when consumed in adequate amounts (10^6-10^7 CFU/g or mL), confer healthy benefits to consumers by FAO/WHO definition. [1] Beneficial effects of probiotic bacteria on human health have been regulation of the gastrointestinal tract, stimulation of the immune system, decrease in serum cholesterol levels and in lactose intolerance, as well as prevention of cancer and cardiovascular disease. [2, 3, 4, 5] A number of food products including dairy products, meats products, beverages products, cereals products, vegetables and fruits products and bread products have been utilized as delivery vehicles for probiotics. [1, 5, 6] Products available on the market that positively influence the intestinal microflora are especially synbiotics. The symbiotic concept can be defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial cells in the gastrointestinal tract. [4] But many factors may affect the viability of probiotic bacteria in foods including the probiotic strains used, pH, the presence of dissolved oxygen, storage temperature, heat treatment, mechanical or osmotic stress, concentration and nature of the added ingredients and food matrices, also the physical circumstances of the human gastrointestinal tract after ingestion strongly influence on the survival of probiotic bacteria. [1, 2, 3, 5, 6, 7] Microencapsulation has proven to be a promising method for bacterial cell protection and several studies have been carried out investigating the protective role of this technique against the adverse conditions to which probiotics can be exposed. [2, 6] Nevertheless, encapsulation does not ensure the total viability of microorganisms. There are reports of encapsulated probiotics with prebiotic with increased viability in capsules compared with capsules without prebiotics. Prebiotics are used as an energy source and as metabolic substrates and micronutrients. Different types of prebiotics have been used to protect probiotics. [1]

Encapsulation of bacteria in calcium alginate beads is one of the most studied systems for probiotic immobilization and protection. Sodium alginate is a water soluble anionic polysaccharide, mainly found in the cell walls of brown algae. This natural polymer possesses several attractive properties such as good

biocompatibility, wide availability, low cost, and simple gelling procedure under mild conditions. Moreover, morphology of alginate microcapsules have suitable porous micro-structure and they also facilitates active components release. [8]

2. MATERIALS AND METHODS

2.1. Chemicals

Alginate sodium salt, calcium chloride, pepsin, pancreatin and bile salts were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other solvents and reagents used in the analysis were of analytical grade.

2.2. Probiotics strains and cultivation conditions

The term ‘‘probiotic’’ includes a large range of microorganisms, mainly bacteria but also yeasts. And the effects of probiotics are strain-specific. [4] In this study were used bacterial strains *Lactobacillus acidophilus* CCM 4833 and *Bifidobacterium breve* CCM 7825^{T}. The strains were obtained from Czech Collection of Microorganisms in Brno and before encapsulation were grown in commercial MRS broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) at 37 °C for 24 h. Viability of bacteria was followed by flow cytometry (Apogee Flow Systems, Hemel Hempstead, UK).

Also ability of probiotic strains to grow on selected prebiotic were investigated. The MRS growth medium contained 10 mg/mL or 100 mg/mL of prebiotic. Sample with free MRS media was used as a control. Criteria for the evaluation of utilization of prebiotics were the growth of probiotics and viability of probiotic cells. Cultivations were carried out in a 10 mL medium at 37°C for 24 and 48 h. The number of cells and viability of bacteria was also measured by flow cytometry.

2.3. Prebiotics and their characterization

As prebiotics were used inulin, psyllium, hemp fiber and apple fiber. Prebiotics were bought from the local market in Brno, Czech Republic. The content of carbohydrates was measured by colorimetric detection using UV-Vis spectrophotometer (Thermo Fisher UK Ltd., Hemel Hempstead, UK)). The content of reducing carbohydrates was determined by Somogyi-Nelson method and content of total carbohydrates was measured by Dubois method. Carbohydrates (especially glucose, fructose and oligosaccharides) from prebiotics were also separated in Rezex ROA-Organic Acid H+ column (Phenomenex Inc., Torrance, CA, USA) using isocratic elution with 5 mM sulfuric acid by high-performance liquid chromatography with refractometric detector (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Co-encapsulation of probiotics with prebiotics

Particles were prepared from alginate according to the procedure from a previous study.[9] The probiotics cells were harvested by centrifugation at 3500 rpm for 10 min at low temperature (4°C) and the cell pellet was washed with sterile distilled water. Cells were next mixed with sterile sodium alginate solutions (1 %, w/v) with or without prebiotic in order to obtain the final concentration of cell cultures 10^{8} CFU/mL. The addition of prebiotics was 0.05 or 0.5 g per 10 mL. Afterwards, suspensions were used for encapsulation. The microcapsules were prepared aseptically using the Büchi Encapsulator B-395 Pro (BÜCHI Labortechnik AG, Flawil, Switzerland) with a 450 µm nozzle size. Prepared particles were followed hardening for 30 min in 1 % (w/v) calcium chloride solution.

2.5. Determination of particle stability and viability of probiotic cells

In prepared particles long-term stability in model acidic conditions was evaluated. As acid conditions was used 2.5 % (w/v) citric acid solution. Five gram of microcapsules was transferred into test tubes containing 50 mL of sterile acid solution and samples were incubated for 1, 3 and 6 week at 5 °C. Mass fractions of released

carbohydrates and cells were determined in regular intervals. Also the viability of encapsulated probiotic cells was during storage performed by light/fluorescence microscopy (Labomed Lx500, Labomed Inc., Los Angeles, CA, USA) using methylene blue or propidium iodide.

Stability of the prepared particles was also tested under physiological conditions. Artificial stomach juice was prepared from 0.25 g of pepsin dissolved in 100 mL of distilled water. To this solution 0.84 mL of 35 % hydrochloric acid was added. Final pH was adjusted to 0.9. Artificial pancreatic fluid was prepared with 0.25 g of pancreatin and 1.5 g of sodium hydrogen carbonate in 100 mL of water (pH=8.9). Bile fluid was composed of 0.8 g of bile acid salts dissolved in 200 mL of phosphate buffer. Incubation of particles was performed at 37°C for 20 min in stomach fluid and pancreatic juice and for 40 or 70 min in bile fluid. After incubation, the mass fractions of carbohydrates and cells released from particles were measured. The viability of probiotic cells during simulated gastrointestinal conditions was performed using microscopy and flow cytometry.

3. RESULTS AND DISCUSSION

3.1. Characterization of prebiotics

In this study content of carbohydrate from selected prebiotics was observed. Mainly content of total carbohydratetes, content of reducing carbohydrates and content of fructose and glucose were measured as described in Materials and Methods. The highest content of total carbohydrates was determined in psyllium, conversely the least content of carbohydrates was detected in hemp fiber (**Table 1**).

Table 1 Quantity of total and reducing carbohydrates, glucose and fructose in the testing prebiotics

sample	total carbohydrates (mg/g)	reducing carbohydrates (mg/g)	glucose (mg/g)	fructose (mg/g)
inulin	535.68±15.23	57.17±1.92	21.12±1.92	5.81±0.51
psyllium	961.47±37.54	72.23±2.33	1.29±0.32	0.57±0.00
apple fiber	97.55±4.63	59.25±1.42	2.11±0.20	0.59±0.05
hemp fiber	30.7±1.56	30.51±1.05	8.09±0.75	2.07±0.33

3.2. Probiotics cultivation with or without additions of prebiotics

Table 2 Comparison of growth of probiotic microorganisms after 24 h cultivation with addition of different types of prebiotics

composition of culture medium	LA		BB	
	number of cells (cells/µl)	live cells (%)	number of cells (cells/µl)	live cells (%)
MRS medium	66680	95	49390	96
MRS medium + 100 mg/ml inulin	55740	100	45840	98
MRS medium + 100 mg/m apple fiber	79090	98	44770	93
MRS medium+ 100 mg/m hemp fiber	116420	98	43600	96
MRS medium + 10 mg/ml inulin	61513	99	49495	100
MRS medium + 10 mg/m apple fiber	54007	98	47236	97
MRS medium+ 10 mg/m hemp fiber	62781	98	50159	96

Prebiotics can be defined as non-digestible food ingredients that, when consumed in sufficient amounts, have health benefits. [4] In this work was evaluated the effect of four selected prebiotics (inulin, psyllium, apple fiber and hemp fiber) on growth and viability of probiotic bacteria. The probiotics were cultivated on medium with or

without prebiotics and number of grown cells was observed. Also the viability of cells was measured. Results indicated significant improvement primarily in grown of strains Lactobacillus acidophilus on hemp fiber (**Table 2**).

3.3. Microcapsules morphology

For co-encapsulation of probiotic cells with prebiotics alginate was used. The capsules were prepared by the method based on the principle of gelation and cross-linking polymers.

Figure 1 Alginate particles with co-encapsulation probiotics and prebiotics: A-particles with apple fiber, B-particles with psyllium, C-particles with inulin

Then the physicochemical evaluation of microcapsules and the viability of probiotics during simulated gastrointestinal conditions and during storage were observed.

3.4. Survival of cells in simulated gastrointestinal conditions

Encapsulated probiotic cells were exposed to model gastrointestinal conditions, *i.e.* artificial stomach, pancreatic and bile juices. The capsules were added to the model fluids and incubated as described in Materials and Methods. After incubation, samples were taken to determine the mass fraction of released carbohydrate and probiotic cells. Also viability of encapsulated and released cells was measured.

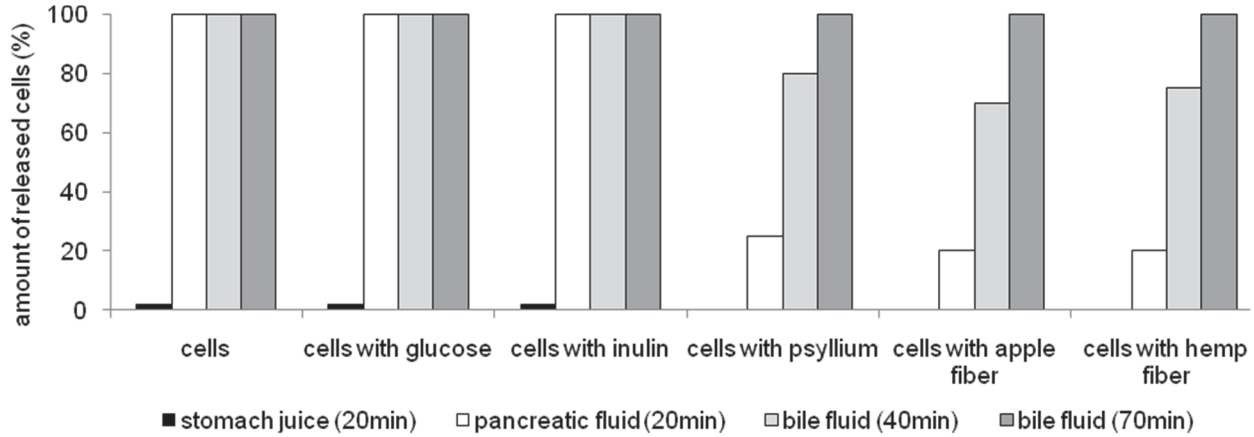

Figure 2 Amount of released components from particles with co-encapsulation probiotics and prebiotics during passage through the simulated gastrointestinal conditions

Particles are able to maintain their integrity during passage through the gastrointestinal tract until they reach their target destination (**Figure 2**), where they break down and release prebiotics and probiotic bacteria. Also the viability of probiotic cells was retained until the target destination. The slow and gradual release of probiotic cells in the intestine environment was primarily detected from particle containing psyllium, apple fiber and hemp fiber.

3.5. Determination of particle stability and viability of probiotic cells during storage

Effect of addition of different prebiotics on viability of probiotic cells during storage was investigated. Effect of prebiotic concentrations in improving the stability of microcapsules and viability of cells was measured as well.

This challenge is investigate in many studies. In this context has been mostly the microencapsulation techniques used. But identification of the proper encapsulating or cell protecting material for different probiotics is a key issue that determines the efficacy of the process. Also, there is increasing interest in the use of combination probiotic and prebiotic due to that when probiotics reach to colon, they could use the prebiotics for survival and implantation that beneficially affect the host. [5]

Table 3 Amount of live probiotic cells in alginate microcapsules during storage

type of particles	live cells (%)					
	LA	BB	LA	BB	LA	BB
	1 week		3 weeks		6 weeks	
cells	95	95	80	80	70	70
cells with glucose	97	100	85	92	75	85
cells with inulin	97	100	90	95	80	90
cells with psyllium	90	97	75	90	65	80
cells with hemp fiber	95	97	90	80	80	75

In all of tested alginate particles with co-encapsulated probiotics with prebiotics viability of cells was measured. After 1 week only 3 % to 10 % of dead cells were detected and about 10 % to 35 % dead cells were found after 6 week storage in model condition (**Table 3**). The highest number of living cells was determined for strains *Bifidobacterium breve* in particles with inulin, conversely the highest viability of *Lactobacillus acidophilus* cells was observed in the particles with hemp fiber. Growth of cells inside particles was observed as well. The highest growth of cells was observed for both of tested strains in particles with hemp fiber (**Figure 3**).

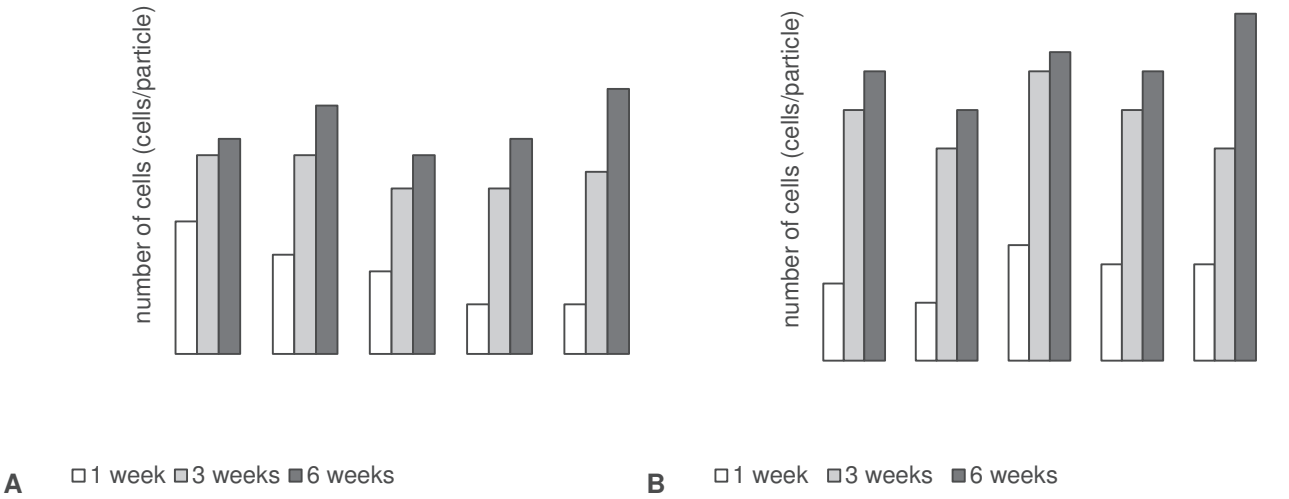

Figure 3 Amount of growth probiotic cells in alginate microcapsules during storage, A) LA=*Lactobacilllus acidophilus*, B) BB=*Bifodobacterium breve*

Moreover, in all of prepared particles with co-encapsulated cells only about 5 % of released cells or prebiotics were detected after 6 week storage in model acidic conditions.

Also co-encapsulation of probiotic cells with antioxidant and another components from natural sources could be positive effect to viability of cells during storage and during digestion or could also provide synergistic health effects. [7] The majority of studies, as well as this study, reported using moist microcapsules. But also the drying of the microcapsules is an important factor. There are various advantages to drying the microcapsules including an improvement in the storage properties and the ease in use. For exaple, it was demonstrated that the process of microencapsulation in alginate microcapsules followed by freeze drying was efficient in protecting the probiotics in their storage and passage through gastrointestinal fluids. [3]

4. CONCLUSION

It can be concluded that the co-encapsulation of probiotics with prebiotics into alginate particles leads to increased tolerance of bacteria to acidic environment. Mainly the hemp fiber exhibited the highest increase of number of probiotics cells and also it contributed to the high viability of encapsulated bacterial cells during long-term storage in model acidic conditions. Particles also maintained their integrity and also viability of probiotic strains during passage through the stomach. Therefore, these particles could be used for targeted transport of probiotic bacteria.

ACKNOWLEDGEMENTS

This work was supported by project "Materials Research Centre - Sustainability and Development" No. LO1211 (Ministry of Education, Youth and Sport of the Czech Republic).

REFERENCES

[1] PEREDO, A.G., C.I. BERISTAIN, L.A. PASCUAL, E. AZUARA a M. JIMENEZ. The effect of prebiotics on the viability of encapsulated probiotic bacteria. *LWT - Food Science and Technology,* 2016, vol. 73, pp. 191-196.

[2] PINTO S. S., VERRUCK S., R.W. VIEIRA C., PRUDÊNCIO E.S, AMANTE E.R., D.M.C. AMBONI R. Influence of microencapsulation with sweet whey and prebiotics on the survival of Bifidobacterium-BB-12 under simulated gastrointestinal conditions and heat treatments. *LWT - Food Science and Technology*, 2015, vol. 64, no. 2, pp. 1004-1009.

[3] HOLKEM A.T., RADDATZ G.C., NUNES G.L., CICHOSKI A.J., JACOB-LOPES E., FERREIRA GROSSO C.R., DE MENEZES C.R. Development and characterization of alginate microcapsules containing Bifidobacterium BB-12 produced by emulsification/internal gelation followed by freeze drying. *LWT - Food Science and Technology*, 2016, vol. 71, pp. 302-308.

[4] BURGAIN, J., GAIANI C., LINDER M., SCHER J. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering*, 2011, vol. 104, no. 4, pp. 467-483.

[5] TRIPATHI M.K., GIRI S.K. Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, 2014, vol. 9, pp. 225-241.

[6] SHORI, A.B. The potential applications of probiotics on dairy and non-dairy foods focusing on viability during storage. *Biocatalysis and Agricultural Biotechnology*, 2015, vol. 4, no. 4, pp. 423-431.

[7] GAUDREAU, H., CHAMPAGNE C.P., REMONDETTO G.E., GOMAA A., SUBIRADE M. Co-encapsulation of Lactobacillus helveticus cells and green tea extract: Influence on cell survival in simulated gastrointestinal conditions. *Journal of Functional Foods*, 2016, vol. 26, pp. 451-459.

[8] BEKHIT M., ÁNCHEZ-GONZÁLEZ L., BEN MESSAOUD G., DESOBRY S. Encapsulation of Lactococcus lactis subsp. lactis on alginate/pectin composite microbeads: Effect of matrix composition on bacterial survival and nisin release. *Journal of Food Engineering,* 2016, vol. 180, pp. 1-9.

[9] MATOUŠKOVÁ, P, et al. Co-encapsulation of probiotic with prebiotics into polysacharide particles and its effect on viability in simulated gastrointestinal fluid. NANOCON 2014 6th International Conference, 2015, pp. 563-568.