MICROENCAPSULATION OF *LACTOBACILLUS FERMENTUM* 39-183 BY SPRAY DRYING IN THE PRESENCE OF PREBIOTICS

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Received: 11 October 2017; Accepted for publication: 12 December 2017

ABSTRACT

The objective of this study was to evaluate the effect of prebiotic substrates on the growth of *Lactobacillus fermentum* 39-183 and to investigate the utilization of these prebiotic substrates as coating materials for microencapsulation. The cell growth of *L. fermentum* 39-183 was significantly increased in the presence of fructooligosaccharide, galactooligosaccharide, lactulose and inulin. The microencapsulation of *L. fermentum* 39-183 cells was carried out by spray drying process using the selected prebiotic substrates and the enteric coating material, whey protein. Scanning and transmission electron microscopy revealed that the particles showed a spherical shape and various sizes between 9.17 and 10.37 µm. During the storage up to 75 days at 25 ºC, powders consisting of WP and FOS afforded better protection to probiotic lactobacilli. Survival rate of *L. fermentum* 39-183 in powder A2 (WP+FOS) was higher than 50% when compared to the powder produced only with whey protein (A1). Using whey as a bacterial substrate and the health benefits associated with both prebiotics and probiotics, it is possible that these powders could be tailored for use in functional food applications.

Keywords: Fructooligosaccharide, galactooligosaccharide, inulin, microencapsulation, probiotics.

1. INTRODUCTION

Probiotics are defined as living microorganisms that contribute to beneficial effects on human health upon ingested in adequate dose [1]. *Lactobacillus* is one of the most commonly studied genus of probiotic bacteria. These bacteria have been found to offer some health benefits include improvement in intestinal disorders and lactose intolerance, altered vitamin content of milk, antagonism against various pathogenic organisms including anti-mutagenic and anti-carcinogenic activities [2, 3]. To exert their benefits, there should be a minimum level of 10^7 CFU probiotic bacteria per ml or g food at the time of consumption. Therefore, these organisms must survive to gastric acid and bile toxicity and enzymes conditions of the human upper gastrointestinal tract. Additionally, probiotic bacteria must survive the adverse conditions, e.g. the presence of acid, oxygen exposure and high temperature during manufacturing, packaging and storage of food [4].

Microencapsulation of probiotic bacteria is currently drawing more and more attention for being a method to improve the stability of probiotic organisms in functional food products [5]. According to Ding and Shah (2007), microencapsulation may improve the survival of these microorganisms, during both processing and storage, and also during

passage through the human gastrointestinal tract [6]. Spray drying is regarded as a microencapsulation method and it has been investigated as a means of stabilizing probiotic bacteria in a number of food matrices, most often composed of proteins, polysaccharides, sugars, and combinations thereof. Spray dried probiotic formulations in various carrier materials have been carried out [7]. Doherty *et al.* (2012) suggested that the presence of a protein in a formulation protects the probiotic against acid stress of the gastrointestinal tract [8]. Whey proteins also have been used by several authors once its use in the micro-particles both in matrix and as a coating agent can promote protection for probiotic microorganisms during gastrointestinal transit [4]. Another approach to increase the viability of probiotic is the use of prebiotics, which are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of bacteria in the colon. According to Corcoran *et al*. (2004), these prebiotics may potentially be exploited as carrier media for spray drying and may be useful for enhancing probiotic survival during processing [9].

In our preliminary studies, 2-hour exposure to simulated gastric juice at 37 ºC (3.5g D-glucose; 2.05 g NaCl; 0.6 g KH_2PO_4; 0.11g $CaCl_2$; 0.37 g KCl; 0.05 g ox-gall bile; 1.33 g L^{-1} pepsin; pH 2.0) resulted in poor survival of *L. fermentum* 39-183, 3.29 ± 0.02 log CFU g^{-1} with 8.67 ± 0.15 log CFU g^{-1} as initial cell count, while with 24-hour continuous exposition to bile salts in pH 6.8 (30 g L^{-1} bile salts), the survival percentage of the probiotic was 27.69 ± 1.14. Considering that, providing *L. fermentum* with a physical barrier against adverse environmental conditions could significantly improve their stability thus ensuring its health effects. Therefore, the aim of this study was to evaluate the effect of partial replacement of whey protein (WP) with prebiotic agents (Inulin, FOS, GOS and Lactulose) on the viability of *L. fermentum* 39-183 after spray drying and viability during storage time as well as to characterize the microcapsules in relation to their physical properties.

2. MATERIALS AND METHODS

2.1. Bacteria and media

Microorganism used in this study was *L. fermentum* 39-183 from our previous research [10].The stock culture was maintained at – 20 ºC in De Man-Rogosa Sharpe (MRS), using 40% (v/v) glycerol as a cryoprotectant. Prior to use, the culture [1% (v/v)] was transferred twice to MRS broth and incubated at 37 ºC for 18 - 20 hours. The encapsulating agents used were commercial whey protein (Molico®, Nestlé, São Paulo, Brazil) and the prebiotic agents Fructooligosaccharide (FOS), Galactooligosaccharide (GOS), Inulin and Lactulose (Sigma-Aldrich, St. Louis, MO, USA). All the reagents were of analytical grade.

2.2. Effect of prebiotic substrates on the growth of *Lactobacillus fermentum* 39-183

Four types of commercially available carbohydrates: Inulin, fructooligosaccharide (FOS), galactooligosaccharide (GOS) and lactulose were used. Incubated 18 - 20 h culture of *L. fermentum* 39-183 were centrifuged at 4000 rpm for 20 min. Ten ml of each basal MRS medium without glucose and beef extract containing various concentrations (0.5%, 1.0%, 1.5% and 2.0%) of each prebiotic substrate in sterile test tubes and tubes were inoculated with active culture containing approximately 8.70 log CFU mL^{-1}. Medium containing and without glucose as sole energy source was taken as positive and negative control, respectively. The inoculated tubes were incubated aerobically at 37 ºC for 24 hours. The viable counts of *L. fermentum* were determined at an interval of 0, 8, 16, 20, and 24 hours by the standard plate method on MRS agar.

2.3. Microencapsultion by spray drying

Three feed solutions were prepared following the procedures described by Ananta *et al*. (2005), with modifications [11]. Whey protein (WP) at a concentration of 50 g L^{-1} was used as the control medium. The WP - prebiotic mixtures were prepared of WP (5%, w/v) and each of the two selected prebiotics (FOS and GOS) (1.5%, w/v). All the media were homogenized into sterile distilled water and heat treated at 80 ºC for 20 min.

The organism was grown and propagated three times successively for activation at 37 ºC for 18 - 20 hours in MRS broth. The cells of *L. fermentum* 39-183 were concentrated by centrifugation of the broth at 4,000 rpm at 4 ºC for 20 min, and the cell pellet was washed twice with 0.85% of sterilized saline solution [12]. The cell pellet was then re-suspended in one fourth of the original volume (5 mL of cell pellet was added to 15 mL of saline solution). The initial population of bacteria in the suspension was 11.88 ± 0.05 log CFU mL^{-1}

The spray drying process was performed with spray dried using a laboratory scale 06AG spray dryer (SD-06AG, Labplant, UK) at constant air inlet temperature of 110 ± 2 ºC and outlet temperature of 65 ± 3 ºC. The flow rate of the spray-drying process was 5.5 mL min^{-1}. The feed solutions containing *L. fermentum* 39-183, WP and/or prebiotics were continuously stirred using magnetic stirrer immediately before feeding into spray drier to maintain homogeneity at room temperature. After completion of spray drying, the dried powder samples were collected from the base of the cyclone and thoroughly mixed with a spatula. The samples were placed in sealed polythene bags and stored at 4 ºC and 25 ºC.

2.4. Survival of microencapsulated *Lactobacillus* after spray drying and viability during storage time

In order to determine the effect of prebiotics on the microencapsulated *L. fermentum* 39-183 after spray drying and during storage, the samples were placed in sealed polythene bags and were stored at 4 and 25 ºC for 75 days, respectively. After the designated storage periods (0, 15, 30, 45, 60 and 75 days), samples were taken and cell survival rates were determined using standard plating techniques on MRS agar. One gram of particles was suspended in 9 mL phosphate buffer solution (pH 7.0) homogenized for 5 min at room temperature. Measurements of viable cells in the suspensions were determined using plate-count method on selective MRS agar with incubation at 37 ºC under aerobic conditions for 48 hours. The average of the results obtained in triplicates was expressed as colony forming units per gram or ml of sample (CFU g^{-1} or CFU mL^{-1}) using following equation [13].

$$a = \frac{a_n \times C_n}{V}$$

Where, a is the number of colonies expressed as CFU mL^{-1}, a_n is the number of colonies determined at dilution n, V is the volume of the sample in mL and C_n is reciprocic value of the dilution n. Afterwards, results calculated in CFU g^{-1} were transformed to log CFU g^{-1} to make the reading to the paper more friendly.

2.5. Physical properties of the microcapsules

The morphology and particle size of the microcapsules were observed with a Jeol scanning electron microscope model JSM 6390 LV (Jeol, Tokyo, Japan) at an accelerating voltage of 10 and 15 kV. Before using the scanning electron microscope (SEM), the samples were placed on a piece of adhesive paper and were coated with gold with a vacuum sputtering coater (Leica, model EM SCD 500, Wetzlar, Germany), as described by Lian *et al*. (2002). To

calculate their diameter, at least 120 particles from each of the different formulations of microcapsules were measured [14].

The moisture content of the spray dried powders was determined through oven drying at 105 ºC until reaching constant weight, according to the International Dairy Federation [15].

2.6. Statistical analysis

All experiments in the present study were carried out in triplicates and the results indicate their mean values. For statistical analysis, the standard errors of the means were calculated and the means with a significant difference ($p < 0.05$) were compared using Duncan Multiple Range Test in Statgraphics Centurion XV.

3. RESULTS AND DISCUSSION

3.1. Effect of prebiotics on the growth of *L. fermentum* 39-183

Lactobacilli preferentially degraded short – or medium – chain oligosaccharides and used for cell growth, rather than long – chain inulin [16]. Therefore, we evaluate the in-vitro growth of *Lactobacillus fermentum* 39-183 in various concentrations of four different oligosaccharide substrates in order to characterize for microencapsulation. The effects of prebiotic type and the prebiotic concentration on the growth of *Lactobacillus fermentum* 39-183 were found to be significant ($P < 0.05$, Table 1).

The initial viable cell numbers of this strain were between 8.53 and 8.72 log CFU mL^1. After the incubation with prebiotics, the increase in the viable cell numbers with prebiotics varied within the range of 0.87 - 2.72 log CFU mL^{-1}. The values after the incubation were 8.66 and 10.78 log CFU mL^{-1} for negative and positive controls, respectively. Based on the counts of viable cells, it was found that the most fermented prebiotic was FOS, followed by GOS, lactulose and inulin. It can be stated that the growth of *L. fermentum* 39-183 in this study was stimulated by all prebiotics tested. However, a statistically significant difference was observed on particular prebiotics (Table 1) with the best growth on FOS at 2% concentration. FOS and GOS enhanced the growth of this strain much more when they were compared with 2% glucose (positive control). These results were consistent with the observations of Rycroft (2001) and Ignatova – Ivanova Ts. (2010), both of them noted that the presence of FOS and GOS contributes to higher growth rate of *L. fermentum* [17, 18]. These two prebiotic substrates were consequently selected for further study, as candidates for the co-encapsulation of *L. fermentum* 38-193.

Table 1. Cell growth of *Lactobacillus fermentum* 39-183 incubated at 37 ºC for 24 hours in MRS broth containing various prebiotic substrates

Prebiotics/ Concentration (%)		Viable cell numbers (log CFU/mL)				
		0h	8h	16h	20h	24h
Negative control		8.56 ± 0.13^{b}	8.61 ± 0.48^{b}	8.63 ± 0.34^{d}	8.64 ± 0.22^{d}	8.66 ± 0.34^{d}
Positive control		8.70 ± 0.32^{a}	9.39 ± 0.22^{ab}	10.16 ± 0.13^{ab}	10.48 ± 0.12^{b}	10.78 ± 0.31^{b}
FOS	0.5	8.72 ± 0.33^{a}	9.34 ± 0.36^{ab}	9.98 ± 0.15^{b}	10.25 ± 0.33^{bc}	10.50 ± 0.35^{b}
	1.0	8.72 ± 0.14^{a}	9.44 ± 0.13^{ab}	10.30 ± 0.22^{b}	10.67 ± 0.24^{ab}	11.01 ± 0.32^{ab}
	1.5	8.72 ± 0.43^{a}	9.55 ± 0.33^{a}	10.50 ± 0.11^{a}	10.92 ± 0.25^{a}	11.30 ± 0.24^{a}
	2.0	8.72 ± 0.35^{a}	9.59 ± 0.24^{a}	10.59 ± 0.40^{a}	11.02 ± 0.25^{a}	11.41 ± 0.14^{a}

Prebiotics/ Concentration (%)		Viable cell numbers (log CFU/mL)				
		0h	8h	16h	20h	24h
Inulin	0.5	8.67 ± 0.13^{a}	9.08 ± 0.22^{b}	9.54 ± 0.45^{a}	9.73 ± 0.08^{c}	9.90 ± 0.18^{c}
	1.0	8.67 ± 0.38^{a}	9.29 ± 0.32^{ab}	9.93 ± 0.37^{b}	10.20 ± 0.09^{bc}	10.45 ± 0.09^{b}
	1.5	8.67 ± 0.23^{a}	9.35 ± 0.31^{ab}	10.10 ± 0.28^{ab}	10.42 ± 0.19^{b}	10.71 ± 0.13^{b}
	2.0	8.67 ± 0.16^{a}	9.39 ± 0.39^{ab}	10.21 ± 0.46^{ab}	10.55 ± 0.31^{ab}	10.86 ± 0.26^{b}
GOS	0.5	8.62 ± 0.19^{b}	9.22 ± 0.31^{ab}	9.84 ± 0.33^{b}	10.10 ± 0.2^{bc}	10.34 ± 0.35^{bc}
	1.0	8.62 ± 0.24^{b}	9.30 ± 0.13^{ab}	10.05 ± 0.13^{b}	10.36 ± 0.41^{b}	10.66 ± 0.21^{b}
	1.5	8.62 ± 0.32^{b}	9.38 ± 0.21^{ab}	10.24 ± 0.42^{ab}	10.61 ± 0.32^{ab}	10.95 ± 0.25^{ab}
	2.0	8.62 ± 0.37^{b}	9.42 ± 0.20^{ab}	10.34 ± 0.40^{ab}	10.74 ± 0.45^{ab}	11.00 ± 0.42^{ab}
Lactulose	0.5	8.53 ± 0.13^{b}	8.92 ± 0.27^{b}	9.27 ± 0.25^{c}	9.35 ± 0.28^{cd}	9.40 ± 0.44^{c}
	1.0	8.53 ± 0.41^{b}	9.12 ± 0.30^{ab}	9.69 ± 0.19^{bc}	9.89 ± 0.29^{c}	10.09 ± 0.32^{bc}
	1.5	8.53 ± 0.43^{b}	9.15 ± 0.29^{ab}	9.80 ± 0.06^{bc}	10.08 ± 0.39^{bc}	10.34 ± 0.20^{bc}
	2.0	8.53 ± 0.13^{b}	9.19 ± 0.12^{ab}	9.90 ± 0.24^{b}	10.20 ± 0.29^{bc}	10.50 ± 0.32^{b}

Data expressed as mean of 3 replicates ± standard error. Means in the same column showing the same letters are not significantly different ($p < 0.05$).

3.2. Survival of microencapsulated *L. fermentum* after spray drying and viability during storage time

Based on our preliminary experiments, the optimal conditions for microencapsulation of *L. fermentum* 39-183 with whey protein (5%) using spray drying process were air inlet temperature of 110 ± 2 ºC, outlet temperature of 65 ± 2 ºC and feed flow of 5.5 mL min^{-1}. Survival of microencapsulated *L. fermentum* 39-183 after spray drying and throughout storage at 4 ºC and 25 ºC is shown on Figure 1(A) and (B), respectively.

The initial viable cell numbers of this strain were between 10.18 - 10.22 log CFU mL^{-1}. After spray drying process, survival rate of *L. fermentum* 39-183 in powders consisting of whey protein (5%, w/v), whey protein (5%, w/v) and FOS (1.5%, w/v), whey protein (5%, w/v) and GOS (1.5%, w/v) was 89.88%, 93.52% and 91.86%, respectively. The gradual decrease of viable cells was when the storage temperature and storage time were increased. According to Ananta *et al*. (2005), since the effectiveness of probiotic consumption on human health is related to their viability, it is of almost importance to not only minimize cell death during the spray drying process but also to ensure minimal loss of viability of the microencapsulated bacteria during storage [11]. After 75 days of storage at 4 ºC, survival rate of powders was higher than 35%. The highest loss was observed in powder produced with Whey protein with 886.000 fold reduction in cell viability. This powder rapidly decreased in viability after 75 days of storage at 25 ºC, surviving to approximate 0.73 CFU g^{-1}. All spray dried microcapsules containing *L. fermentum* showed survival for a period of up to 75 days of storage at both temperatures. The powders produced with Whey protein and FOS (A2), whey protein and GOS (A3) showed higher ($P < 0.05$) initial count (after spray drying), when compared to the powder produced only with whey protein (A1). This fact suggests that FOS and GOS had a positive effect on the protection of *L. fermentum* during the encapsulation process, probably because it acted as a thermo-protector for the cells undergoing the drying process. Lian *et al*. (2002) reported that besides difference in chemical characteristics, the

encapsulating agents have different physical properties [18]. Therefore, it is reasonable to expect that these agents tested in this study may exert different degrees of protective effect on the entrapped cells of a test organism when subjected to heat inactivation during spray drying and, thus, take survival of *L. fermentum* to a different level.

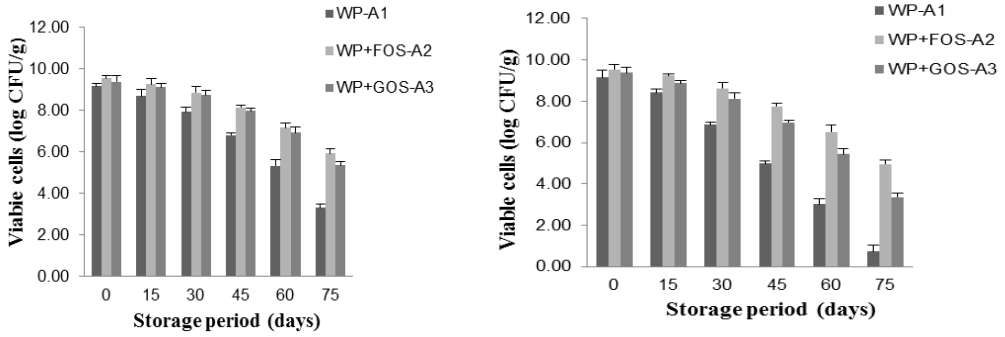


Figure 1. Viability of encapsulated *L. fermentum* 39-183 after spray drying and during cold storage for 75 days of microparticles obtained by spray-drying method and whey protein particles using protective agents, fructooligosaccharide (FOS) and galactooligosaccharide (GOS) at 4 ºC (A) and 25 ºC (B). Values are presented as averages (n = 3) ± standard error.

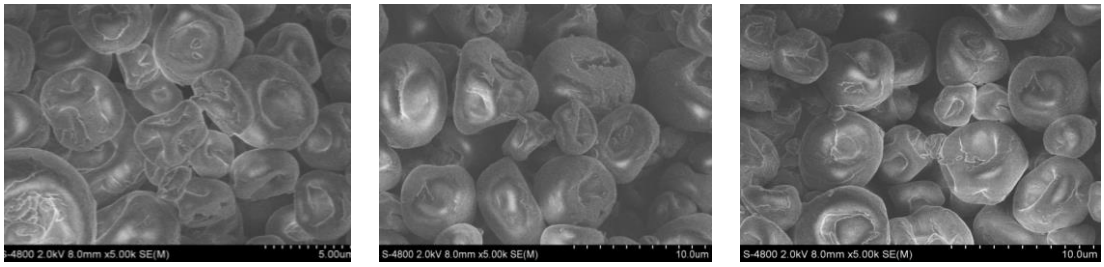
After 75 days of storage, at temperature 25 ºC, the powder A2 was found to exhibit higher survival rates than the other powders ($P < 0.05$). Survival rate of powder A2 was higher than 50%, surviving to approximate 4.96 log CFU g^{-1}, while during the same time-frame, 64% reduction in probiotic viability was observed in powder A3. These results were consistent with the observations of Teixeira *et al*. (1995a), Gardiner *et al*. (2000) both of whom noted that temperature is critical for microbial survival during storage, and higher survival rates have been maintained at lower storage temperature, which restricts the possible applications for many probiotic products [19, 20]. Therefore, the improved survival rates observed in the bacteria microencapsulated with prebiotic substrates suggest that these preparations may be used to improve cell viability during ambient storage conditions. *L. fermentum* 39-183 appears to lend itself well to the spray-drying process and was relatively stable during powder storage in comparison with other strains of probiotic lactobacilli studied. Exploiting whey as a bacterial substrate and encapsulation matrix within spray-drying process offers an efficient option for industrial production of vital probiotic. Powders consisting of WP and FOS afforded better protection to probiotic lactobacilli during storage than WP/GOS combinations and WP alone.

3.3. Physical properties of the microcapsules

Figure 2 shows the SEM micrographs of the *L. fermentum* 39-183 microcapsules produced with different encapsulating agents. The SEM revealed the absence of free bacteria confirming the formation of microcapsules, for all the encapsulating agents. The particles showed a spherical shape and various sizes, with concavities typical of materials produced by spray drying. The control particle (A1), produced with whey protein, and the particles produced with prebiotics (A2 and A3) showed similar morphologies, thus indicating that the encapsulating agents did not affect morphology. According to Saénz *et al*. (2009), the formation of concavities in the surface of atomized particles can be attributed to the shrinkage of the particles during the drying process because of the rapid evaporation of the liquid drops [21]. The external surfaces showed walls free of fissures or disruptions, which is fundamental for guaranteeing higher protection and lower permeability of gases. Similar results were noted by Desmond *et al*. (2001), Rodríguez- Huezo *et al*. (2007) in

Lactobacillus paracasei spray dried with RSM and in *Bifidobacterium bifidum* spray dried with aguamiel (a kind of prebiotic), respectively [22, 23].

The microcapsules were of assorted sizes, between 9.17 and 10.37 μm (Table 2). Such values are expected for powders obtained through spray drying, which may vary from 5 to 50 μm. However, the capsules produced in this study showed a smaller particle size ($P < 0.05$) than the other powders described by Fang and Bhandari (2010), the molar mass of FOS, GOS is lower, has a small DP, ranging from 2 to 8, therefore, showing shorter chains [24]. The powder produced only with whey protein (A1) showed higher ($P < 0.05$) moisture content than the powders produced with the prebiotics (Table 2). However, our results were higher than the results were observed by Corcoran *et al.* (2004) in microcapsules of *Lactobacillus rhamnosus* GG spray dried with skim milk (RSM) and polydextrose (PD) [9]. According to Simpson (2005) and Tonon (2009) to guarantee microbiological stability, i.e., the moisture content of powders is equal to or smaller than 4 %, is very positive for powder stability since it represents less free water available for biochemical reactions and hence longer shelf life [5, 25].



A B C

Figure 2. SEM micrographs of *L. fermentum* 39-183 spray-dried microcapsules with different encapsulating agents
A = Whey protein, B = Whey protein and FOS, C = Whey protein and GOS.

Table 2. Properties of the *Lactobacillus fermentum* 39-183 microcapsules produced with different prebiotic substrates

Properties	Microcapsules		
	A1	A2	A3
Particle size (μm)	10.37 ± 6.61	9.17 ± 6.55	9.28 ± 7.48
Moisture (g $100g^{-1}$)	8.13 ± 0.03	8.08 ± 0.04	8.01 ± 0.04

Data expressed as mean of 3 replicates ± standard error.

A1: microcapsules with Whey protein.

A2: microcapsules with Whey protein and FOS.

A3: microcapsules with Whey protein and GOS.

4. CONCLUSION

In conclusion, we found that the effective preservation of the probiotic cells applying spray drying as encapsulation methods and appropriate selection of prebiotic materials provided the survival rate of microencapsulated *L. fermentum* 39-183 in whey protein microparticles to be above the therapeutic level within 2 and 3 months of cold storage, respectively. Furthermore, probiotic cultures retained good viability during storage in powders containing WP/prebiotics at 4 ºC and 25 ºC. Powders consisting of WP and FOS afforded better protection to probiotic *L. fermentum* 39-183 during storage than WP/GOS

combinations and WP alone. Storage survival was affected by appropriate selection of prebiotic materials with FOS (2%) best, followed by GOS. *L. fermentum* 39-183 appears to lend itself well to the spray-drying process and was relatively stable during powder storage in comparison with other strains of probiotic lactobacilli studied. Given the broad applicability of whey protein powders and the health benefits associated with both prebiotics and probiotics, it is possible that these powders are potential candidates to be further used as functional ingredients in pharmaceutical and food industry.

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TÓM TẮT

VI GÓI VI KHUẨN *LACTOBACILLUS FERMENTUM* 39-183 BẰNG PHƯƠNG PHÁP SẤY PHUN VỚI SỰ CÓ MẶT CỦA CÁC PREBIOTIC

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Mục tiêu của nghiên cứu là đánh giá ảnh hưởng của các prebiotic đến sự phát triển vi khuẩn *Lactobacillus fermentum* 39-183 và hiệu quả bảo vệ vi khuẩn trong quá trình vi gói. Sự phát triển của vi khuẩn *Lactobacillus fermentum* 39-183 gia tăng đáng kể khi có mặt của các prebiotic: fructooligosaccharide, galactooligosaccharide, lactulose và inulin. Vi khuẩn *Lactobacillus fermentum* 39-183 được vi gói bằng phương pháp sấy phun với vật liệu Whey protein. Kết quả chụp SEM cho thấy, vi hạt hình cầu và kích thước khác nhau trong khoảng 9.17 đến 10.37 µm. khi bảo quản ở 25 ^{o}C trong 75 ngày, vi hạt chứa WP và FOS bảo vệ vi khuẩn probiotic lactobacilli tốt hơn. Tỷ lệ vi khuẩn *L. fermentum* 39-183 sống trong mẫu A2 (WP + FOS) cao hơn đến 50% khi so sánh với mẫu chỉ sản xuất với whey protein (A1). Việc sử dụng whey làm chất nền và lợi ích về sức khoẻ của prebiotic và probiotic, các chế phẩm bột này có thể được điều chỉnh để sử dụng trong các ứng dụng thực phẩm chức năng.

Từ khóa: Fructooligosaccharide, galactooligosaccharide, inulin, probiotics, vi gói.