Biotechnology and Bioprocess Engineering 25: 287-292 (2020)
DOI 10.1007/s12257-019-0406-x

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Evaluating the Cryoprotective Encapsulation of the Lactic Acid Bacteria in Simulated Gastrointestinal Conditions

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Received: 30 October 2019 / Revised: 24 December 2019 / Accepted: 1 January 2020

Abstract To expect the positive physiological functions from probiotic consumption, the lactic acid bacteria should survive, partially, in the gastrointestinal (GI) tract. This study aimed to evaluate the viable stability of probiotic bacterial cells (*Lactobacillus plantarum* MG989, *L. fermentum* MG901, *Streptococcus thermophilus* MG5140, *Lactococcus lactis* MG5125, and *Enterococcus faecium* MG89-2) that were coated with sodium alginate and pumpkin powder (SP) by comparing their resistance in simulated intestinal fluid (with pancreatin) and simulated gastric fluid (with pepsin) *in vitro* with that of the non-coated free cells. The viable stability was determined by counting cells with colony forming unit (CFU) from agar plate culture of SP coated and non-coated free cells in simulated GI conditions. Survival rate enhanced up to 28.7% and 14.0% in the condition of simulated gastric fluid and simulated intestinal fluid, respectively. The results showed that the SP coated cells exhibited considerably greater resistance to the simulated gastric fluid than the activated cells ($p < 0.001$), showing that the SP coating may enhance the survival of probiotic bacteria after consumption during their transit through the GI tract after freeze-drying process.

Keywords: probiotic, sodium alginate, pumpkin, gastrointestinal tract

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1. Introduction

Probiotics are defined as living microorganisms which when administered in adequate amounts, confer health benefits to the host [1]. Probiotics contribute toward maintaining the balance of the host's intestinal microbiota [2-4], and have provided various curative effects in the form of diarrhea prevention, immune system stimulation, intestinal microflora balance, lactose intolerance, anticarcinogenic properties, and neurological diseases [5-13]. Besides the therapeutic health benefits of probiotics in food, commercial interests are also rising for cosmetic and pharmaceutical applications [14]. According to Global Market Insights, Inc., the global market for probiotic supplements is increasing and is predicted to surpass three billion USD by 2024 (Global Market Insights, accessed on 15 October 2019).

Positive physiological functions could be expected through the intake of appropriate amounts of viable probiotics. The viability of probiotics at the time of consumption should be at least 10^7 CFU/g to survive the gastrointestinal tract environment and exert their positive physiological functions on the human body [8,15-17]. Thus, a key characteristic of probiotics is that they should be able to survive in the gastrointestinal tract after processing and product intake [18]. However, improving the probiotics viability remains a challenge during food processing and long-term storage [19].

Among the various cell preservation methods, freeze-drying is a well-known dehydration process in the food and dairy industry. Lyophilized or freeze-dried bacteria have been used for many decades, and freeze-drying methods have proven to be the most convenient and effective means

of bacteria preservation [20,21]. However, freeze-drying requires an adequate stabilization processes to avoid cell membrane damage or death [22]. Thus, manufacturing and maintenance methods have been adopted to develop cryoprotectant agents to maximize the storage stability, viability, and activity of the bacterial cells.

To protect the cells from freeze-drying, various cryoprotectants are used to mitigate the damage caused by ice crystallization, membrane rupture, and osmotic stress [23-25]. Although the mechanism of cryoprotective actions depends on the ingredients, an ideal cryoprotectant would increase the cell viability after freeze-drying, be easily removed, and available on a large scale [26]. In addition, the cryoprotectants considered must be non-toxic and biocompatible.

Previously, we screened the effectiveness of various cryoprotectants including natural ingredients by investigating the cell survival rate [27-29]. Based on these results, we concluded that mixture of seaweed and pumpkin is a material of interest to serve as a cryoprotective agent. To the best of our knowledge, there are no reports of encapsulating probiotic bacterial cells with sodium alginate and pumpkin mixture. Thus we evaluated and compared the efficacy of the mixture as cryoprotectants through *in vitro* acid and bile resistance of probiotic bacteria in this study.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Five probiotic bacteria (*Enterococcus faecium* MG89-2, *Lactobacillus plantarum* MG989, *L. fermentum* MG901, *Lactococcus lactis* MG5125, and *Streptococcus thermophilus* MG5140) which are commonly used in foods and supplements were provided by MEDIOGEN Co. (Jecheon, Korea). For experimental purposes, the strains were activated by growing at 37°C for 18 h in an MRS (de Man, Rogosa and Sharpe, Difco, USA) broth two times, successively. This culture was transferred to 4 L of MRS broth in a 7 L fermenter (KoBioTech, Korea) and incubated at 37°C. After 18 h of incubation, the probiotic bacterial cells were harvested by centrifugation at 4,000 × *g* for 20 min at 4°C.

2.2. Viable cell counting

Cell viability was determined by cell counts on MRS agar plates using colony forming unit per mL (CFU/mL). Freeze-dried non-coated cells were excluded from the cell viability test due to cell damage following freeze drying process. Thus freeze-dried SP coated bacterial cells were compared with non-coated activated free cells. This would be a result that can show whether the freeze-dried SP coated cells can guarantee the activity as much as the actually activated cells.

2.3. Cryoprotective agents and encapsulation of bacteria

To identify the cryoprotective effectiveness, pumpkin and sodium alginate were selected based on a previous study [28]. The sodium alginate (a seaweed derivative) was obtained from KunPoong Bio Co. Ltd. (Seoul, Korea), and pumpkin powder from Cedenco Foods New Zealand Ltd. (Auckland, New Zealand). Equal amounts of sodium alginate and pumpkin powder were mixed (SP powder). SP powder (2.5 g) was mixed with 100 mL of sterile distilled water (DW) in a beaker, and the solution was stirred gently using a magnetic stirrer until completely dissolved (SP solution). Freshly harvested bacterial cell pellets were added to the SP solution in a ratio of 1:2.5 (w/w) and mixed well for 5 min before freeze-drying.

2.4. Freeze-drying process

The bacterial cell suspended SP solutions were dispersed in wall materials and freeze-dried (Heto drywinner, Allerod, Denmark). Primary drying was performed to a shelf temperature of -40°C for 1 h. Secondary drying was performed stepwise up to 20°C, for a total 24 h. The freeze-dried cells were harvested and collected in polythene bags, wrapped in aluminum foil, and stored at 4°C until further use. Along with each process, the activated probiotic cells were used as controls for comparison because the survival rates of non-coated freeze dried cells were considerable low (data not shown). All experiments were carried out in triplicates.

2.5. Response to simulated gastrointestinal tract conditions

To simulate the human gastric juice, low pH conditions were induced according to the methods described by Dolly *et al*. [30] with slight modifications. Briefly, simulated gastric fluid (SGF) was prepared using MRS broth (pH 2 adjusted with 1 N HCl) with 3 g/L of pepsin (Sigma-Aldrich). The powdered freeze-dried cells (0.5 g) were dissolved in sterile SGF (9.5 mL) and incubated under anaerobic conditions at 37°C. From the SGF culture, 100 μL of sub-amples were obtained at 0, 1, 2, and 3 h, and homogenized with MRS agar. Petri dishes remained on a flat surface until complete agar solidification. And the plates were incubated at 37°C for 24 h, but the plates for *S. thermophilus* MG5140 were incubated at 42°C for 24 h.

Simulated intestinal fluid (SIF) was prepared using MRS broth at pH 7 (adjusted with 1 N NaOH) and adding pancreatin from porcine pancreas (Sigma-Aldrich) with a concentration of 1 g/L to determine the bile tolerance of the five probiotic strains. The powdered freeze-dried cells (0.5 g) were dissolved in sterile SIF 9.5 mL and incubated

under anaerobic conditions at 37°C. Samples were obtained from the SIF culture at 0, 1, 2, 3, and 4 h, and poured into MRS agar plates to count cells. The agar plates were incubated at 37°C for 24 h, but the plates for (*S. thermophilus* MG5140 were incubated at 42°C for 24 h.

2.6. Scanning electron microscope

The morphological characteristics of the freeze-dried cells were determined by scanning electron microscopy (SEM) (Hitachi TM3000, Japan) performed at Pusan National University. The freeze-dried samples were mounted on a stub using double sided adhesive metallic tape and coated with gold. The beam energy operated at an accelerating voltage of 15 kV. The cells were viewed at 1,000 and 5,000 × magnifications.

2.7. Statistical analysis

All experiments were performed in triplicate and the results were represented as means value ± standard deviation. ANOVA was performed to evaluate statistical differences between SP coated cells or not using R (version 3.6.2; The R Foundation for Statistical Computing). Significant differences between the results were evaluated using Tukey's honestly significant difference test.

3. Results

3.1. Viability under simulated gastric condition

Five strains were exposed to simulated gastric fluid condition (pH 2). Both freeze-dried SP coated probiotic bacteria and non-coated free probiotic bacteria decreased with time (Table 1). The average survival rates of activated non-coated free cells were 59.9%, 49.1%, and 35.5% at point 1, 2 and, 3 h, respectively. In SP coated cells, the survival rates were 77.4%, 68.5%, and 57.7% at point 1, 2, and 3 h, respectively. Overall, the viability of SP coated cells was significantly higher ($p < 0.001$) than that of the activated non-coated cells (Table 2).

3.2. Survival of bacteria under simulated intestinal condition

Simulated intestinal conditions (0.1% of pancreatin) at pH 7, representing the human digestive system helped to determine whether the freeze-dried SP coated cells and activated non-coated free cells could survive in human intestinal conditions. All strains showed a steady reduction of viability (Table 3). The average survival rate of the activated non-coated free cells was 94.3% after 4 h of exposure. In contrast, the SP coated cells showed a slightly

Table 1. Viability of probiotic bacterial cells under simulated gastric conditions (pH 2)

	Strains	Exposure time				Survival (%) after 3 h
		0 h	1 h	2 h	3 h	
Activated non-coated free cells	*L. plantarum* MG989	7.98 ± 0.41	5.75 ± 0.04	5.11 ± 0.17	3.83 ± 0.10	47.99
	L. fermentum MG901	8.26 ± 0.21	4.96 ± 0.15	3.39 ± 0.11	2.48 ± 0.14	30.02
	Lac. lactis MG5125	8.27 ± 0.20	5.62 ± 0.31	4.94 ± 0.09	3.78 ± 0.21	45.71
	S. thermophilus MG5140	8.06 ± 0.15	2.78 ± 0.15	2.23 ± 0.06	1.07 ± 0.16	13.28
	E. faecium MG89-2	8.13 ± 0.31	5.26 ± 0.11	4.33 ± 0.27	3.30 ± 0.19	40.59
SP coated cells	*L. plantarum* MG989	8.91 ± 0.24	7.75 ± 0.27	7.04 ± 0.36	5.70 ± 0.41	63.97
	L. fermentum MG901	8.03 ± 0.17	6.04 ± 0.41	4.62 ± 0.12	4.14 ± 0.45	51.56
	Lac. lactis MG5125	8.54 ± 0.29	7.06 ± 0.20	6.87 ± 0.49	5.85 ± 0.19	68.50
	S. thermophilus MG5140	9.12 ± 0.11	6.24 ± 0.21	5.16 ± 0.21	3.83 ± 0.11	42.00
	E. faecium MG89-2	8.81 ± 0.16	6.50 ± 0.29	6.06 ± 0.27	5.52 ± 0.28	62.66

The results are expressed as mean ± standard deviation; each data point is the average of 3 repeated measurements from 3 independently replicated experiments. *L., Lactobacillus*; *Lac., Lactococcus*; *S., Streptococcus*; *E., Enterococcus.*

Table 2. Summary of survival ability of five probiotic bacterial strains under simulated gastrointestinal conditions

Strain	SGF			SIF		
	SP coated (%)	Non coated (%)	*p* value	SP coated (%)	Non coated (%)	*p* value
L. plantarum MG989	63.97	47.99	$< 0.001^{***}$	98.99	95.99	$< 0.001^{***}$
L. fermentum MG901	51.56	30.02	$< 0.001^{***}$	97.26	95.88	0.0132^{*}
Lac. lactis MG5125	68.50	45.71	$< 0.001^{***}$	97.31	97.58	$< 0.001^{***}$
S. thermophilus MG5140	42.00	13.28	$< 0.001^{***}$	98.25	84.24	$< 0.001^{***}$
E. faecium MG89-2	62.66	40.59	$< 0.001^{***}$	99.77	97.91	$< 0.001^{***}$

Significant correlation between the SP coated and activated non-coated cells is shown in asterisk. *, $p < 0.05$; ***, $p < 0.001$. *L., Lactobacillus*; *Lac., Lactococcus*; *S., Streptococcus*; *E., Enterococcus.*

Table 3. Viability of probiotic bacterial cells under simulated intestinal conditions (pH 7)

Strains		Exposure time					Survival (%) after 4 h
		0 h	1 h	2 h	3 h	4 h	
Activated non-coated free cells	*L. plantarum* MG989	7.98 ± 0.41	7.81 ± 0.21	7.79 ± 0.37	7.69 ± 0.39	7.66 ± 0.31	95.99
	L. fermentum MG901	8.26 ± 0.21	8.02 ± 0.41	7.99 ± 0.21	7.98 ± 0.21	7.92 ± 0.17	95.88
	Lac. lactis MG5125	8.27 ± 0.20	8.16 ± 0.29	8.18 ± 0.41	8.10 ± 0.28	8.07 ± 0.25	97.58
	S. thermophilus MG5140	8.06 ± 0.15	7.60 ± 0.21	7.33 ± 0.39	7.03 ± 0.24	6.79 ± 0.07	84.24
	E. faecium MG89-2	8.13 ± 0.31	8.06 ± 0.19	8.04 ± 0.31	8.01 ± 0.11	7.96 ± 0.10	97.91
SP coated cells	*L .plantarum* MG989	8.91 ± 0.24	8.91 ± 0.37	8.89 ± 0.48	8.87 ± 0.31	8.82 ± 0.34	98.99
	L. fermentum MG901	8.03 ± 0.17	7.97 ± 0.21	7.91 ± 0.41	7.90 ± 0.09	7.81 ± 0.21	97.26
	Lac. lactis MG5125	8.54 ± 0.29	8.47 ± 0.11	8.42 ± 0.38	8.41 ± 0.27	8.31 ± 0.38	97.31
	S. thermophilus MG5140	9.12 ± 0.11	9.12 ± 0.26	9.07 ± 0.47	9.06 ± 0.11	8.96 ± 0.34	98.25
	E. faecium MG89-2	8.81 ± 0.16	8.83 ± 0.10	8.81 ± 0.10	8.80 ± 0.06	8.79 ± 0.26	99.77

The results are expressed as mean ± standard deviation; each data point is the average of 3 repeated measurements from 3 independently replicated experiments. *L., Lactobacillus*; *Lac., Lactococcus*; *S., Streptococcus*; *E., Enterococcus.*

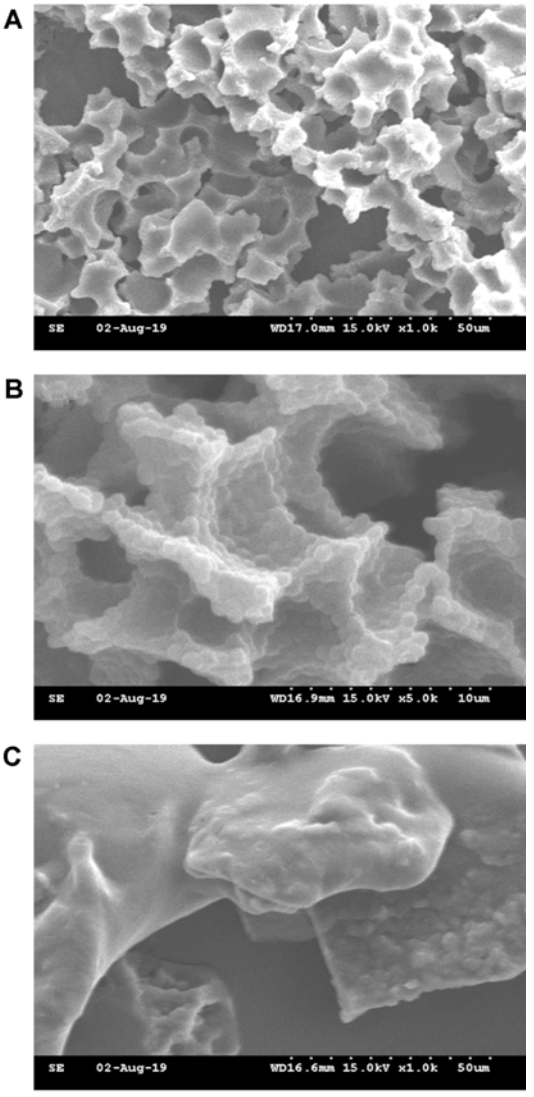

Fig. 1. Scanning electron microscopy images of freeze-dried cells. Non-coated free cells at 5,000 × magnification (A) and 1,000 × magnification (B), and sodium alginate and pumpkin powder (SP) coated cells at 1,000 × magnification (C).

higher survival rate (98.3%) (Table 3). All SP coated freeze-dried cells were significantly higher than that of the activated non-coated cells in survival rate (Table 2).

3.3. Morphological characterization of cells by scanning electron microscopy

The scanning electron microscope photomicrographs showed the appearance of the freeze-dried cells (Fig. 1). The shape of the cells depended on whether or not the cryoprotectant SP solution was processed. SP solution encapsulation formed thin layers of embedded bacterial cells and showed a smooth surface structure (Fig. 1C). In contrast, non-coated free cells showed a porous rough surface (Fig. 1A and B).

4. Discussion

A main property for the effective inclusion of probiotics in functional foods is the capacity of tolerance to digestive stress [31]. The probiotic bacteria must survive in harsh gastrointestinal environments enduring gastric juice, bile, and pancreatic enzymes, to settle at the epithelium of intestinal tract. Many studies have investigated the encapsulated lactic acid bacterial cells in dairy products [32,33]. However, little data is available about the use of encapsulated microorganisms and their resistance to gastrointestinal stress. Here, to identify the efficacy of the SP solution as a cryoprotectant, we observed the bacterial cell viabilities after exposure to simulated gastric and intestinal conditions.

The majority of tested SP coated probiotic bacteria were found to be more resistant to simulated gastrointestinal conditions than that of activated non-coated. Our results were consistent with studies of cell coating technology that discovered the increased survival of probiotic bacteria under acidic conditions [34,35]. Notably, acid-sensitive strain

such as *S. thermophilus* MG5140 showed an approximate 15 times improved viability with SP coating treatment in simulated gastric fluid, and showed an enhanced survival rate from 84.3% to 98.2% after 4 h incubation in simulated intestinal fluid (Table 1 and 3). Although it is hard to compare the results between SP coated freeze-dried cells and non-coated freeze-dried cells in this study, our results indicated that the SP coated freeze-dried cells have a higher or as much survival rate than activated free cells.

The extent of bacterial survivability could be influenced by cryoprotective agents. For example, the survival rate of *L. acidophilus* encapsulated using chitosan, high amylose corn starch (Himaize™; Starch Australasia Ltd., Lane Cove, Australia), and alginate was greater [36]. Picot and Lacroix [37] observed an increase in cell survival under simulated gastrointestinal conditions by encapsulating *Bifidobacteria* in whey protein. In this study, two kinds of polysaccharides, sodium alginate and pumpkin, would be a good wall materials for bacterial cells to avoid direct contact with the external environment. Sodium alginate forms insoluble alginate gel through complexation with divalent ions. Thus sodium alginate widely used to encapsulate macromolecular bioactive agents [38]. Pure pumpkin powder might act as polysaccharides like a sodium alginate as mentioned above. Pumpkin is a good material of being non-toxic and biocompatible. However, the mechanism of pumpkin as cryoprotectant is poorly understood.

Although cryoprotectant agents could protect cell viability, all biopolymers do not improve survival rate of all bacterial strains. For example, studies by Sun and Griffiths [39] and Sultana *et al*. [33] using gellan-xanthan beads and alginate-starch for *Bifidobacteria* encapsulation, respectively, did not observe a significant increase in survival when the cultures were subjected to high acidity *in vitro* conditions. Favaro-Trindade and Grosso [40] presented that alginate immobilization was not efficient at increasing the tolerance of *L. acidophilus* or *Bifidobacterium lactis* to gastrointestinal secretions. These indicate that the cell viability after freeze-drying depends on the type of cryoprotectant agents.

Another factor that may affect survival rates is the physical properties of the SP solution. SP solution encapsulation formed thin layers of embedded bacterial cells and showed a smooth surface structure (Fig. 1C). In contrast, non-coated free cells showed a porous rough surface (Fig. 1A and B). Our results agree with other studies which observe the encapsulated matrix type of cryoprotectants [41,42]. The variations in microstructures in encapsulated cells can be attributed to the differences in viscoelastic and film forming characteristics of each wall material [43]. Encapsulation could block direct contact between bacterial cells and harsh gastrointestinal environments. The capsule that encloses the probiotic bacterial cells dissolves over time, helping to settle bacteria in human intestinal tract.

5. Conclusions

The aim of this study was to evaluate the efficacy of natural materials such as alginate and pumpkin as cryoprotectants. Results from this study demonstrated that SP coating is an optimized cryoprotectant, showing the survivability of SP coated probiotic bacterial cells (*L. plantarum* MG989, *L. fermentum* MG901, *L. lactis* MG5125, *S. thermophilus* MG5142, and *E. faecium* MG89-2) improved after freeze-drying and simulated gastrointestinal conditions. Therefore, our results expected that the non-toxic and biocompatible cell coating technology developed in this study will contribute toward enhancing cell stability in delivery systems.

Declaration

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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