Vietnam Journal of Science and Technology ***56*** *(2) (2018) 208-215*

DOI: 10.15625/2525-2518/56/2/9850

USE OF WHEY PROTEIN FOR ENCAPSULATION AND CONTROLLED RELEASE OF PROBIOTIC BACTERIA FROM PROTEIN MICROENCAPSULATE IN *EX VIVO* PORCINE GASTROINTESTINAL CONTENTS

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Received: 26 May 2017; Accepted for publication: 10 March 2018

Abstract. The aim of this study was to evaluate the efficacy of whey protein isolate (WPI) as an encapsulation matrix for improvement of *L. fermentum* 39-183 viability to low pH and bile and releasing the encapsulated bacteria in *ex vivo* porcine gastrointestinal (GI) contents. 1g of protein microcapsules (≈ 10^8 CFU of *L. fermentum* 39-183 or *E. coli* GFP^+) were incubated in *ex vivo* porcine GI contents (9 mL) under anaerobic conditions at 37 ^{0}C. Results showed that there was higher than 86 % cell survival for encapsulated *L. fermentum* 39-183 after 3 h incubation in pH 2.0, whereas free cell experienced complete viability loss. Encapsulated *L. fermentum* 39-183 showed only about 0.86 log reduction for all bile salt levels tested (0.5 ÷ 2.0 %), while 3.34 log decrease of free cell after 6 h of incubation. There was almost a complete release (3.9 × 10^8 CFU) of microencapsulated bacteria in the ileal contents within 2 h, while there was no significant release of encapsulated bacteria in the gastric contents even after 8 h of incubation. This study led to the development and design of a protein capsulation for reinforced probiotic protection during the stressful conditions of gastric and controlled release at a defined location.

Keywords: protein capsules, *ex vivo* porcine gastrointestinal contents, lactobacillus fermentum 39-183, probiotics.

Classification numbers: 3.7.2; 2.7.1.

1. INTRODUCTION

Microencapsulation with respect to a food application involves reversible of active bio-molecules in stable core and releasing it at desired sit (such as intestine or colon). Probiotics, minerals, vitamins, phytosterols, fatty acids, lycopene and antioxidants are some of the compounds which have been delivered through microencapsulation techniques. Probiotics are defined as living microorganisms that contribute to beneficial effects on human health upon

ingested in adequate dose and have been widely incorporated in various dairy products and marketed as functional foods [1]. However, there is a considerable loss of viability as probiotic bacteria pass through the low pH of the stomach and high bile salt conditions of the intestine. Choice of the capsule materials is a major element for successful microencapsulation of probiotics and the use of microencapsulation probiotics in functional foods [2]. As a biopolymer used for a coating agent of probiotic live bacteria, whey protein also appears as a potential candidate because it is entirely biodegradable and frequently used in many types of food product. Whey protein isolate (WPI) have been shown to increase the survival of probiotics in the Simulated Gastric Juice (SGJ) by the addition of the isolate to the bacterial culture or as a wall material for encapsulation. *Bifidobacterium infantis* was subjected to SGJ at pH 2.0 for 3 h in the presence of 1 gL^{-1} whey protein isolate [3]. The presence of whey protein isolate significantly improved survivability when compared to bacteria incubated in SGJ without WPI. *Lactobacillus rhamnosus* was encapsulated by extrusion using a 7:3 mixture of 12 % WPI:bacteria. This material was subjected to a dynamic gastrointestinal model which varied in pH from 4.4 to 2.0 over 90 min [4]. However, the survival level was strain – dependent and once the encapsulated, bacteria reach the targeted organs, it is ideal for the microencapsulated matrix to release them in a controlled fashion. There has little published data on the conditions and profile of release of probiotics from protein matrices in *ex vivo* and *in vivo* GI condition. Such the objectives of this research were to assess acid and bile salt resistance of whey microcapsule and investigate the release profile of *Lactobacillus fermentum* 39-183 from whey microcapsule in *ex vivo* porcine gastrointestinal contents.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

Lactobacillus fermentum 39-183 and green fluorescent tagged *Escherichia coli* (*E. coli* GFP^+) were used in this study. *L. fermentum* 39-183 sourced from our previous research [5].The stock culture was maintained at – 20 ^{0}C in MRS broth (Merck Darmstadt, Germany) supplemented with 50 % sterile glycerol. Prior to use, the culture [1 % (v/v)] was transferred twice to MRS broth and incubated at 37 ^{0}C for 18 ÷ 20 h.

E. coli GFP^+ K12 was supplied by the Department Biotechnology of Ho Chi Minh City University of Science. Prior to use, the culture [1 % (v/v)] was transferred twice to LB broth (Oxoid, Sydney, Australia) containing ampicillin (100 mg/ml) and incubated at 37 ^{0}C for 24 h.

2.2. Microencapsulation of microorganisms

The preparation of the whey microcapsule was carried out according to the method described by O'Neill [6] with some modification. WPI powder (6 % w/v) was rehydrated in distilled water, agitating the solution for 1 hour at room temperature and then allowed to stand for 2 hours to ensure complete hydration of the proteins. Sodium azide was added (0.02 %) to the whey protein solution and heated at 90 ^{0}C for 30 minutes. The denatured WPI solution was then cooled and held at room temperature for 2 hours. To form microcapsules, the encapsulation matrix (6 % (w/v) WPI) and the cell suspension mixture (7:3) was injected through in a 25G needle into a filter sterilized cross – linking solution (5 % (w/v) $CaCl_2$ + 10 % (w/v) Tween 80), which was stirred at 250 rpm by a magnetic stirrer. The resulting capsules were allowed to harden in the cross – linking solution for 1 hour, and were then collected by filtration using

cheesecloth, which was sterilized in boiling water for 12 min prior of use. The microcapsules were then washed with distilled water and collected for the following tests.

2.3. Characterization of microparticles

The shape and surface morphology of the microcapsules was observed with a scanning electron microscope (SEM) and the average size of the microcapsules was evaluated by Particle Size Distribution Analyzer LA-920 (HORIBA, JAPAN).

2.4. Determination of *L. fermentum* 39-183 viability in whey microcapsule

The viability of the encapsulated *L. fermentum* 39-183 (containing 8.64 log CFU/g) in whey microcapsule was determined by vigorously homogenizing 1 g of the micro-bead in 9 mL of sterile phosphate buffer solution (PBS) pH 7.0 for in 10 min at room temperature. Viable cell (CFU/g or CFU/mL) was determined by plating on MRS plates and incubating at 37 ^{0}C for 48 hours.

2.5. Survival of free and encapsulated in simulated gastric juice (SGJ) and bile salts

The Simulated Gastric Juice (SGJ) were prepared by suspending of 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH_2PO_4, 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g oxgall bile (MI, Sigma) and 13.3 g pepsin in 1000 ml distilled water according to the method of Kim *et al*. [7]. The artificial gastric juice was adjusted to different pH values (2, 4 and 7) using 1M HCl. MRS broth without addition of bile salt was used as a control. Either wet whey microcapsule (1 g) containing *L. fermentum* 39-183 or 1 mL of washed cell suspension were added into the prepared tubes (9 mL prepared solutions/tube) and incubated at 37 ^{0}C for 0, 6, 12, 18 and 24 h. The whey microcapsule was then removed and placed in 9 mL of sterile phosphate buffer solution (PBS) pH 7.0 for in 10 min at room temperature. Total viable cells numbers were determined by the plate count method. The resistance to bile salts was determined by inoculating free and encapsulated cell in MRS broth containing 0.5 %, 1.0 %, 2.0 % (w/v) Ox-bile (Biochemika, Fluka; Sigma-Aldrich) after 6 h incubation at 37 ^{0}C. All samples were treated in triplicates.

2.6. Release profile of microencapsulated bacteria in porcine gastrointestinal contents (*ex vivo*)

Gastrointestinal contents (gastric, duodenum, jejunum, ileum and colon) from three different pigs (8 months old) were collected and used within 1 ÷ 2 h after slaughtering. Wet whey microcapsule (1 g) (containing 8.60 log CFU *L. fermentum* 39-183 or *E. coli* GFP$^+$/ 1 g microcapsule) were incubated in different sections of intestinal contents (9 ml) for 3 h at 37 ^{0}C under anaerobic conditions. Samples of 1 ml were collected at different time intervals (0, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 h), and enumerated for *L. fermentum* 39-183 or *E. coli* GFP$^+$ by spread plating on Lactitol-Lactobacillus-Vancomycin (LLV) agar or LB-ampicillin/arabinose media, respectively. The fluorescent bacteria were plated on LB agar (Oxoid, Australia) containing 100 mg/ml ampicillin and 1.2 mg/ml arabinose and enumerated after 24 h incubation by observing green fluorescent colonies under a UV illuminator. All samples were treated in triplicates.

2.7. Statistical analysis

Results of three independent assays are presented as mean values ± standard deviation (SD). Data were analyzed by ANOVA and Turkey's test. Statistical analysis was carried out

with the Statgraphics Centurion XV program (Statgraphics, USA). Results were considered significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characteristics of whey microcapsule

Extrusion is the oldest and most common technique used for microencapsulating probiotics in hydrocolloid gel matrices. The size and shape of the capsules are influenced by many factors. In this study, *L. fermenrum* 39-183 was microencapsulated with whey protein isolate by extrusion method and the shape and surface morphologies of the whey protein microcapsule were investigated using SEM and shown in Figure 1. Whey protein microcapsule exhibited a spherical shape with a wrinkled surface (a round, flattened shape – not completely smooth – without visible cracks or pores on the surface). Extrusion method has been used for producing capsules with 0.2 to 5 mm. Microcapsule size is an important consideration since the microcapsules must have a high volume-to-surface ration for increasing the protective effect and be sufficiently small to avoid a negative sensory impact [8]. In this study, the mean whey protein microcapsule size was 311.9 µm, so formulation cannot be discriminated by the size criterion.

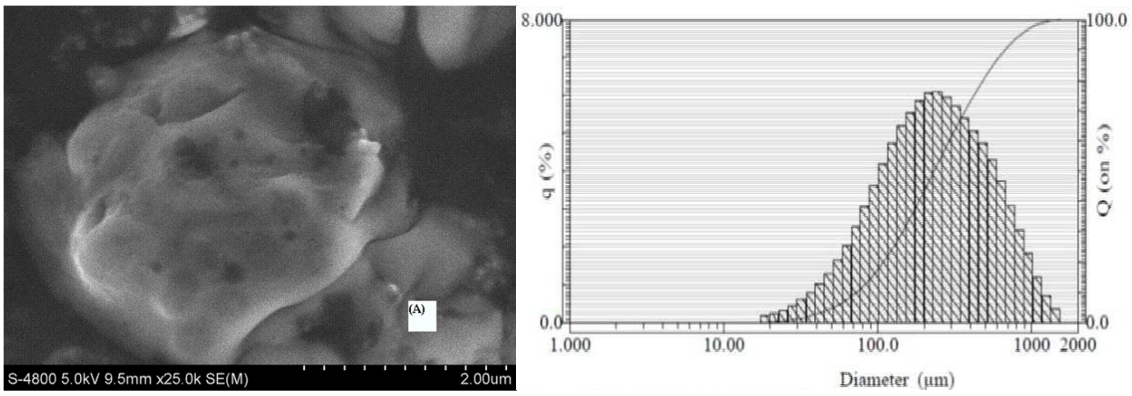

Figure 1. Scanning electron microscopic (SEM) observation of whey microencapsule.
Sympols: (A) – shape morphology of whey microcapsule, (B) – Size of whey microcapsule.

3.1. Simulated gastric juice tolerance of whey protein microcapsule

One of the main barriers for oral probiotic bacteria is the stomach low pH, which is related to the high hydrochloric acid concentration of the gastric acid. To test the performance of the encapsulated and free cell *L. fermentum* 39-183 at different pH values, they were incubated in the artificial gastric juice adjusted pH 2.0, 4.0 and 7.0 after 6, 12, 18 and 24 hours. Viability of *L. fermentum* 39-183 at pH 2.0 and 4.0 appeared to decrease as the incubation period increased (Fig. 2). However, *L. fermentum* 39-183 showed growth over the incubation period at pH 7.0, with both encapsulated and free cell. Encapsulated cell increased 2.18 log CFU/mL, while free cell increased 0.65 log CFU/mL. After 24 hours of exposure, encapsulated cell was highly tolerant and retained their viability under acidic conditions at pH 4.0. Encapsulated cell decreased 2.25 log less than free cell. At pH 2.0, statistical analysis showed a significant difference ($p < 0.05$) between reductions obtained with encapsulated and free cells. The encapsulated cell showed a 2.63 log reduction, while the free cell decreased by 4.78 log after 6

hour of incubation. This suggested that whey protein isolated protected and significantly improved survivability of *L. fermentum* 39-183 in the SGJ. According to Lundin *et al.*, during digestion the microcapsule could be influenced as follows: hydrolyzation by acid, proteolysis by pepsin, shearing forces by peristaltic stomach movements and finally body temperature [9]. Gastric pepsin enzyme may cause the protein hydrolysis into polypeptides, oligopeptides and some free amino acids. One of the reasons explaining the good resistance of whey protein capsule could be that the cleavage sites were partially hidden in the structure. In addition, it has been demonstrated that low pH had no effect on the composition and structure of whey protein [10]. However, the survival both types of cell (encapsulated and free) rapidly decreased and did not survive after 24 hours of incubation.

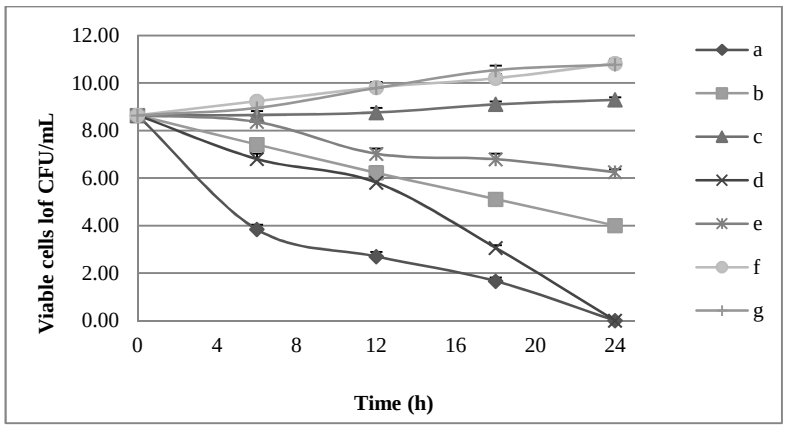

Figure 2. Survivability of encapsulated and free *L. fermentum* 39-183 over 24 hours of incubation at different pH values. Symbols: a – free cell (pH 2); b – free cell (pH 4); c – free cell (pH 7); d – encapsulated cell (pH 2); e – encapsulated cell (pH 4); f – encapsulated cell (pH 7); g – control.

Bile salt tolerance of whey microcapsule

Table 1. Reduction in viable counts of free and encapsulated *L. fermentum* 38-183 over 6 hours of incubation in bile salt conditions.

Bile salt (%)	Free cell (log CFU/mL)			Encapsulated cell (log CFU/mL)		
	Initial	6 h	Reduction	Initial	6 h	Reduction
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0.5	8.60 (0.17)	6.89 (0.17)	1.71 (0.03)	8.60 (0.17)	8.01 (0.18)	0.59 (0.03)
1.0	8.60 (0.17)	5.64 (0.11)	2.96 (0.22)	8.60 (0.17)	7.85 (0.15)	0.75 (0.19)
2.0	8.60 (0.17)	2.93 (0.14)	5.67 (0.15)	8.60 (0.17)	7.47 (0.21)	1.13 (0.21)

* Mean and standard deviation were obtained from triplicate samples.

After microorganisms pass through the stomach, they enter the upper intestinal tract where bile salts are secreted into the gut. As a surface active compound, bile penetrates and reacts with lipophilic side of bacterial cytoplasmic membrane causing a damage of membrane structure [7,

11]. Bile also affects the structure and function of large macromolecules such as DNA and proteins leads to the damage of molecule. To test bile salt tolerance, encapsulated and free cell *L. fermentum* 39-183 were exposed to solutions containing different levels of bile salts after 6 hours of incubation. Results shown in Table 1 indicate that viable cells of encapsulated and free cells gradually decreased when the concentration of bile salt was increased up to 2.0 %. Encapsulated *L. fermentum* 39-183 with initial cell load of 8.60 log CFU/g showed 0.58 log and 0.75 log reduction when exposed to 0.5 % and 1.0 % bile salt broth, respectively, while the free cells decreased by 1.70 log and 2.97 log, respectively. Encapsulated *L. fermentum* 39-183 was observed resistant to 2.0 % bile salt, which remained survival rate higher than 50 % after 6 hours of incubation, whereas free cell showed a decrease of 5.65 log. The results obtained also showed that encapsulation provided protection for cells, since the survival of encapsulated cells was significantly better ($p < 0.05$) than that of free cells. The results related to improve survivability of encapsulated cells treated with bile salt obtained in this study are in accordance.

3.4. Release profile of microencapsulated bacteria in *ex vivo* porcine gastrointestinal contents

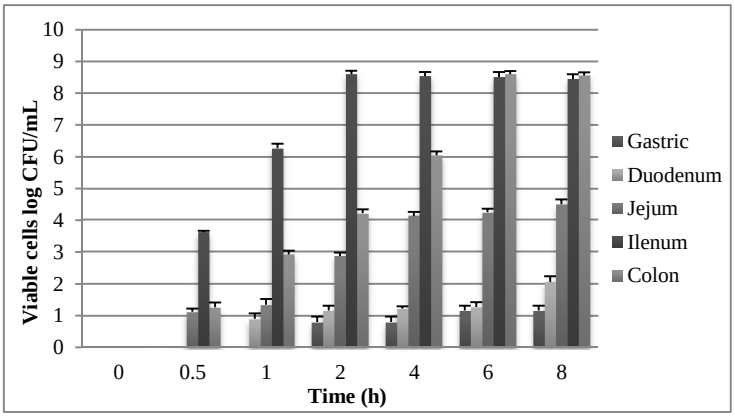

Figure 3. Release profiles of microencapsulated *E. coli* GFP^+ in porcine gastrointestinal contents. The error bars represent standard deviation of mean (n=3).

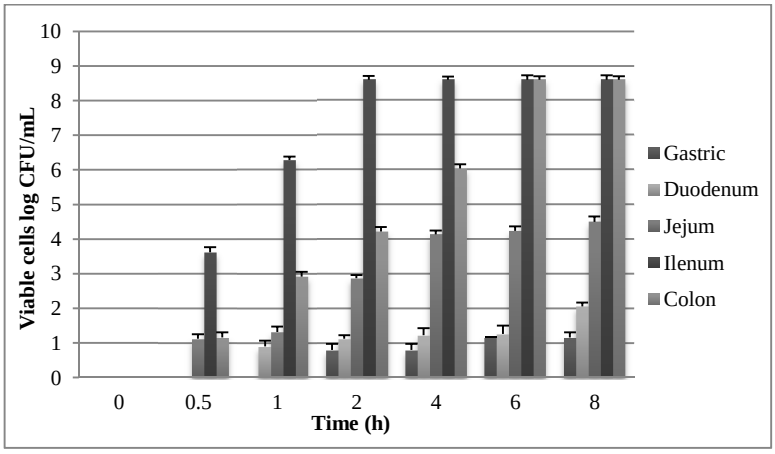

Figure 4. Release profiles of microencapsulated *L. fermentum* 39-183 in porcine gastrointestinal contents. The error bars represent standard deviation of mean (n = 3).

Figure 3 and 4 show the release profile of *E. coli* GFP^+ and *L. fermentum* 39-183 from protein capsules in *ex vivo* porcine GI contents, respectively. The release profile of bacteria from whey protein capsules varies in different GI conditions. A mount of 2.06 ± 0.11 CFU10 mL^{-1} was counted in the duodenal contents (pH = 5.2), while there was greater amount of released bacteria in the jejunum contents (pH = 6.5) around 4.50 ± 0.15 CFU10 mL^{-1} after 8 h of incubation. There was a complete release of *L. fermentum* 39-183 or *E. coli* GFP^+ (8.60 ± 0.11 CFU10 mL^{-1}) from whey protein capsules in ileum (pH = 7.2) after 2 h of incubation, while the cell count of both of strains gradually increased from 1.16 ± 0.15 CFU10 mL^{-1} at 0.5 h to complete release (8.60 ± 0.10 CFU10 mL^{-1}) after 6 h in colon. In contrast, there was no significant release of *L. fermentum* 39-183 or *E. coli* GFP^+ in the gastric contents (pH = 2.5) after 8 h. This suggests that the bacteria were either dead or trapped in the capsules. However, addition of phosphate buffer (after 8 h) increased the viable counts of microencapsulated bacteria (*L. fermentum* 39-183 and *E. coli* GFP^+) to nearly 8.60 CFU10 mL^{-1} with in 15 min in gastric, duodenal and jejunum contents. This shows that the bacteria were alive but not released completely from capsules. However, there was no significant decrease in the viable cell of *E. coli* GFP^+ in ileum and colon contents after a complete release form microcapsules. This suggests that *E. coli* GFP^+ strain K12 is not a native gut bacterium, therefore is not able to survive in porcine gut contents. Our results are similar with Iyer *et al*. [12] who reported that *L. casei* Shirota was completely released from chitosan-coated alginate-starch capsule in ileum and colon. However, in our study, the time release of *L. fermentum* from whey protein capsule in in ileum and colon was shorter.

4. CONCLUSIONS

These results demonstrate that whey proteins can be used as a convenient material for improving *L. fermetum* 39-183 protection. Whey protein microcapsule has an excellent capacity to encapsulate bioactive organisms that are sensitive to stomach circumstances, with concomitant controlled release at a defined location. Whey protein encapsulation efficiently minimizes the bacteriocidal effects of the gastric pH and maximizes the number of probiotics reaching the ileum and subsequently the colon. Thus, this encapsulation technique may act as a platform technology for promoting targeted delivery of probiotics with potential applications within the food and pharmaceutical industries.

Acknowledgement. We thank Dr. Tran Van Hieu and his staff at Department Biotechnology of Ho Chi Minh City University of Science for providing *E. coli* GFP^+ K12 and analyses of images of encapsulated *E. coli* GFP^+ by fluorescence microscopy.

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