

## STUDY ON THE EFFECT OF CALCIUM-ALGINATE AND WHEY PROTEIN ON THE SURVIVAL RATE OF *Bifidobacterium bifidum* IN MAYONNAISE

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Received: 3 August 2019; Accepted for publication: 3 November 2019

**Abstract.** The functional food development by adding probiotic bacteria is getting a lot of concern. In this study, *Bifidobacterium bifidum* AS 1.1886 was encapsulated in calcium-alginate 2 % w/v (C sample) or the mix of calcium-alginate 2 % (w/v) and whey protein 1 % (w/v) (CW sample) or calcium-alginate 2 % (w/v) coated by whey protein 1 % (w/v) (CcW sample) by extrusion method, and added to mayonnaise product. The pH changes, the survival rate of probiotic bacteria, and total yeast and mold count during storage, as well as the probiotic survival in simulated gastric medium, were evaluated. The result showed that the pH changes were not significantly different in all mayonnaise samples in this test. The viability of the free probiotic cell was significant decrease by 5.85 log CFU/g compared to 0.26 ÷ 1.14 log CFU/g in encapsulated cell samples after four weeks of storage. None of the free cells survived after six weeks of storage. The total yeast and mold count in samples related to the probiotic count, the viability of probiotic cells higher 6 log CFU/g might be controlling the growth of yeast and mold in mayonnaise. Whey protein has been shown to significantly improve the survival rate of *B. bifidum* and calcium-alginate coated by whey protein, indicating the most effective protection.

**Keywords:** calcium-alginate, mayonnaise, microencapsulation, probiotic bacteria, whey protein.

**Classification numbers:** 1.4.4, 1.4.8.

### 1. INTRODUCTION

The benefits of probiotic bacteria were investigated in a lot of previous studies [1, 2]. Nowadays, the application of probiotic bacteria in food is a trend to experiment. Products containing viable probiotic bacteria were multiform, not only dairy products such as yogurt, ice cream [3, 4] but mayonnaise was also interested in the market. Addition of probiotic into mayonnaise could make a new product that gives the health benefit of the customer. However,

storage condition of mayonnaise was causing a remarkable reduction in the survival rate of probiotics [5]. These probiotic foods only bring health benefits if probiotics must be alive and maintain over 6 log CFU/ml (CFU/g) until the consumption [2]. Furthermore, gastrointestinal and bile salt conditions were the agent causing the death of probiotics, which were sensitive in this environment [6, 7]. Therefore, many studies to enhance the viability of probiotic were carried out, and encapsulated probiotic can improve the high survival rate of bacteria. In the encapsulated technique, the matrix materials play an important role for probiotic protection. Among the matrix materials such as alginate, chitosan, carrageenan etc., alginate is the high applicability due to convenience, non-toxic, bacteria protection ability compared to free cells bacteria [8]. However, previous studies indicated that the probiotic viability was not protected completely by the calcium-alginate capsule, because its porous structure facilitated the penetration of ion H of the low pH medium into the capsule [9]. The proposed solution including, combination of calcium-alginate and supplement wall materials such as whey protein, starch etc. by mixing or coating that would improve significantly the viable probiotic than pure calcium-alginate [6, 7, 10]. It has indicated that both methods gave high effects on enhancement of the viable probiotic bacteria in calcium-alginate. However, the study on the protective effect of these encapsulation ways in mayonnaise was poorly reported. Therefore, in this study, *Bifidobacterium bifidum* was microencapsulated in calcium-alginate and whey protein by mixing and coating by the extrusion method. Preparations supplied in mayonnaise was determined the change in storage condition concluding pH change, *B. bifidum* viability, yeast, and molds counts and the viability of probiotic in simulated gastrointestinal fluid in this study.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains and encapsulation

*Bifidobacterium bifidum* AS 1.1886 strain was cultured in 10 ml of MRS (de Man, Rogosa and Sharpe) broth at 37 °C for 24 h before being transferred to 90 ml of MRS broth and incubated under the same conditions. Biomass was collected by centrifuging and used for the next experiments.

Extrusion encapsulation method was carried out by the following steps [8]. Briefly, 10 ml cell suspension was added into 40 ml sodium alginate 2.5 % w/v (C sample) or the mix of sodium alginate 2.5 % w/v and whey protein 1.25 % w/v (CW sample). Then the mix was injected through an aseptic syringe into 50 ml solution CaCl<sub>2</sub> 0.05 M and incubated for 15 minutes. The encapsulated beads were collected in case of C and CW samples. In case of coating samples (CcW), a part of C sample then incubated in whey protein 1 % (w/v) by using shaking incubator (Techlab LSI-3016R) at 150 rpm for 10 min. The encapsulation yield calculated according to the following formula:

$$\text{Encapsulation yield(\%)} = \frac{\sum \log CFU_{after}}{\sum \log CFU_{before}} \times 100 \%$$

The particles were measured average size by electrical caliper and storage at 4 °C.

### 2.2. Mayonnaise preparation

Mayonnaise was carried out according to a previous description [11]. Briefly, soybean oil (Simply) 74 %, egg 14 %, vinegar (Maille) 10 %, salt (Xuan Hong) 1 %, sugar (Bien Hoa) 1 %, and white pepper (Xuan Hong) 0.2 %. Sugar and vinegar mingled, and then all other ingredients

were added and stirred homogeneously (Digital Ultra-Turrax T25, at 2.000 rpm/1 min). The oil was added very slowly while stirring continuously (5.000 rpm/5 min). Finally, encapsulated probiotic or free probiotic cells were added into mayonnaise and storage at 4 °C.

The pH changing of mayonnaise samples and the survival rate of *B. bifidum* during storage were determined after storing immediately as well as the end of every two weeks until ten weeks of storage at 4 °C. The total number of yeast - mold spores were quantified according to ISO 21527-1: 2008. Briefly, the sample was diluted in saline water and then spread on DRBC (Dichloran Rose Bengal Chloramphenicol) medium at 30 °C ± 1 °C for five days.

### **2.3. Effects of simulated gastric fluid (SGF) and intestinal fluid (SIF) on *B. bifidum* after 8 weeks of storage**

The experiments were carried out according to the method described by Lieu *et al.* [11] with slight modifies. Briefly, 5-grams of the mayonnaise after eight weeks of storage were incubated in 45 ml of Simulated gastric fluid (SGF) medium (9 g/l NaCl + 3 g/l pepsin (Himedia) adjusted to pH 2 with 5N HCl), at 37 °C and shaking speed 100 rpm for 120 minutes. Similarly, 5-grams of mayonnaise were incubated in 45 ml of (simulated intestinal fluid) SIF medium (0.85 % NaCl, 0.3 % bile salts, adjusted to pH 6.5 with 5N NaOH) at 37 °C for 240 minutes. The viability of *B. bifidum* in the mayonnaise was immediately assayed by plating on MRS media.

### **2.4. Enumeration of free and encapsulated probiotics**

The viability of *B. bifidum* was carried out according to Sultana *et al.* [6] with slight modifications. 5-grams were resuspended in 45 mL of phosphate buffer (0.1 M, pH 7.0), followed by homogenizing in a stomacher (IUL-Spain) for 10 min. The probiotic viability (CFU/mL) was determined by spreading on MRS-agar at 37 °C for 48 h. The process of similar samples was used for free samples.

### **2.5. Statistical analysis**

All data obtained will be expressed as ± mean value (SD) of at least three replicates for each treatment using a Turkey test (SPSS20, IBM Inc.) and on Excel 2013 (Microsoft Inc.).

## **3. RESULTS AND DISCUSSION**

### **3.1. Encapsulation efficiency**

The effect of the microencapsulation method on encapsulation efficiency (EE) is shown in Figure 1. The results indicated the encapsulation efficiency of all surveyed samples was over 94 %, wherein the highest yield was 97 % microencapsulated probiotic of C, whereas CW and CcW samples were 96 % and 94.5 %, respectively. However, this difference was not significant ( $p > 0.05$ ).

The encapsulation efficiency was an important parameter to evaluate the effect of the encapsulated process [10]. The higher encapsulation yield decreased the content of preparations supplying foods leading to reduce the sensory affecting by encapsulated bead. The extrusion method was prevalent research due to the facility, low fee, and lightly process condition and

friendly with probiotic [8]. The viability of probiotics in microcapsules increased with an increase in gel concentration [12]. Besides, the adding of material walls such as resistant starch (2 % w/v) in calcium-alginate gel could improve encapsulation efficiency [6]. In the present study, the compound of material walls with alginate (2.5 % w/v) with or without whey protein (1.25 % w/v) gave a high encapsulation efficiency compared to other material walls (data not shown). The results in this study also showed that coating with whey protein had the lowest efficiency of others (Figure 1). A similar result was reported by Ji *et al.* [10], the EE of *B. longum* in calcium-alginate coating by chitosan was  $90 \pm 3.4$  % compared with  $95 \pm 2.5$  % that of *B. longum* in pure calcium-alginate. This difference might be due to shaking incubation process in whey protein for making the coating layer would leak *B. bifidum* cell from the Ca-alginate matrix leading to decrease of the encapsulation yield (Figure 1).

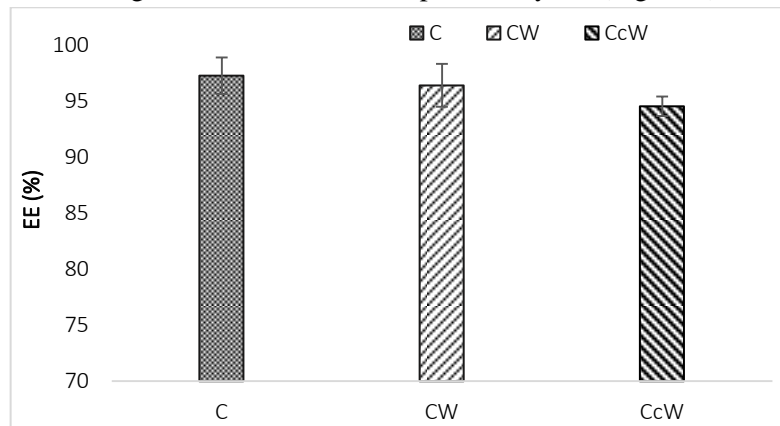


Figure 1. Encapsulation efficiency (C: alginate; CW: alginate + whey; CcW: whey coating alginate).

### 3.2. pH changes during storage of mayonnaise

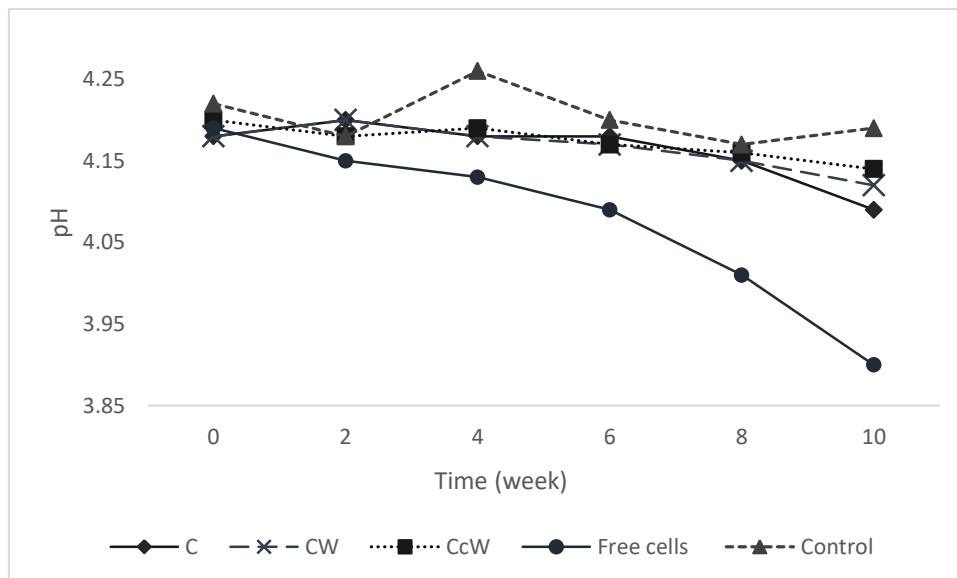


Figure 2. pH changes in mayonnaise during storage condition (C: alginate; CW: alginate + whey; CcW: whey coating alginate).

The results from Figure 2 show that the pH values of samples have a slight decrease during the 10-weeks of storage. In general, the pH values of all probiotic supplemented samples tended to decrease to be lower than that of the non-supplemental control sample, in which the mayonnaise supplement with a free probiotic showed the lowest pH value.

The change in pH value during storage would affect the product's sensory. Fahimdanesh *et al.* [13] reported that the pH value of the probiotic added mayonnaise and control samples was not different during storage time. Whereas, the study of Khalil *et al.* [5] indicated that the pH value of the mayonnaise containing encapsulated bead was lower than the control sample. However, the result from this study (Figure 2) agrees with the study of Ding *et al.* [14] that the pH value of the control sample was higher than that of the probiotic supplement sample. During storage, free probiotic bacterial cells can still use carbohydrates and produce small amounts of acid, which causes the product pH to be decreased gradually during storage [14]. Meanwhile, the uptake of nutrients and metabolic release processes take place more slowly through the microscopic shell of alginate [6] that leading to the pH value of the encapsulated and control samples was slightly higher than that of free probiotic cell sample (Figure 2).

### 3.3. Survival of *B. bifidum* during mayonnaise storage

The effect of storage conditions on the viability of *B. bifidum* in mayonnaise is shown in Figure 3. The results showed that free and encapsulated *B. bifidum* all tend to decrease during ten weeks of storage at 4 °C. In the sample containing free cells, the viability of *B. bifidum* decreased rapidly with more than 5.8 log CFU lost after 4<sup>th</sup> weeks of storage and no cells were recorded after 6<sup>th</sup> weeks of storage (Figure 3). In samples containing encapsulation cells, the viability of *B. bifidum* in C samples decreased sharply to less than 6 log CFU/g while CW and CcC samples were all higher. At the 10<sup>th</sup> week of storage, the probiotic density at all three samples decreased to  $4.17 \pm 0.12$ , respectively;  $5.15 \pm 0.09$ , and  $5.33 \pm 0.12$  log CFU/g.

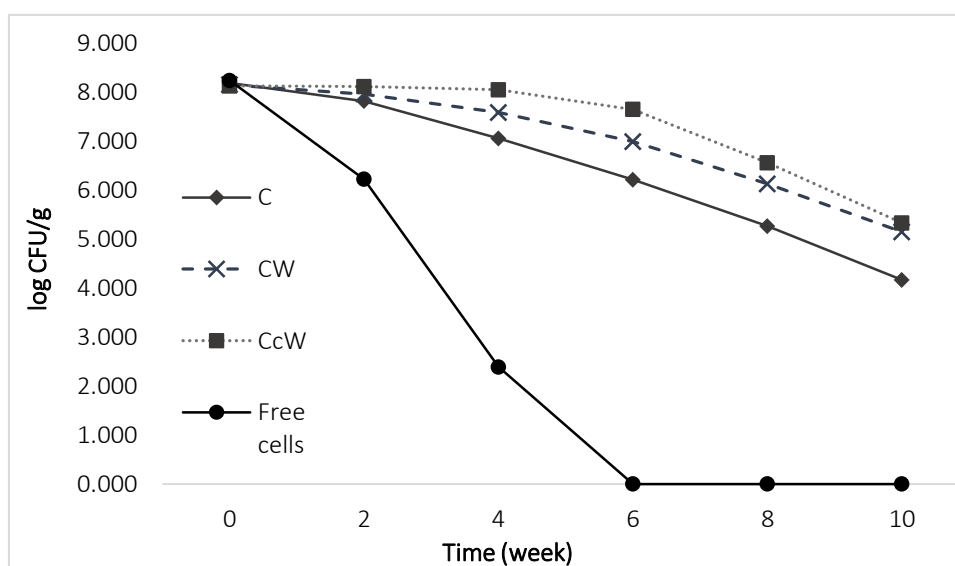


Figure 3. Survival of free and microencapsulated cells in mayonnaise during storage condition (C: alginate; CW: alginate + whey; CcW: whey coating alginate).

The study also showed that CcW samples maintained a stable amount of *B. bifidum* during the first 4-weeks of storage (Figure 3).

Storage conditions in which low pH value products are agents that significantly affect the viability of probiotic bacteria. Besides, the activity of probiotic bacteria still occurs under storage conditions [3], produce acid, and result in pH reduction. These effects are responsible for the decreased sharply in *B. bifidum* density during storage (Figure 3). In previous studies, calcium-alginate showed a higher ability to protect Bifidobacteria in mayonnaise than in free cells. Microcapsules structure helps to limit the impact of the environment inside of the composition [10]. The results from Figure 3 showed that calcium-alginate improved *B. bifidum* survival rate compared to control, and the protective effect was significantly improved when combined with whey protein (Figure 3). The porous structure in the calcium-alginate gel network facilitates inhibitors to enter and cause cell death [9]. High buffering whey protein [15], when combined with calcium-alginate, may help to limit the porous structure of the composition, which improves the *B. bifidum* survival rate (Figure 3). Besides, the concentration of *B. bifidum* in stable CcW samples during the first weeks of storage showed that calcium-alginate coated by whey protein had a better protective effect than the mixed form. The results also show that the 8-week storage period is suitable to ensure the requirement of probiotic food [2].

### 3.4. The growth of yeast and molds during mayonnaise storage

The growth of yeast and molds during 10 weeks of mayonnaise storage was presented in Table 1. These results showed that total yeast and molds counts in a sauce containing free cells were not discovered after 4 weeks, however, the presence of total yeast and molds was recorded after 6 weeks storage. In tenth weeks, the growth of yeast and molds in mayonnaise adding microencapsulated probiotics was remarked (Table 1)

Table 1. The growth of yeast and molds (log CFU/g) in mayonnaise sauce at 4 °C.

Week	C sample	CW sample	CcW sample	Free cells	Control
0	Na	Na	Na	Na	Na
2	Na	Na	Na	Na	Na
4	Na	Na	Na	Na	Na
6	Na	Na	Na	2.14 ± 0.11	2.25 ± 0.18
8	Na	Na	Na	2.81 ± 0.14	2.67 ± 0.22
10	2.22 ± 0.18	2.14 ± 0.16	2.17 ± 0.10	3.16 ± 0.16	3.54 ± 0.19

Na: Not noticed when the density is below 2 logs CFU/g.

Evaluation of yeast and molds growth was to determine the contamination level of food products and control harmful bacteria, which last the shelf life of products. Probiotic bacteria not only contribute health benefits for the consumer but also be an antibacterial agent due to the production of bacteriocin [16]. The results showed that total yeast-mold was recorded in samples containing free probiotic cells at 6<sup>th</sup>-week when *B. Bifidum* was not recorded in these mayonnaise samples (Table 1). The same result for encapsulated *B. bifidum* samples that the total yeast and molds only detected in 10<sup>th</sup> weeks of storage when the viability of *B. bifidum* decreased drastically compared to the initial density (Table 1). A similar result was reported by Khalil *et al.* [5], that the probiotic bacteria significantly affected the growth of the total yeast and molds in mayonnaise. This result showed that the viability of probiotic cells higher 6 log CFU/g would be controlling the growth of yeast and molds in products (Figure 3, Table 1). This finding

also investigated that 8-weeks storage was suitable for ensuring the number of probiotic bacteria in products [2] and controlling yeast and molds growth in products (Figure 3, Table 1).

### 3.5. The viability of *B. bifidum* in SGF and SIF after 8 weeks of storage

The survival of *B. bifidum* in SGF and SIF after 08 weeks of storage is shown in Figure 4. The result showed that there was a significant difference among these samples, in which the viability of *B. bifidum* in C samples was the lowest ( $p < 0.05$ ) in the study. Whey protein has been shown to significantly improve the survival rate of *B. bifidum* and calcium-alginate coated by whey protein, indicating the most effective protection (Figure 4). The condition of digestive fluids is a lethal agent for probiotic bacteria [10]. Calcium-alginate beads significantly improve the viability of probiotic bacteria [5]. However, at pH conditions below 4, the calcium-alginate structure network is easily converted into the soluble alginate form [17], leading to *B. bifidum*, the bacterium that was unable to grow under pH below 4 [18], in the capsules rapidly exposed to pH and cell death conditions (Figure 4). Whey protein with high buffering properties has shown to significantly improve the probiotic microbial viability compared to non-supplemental samples (Figure 4). Restricting the porous structure of calcium-alginate with the addition of other microcapsules published in previous studies has shown to significantly improve the viability of probiotic bacteria [6, 7]. Rajam *et al.* [7] demonstrated that the combination of whey protein and sodium alginate significantly improves the viability of probiotic bacteria in simulated gastrointestinal conditions. However, studies on the protection effectiveness of capsules in mixed and coating ways were poorly reported. The present study showed that capsules with whey protein as the coating layer that shows greater efficacy than mixed forms (Figures 3, 4). This would be because when whey protein added during calcium-alginate microcapsule production has affected the gel structure network. Whereas, the gel structure network was not affected by the whey protein coating process.

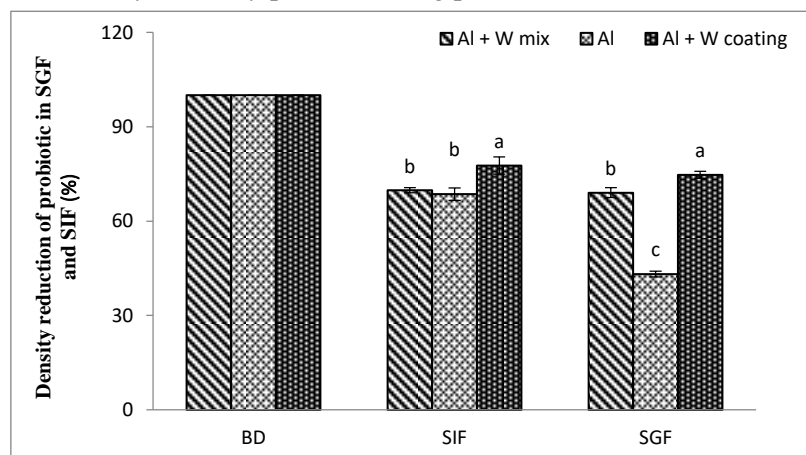


Figure 4. Survey of probiotic density in SFG and SIF.

## 4. CONCLUSIONS

The results of this study indicated that the encapsulation way (mix or coat) did not affect considerably on the encapsulation efficiency of the extrusion method. The storage condition of mayonnaise affected significantly on the survival of *B. bifidum* meanwhile encapsulation technique would improve the viability of this probiotic bacteria. Besides, the pH value of free

cell samples had a trend to decrease lower than that of encapsulated and control samples. Moreover, the development of yeast-molds was related to the density of probiotic in mayonnaise. This result showed that the viability of *B. bifidum* higher 6 log CFU/g would be controlling the growth of yeast and molds in mayonnaise. The supplement of whey protein into calcium-alginate encapsulation improved significantly the survival of *B. bifidum* cells during storage time and in the simulated gastrointestinal conditions. Whey protein coating calcium-alginate identified the best *B. bifidum* protection efficiency compared to others. The results of this study indicated a potential applicability of encapsulated probiotic cells in mayonnaise product.

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