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BIFIDOBACTERIUM BIFIDUM BB28 MICROENCAPSULATED WITH CA-ALGINATE: SURVIVAL UNDER SIMULATED GASTROINTESTINAL CONDITIONS AND STABILITY DURING STORAGE

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The present study was to evaluate the survival rate of free and encapsulated *Bifidobacterium bifidum* BB28 under simulated gastrointestinal conditions and its stability during storage. Results showed that non-microencapsulated *Bifidobacterium bifidum* BB28 was more susceptible to simulated gastrointestinal conditions than microencapsulated bacteria. Microencapsulated *Bifidobacterium* BB28 exhibited a lower population reduction than free cells during exposure to simulated gastrointestinal conditions, the viable count of monolayer microcapsules, double layer microcapsules, and triple layer microcapsules decreased by nine magnitudes, four magnitudes, and one magnitude after 2 h, respectively. The enteric test showed that the microorganism cells were released from the monolayer, double layer, and triple layer microcapsules completely in 40 min. Moreover, the optimum storage times of free *Bifidobacterium* BB28, monolayer microcapsules, double layer microcapsules, and triple layer microcapsules were 21 days, 21 days, 28 days, and more than 35 days in orange juice, pure milk, and nutrition Express (a commercially available milk based drink), and the viable counts were maintained at 1×10^6 CFU g^{-1} or more, which means that the double layer and triple layer of microcapsules of *B. bifidum* BB28 have great potential in food application.

Keywords: *Bifidobacterium bifidum* BB28, stability, survival, microencapsulation, simulated gastrointestinal

Probiotics are living microorganisms, which are beneficial to human health (FAO/WHO, 2002). Lactobacilli and bifidobacteria species have shown beneficial effects on immunomodulation and on susceptibility to various intestinal diseases (Shah. 2007; Denkova et al., 2011). However, these probiotics are also fastidious and obligate anaerobes, which pose a technological challenge for the dairy industry. To utilize their beneficial properties, probiotics must be able to tolerate the acidic conditions in the stomach environment as well as bile in the small intestine (Doleyres et al., 2004). The acidic environment in the stomach and bile salts secreted into the duodenum are the main obstacles to the survival of the ingested bacteria. In general, bifidobacteria have a relatively lower tolerance for the pH of the gastric juice (Collado & Sanz, 2006). Moreover, the survival rate of probiotics during processing and storage of food is also essential for the development of products that have an adequate number of viable cells (Anal & Singh, 2007). The benefits promoted by probiotic bacteria are increasingly explored in different uses in various types of foods (Souza & Saad, 2009). Nowadays, the application of non-milk-based probiotic preparations used to obtain beverages or directly as probiotic tablets, capsules, or lyophilized preparations increases (Denkova et al., 2014).

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Microencapsulation is a promising technique to render physical protection and improve the stability of probiotic organisms in functional food products (Gouin, 2004; Anal & Singh, 2007; Brinques & Maz, 2011). However, results showed that the survival rate of probiotics after exposure to acid are rarely satisfactory, and when a high protection against gastric juice was noted, the survival of the cells dramatically decreased within a few weeks during storage (Albertini et al., 2010).

The aim of this paper is to evaluate the survival rate of Ca-alginate microencapsulated *Bifidobacterium bifidum* BB28 (*B. bifidum* BB28) in artificially simulated gastric juices, bile salt, and intestinal tract, and to study the stability of microencapsulation of *B. bifidum* BB28 in food.

1. Materials and methods

1.1. Materials

The strain of *B. bifidum* BB28 was obtained from School of Food & Biology Engineering, Shaanxi University of Science & Technology. Alginate (Luo Senbo Technology Co., Ltd. Xi'an), was used as carrier agent and MRS broth (Hope Bio-Technology Co., Ltd. Qingdao) as cultivation medium. All chemicals used were of analytical grade. Centrifuge (LG10-2.4) was used to obtain microcapsules.

1.2. Microorganism

B. bifidum BB28 was cultured for 24 h in MRS medium at 37 °C, the cells were harvested by centrifugation at 1500×*g* for 10 min at 4 ºC, and washed twice before suspending them in 5 ml normal saline. The final cell concentration was adjusted to 1.0×10^{11} CFU ml^{-1}.

1.3. Microencapsulation

B. bifidum BB28 was encapsulated in sodium alginate matrix. Sodium alginate solutions (2% w/v) and chitosan solution (1% w/v, pH 5.3) were prepared, sterilized by autoclaving (120 ºC for 15 min) and cooled to 38–40 ºC. Palm oil (food grade) was prepared, melted for 15 min at 37 ºC. 2% sodium alginate solutions (10 ml) and 1 ml of free cell suspension were transferred into a centrifuge tube, and the content was vortexed to homogeneity. The alginate–cell mixture was added dropwise to a beaker (300 ml) containing an emulsion of oil and water in ratio 5:1, containing 0.4% Tween 80, while stirring magnetically. After 15 min, a uniformly turbid emulsion was obtained where 2% calcium chloride was quickly added to harden microcapsules and break the emulsion. The monolayer microcapsules (MM) were harvested by centrifugation at 1500×*g* for 10 min. To form chitosan coated sodium alginate double layer microcapsules (DM), 1% chitosan solution was added and mixed with the above-mentioned monolayer microcapsules for 30 min, and washed with sterile saline solution for three times. Finally, the palm oil coated sodium alginate-chitosan beads were formed by adding palm oil, mixing for 30 min, and washing with sterile saline solution for three times. Thus, free *B. bifidum* BB28, alginate sodium encapsulated *B. bifidum* BB28 cells (MM), alginate-chitosan encapsulate *B. bifidum* BB28 (DM), and alginate-chitosan-palm oil encapsulate *B. bifidum* BB28 (TM) were obtained for further evaluation of survival and storage stability.

1.4. Viable count

The 1 g capsules were transferred into test tubes containing 9 ml tri-sodium citrate to free the encapsulated bacteria. The samples were diluted tenfold by sterile saline solution, and the appropriate dilutions were transferred to anaerobic tubes containing MRS agar, then incubated at 37 ºC for 48 h. The method for counting viable bacteria was described by CHEN and co-workers (2012).

1.5. Survival of microencapsulated and non-microencapsulated B. bifidum *BB28 under simulated gastrointestinal conditions*

1.5.1. Gastric juice tolerance test. The simulation of gastric juice conditions was done in HP medium (0.16% hydrogen chloride, 0.1% pepsin) with pH adjusted to 1.2. One gram of microcapsules or 1.0 ml of free suspended cells was added to test tubes containing 9 ml of HP medium. The tubes were incubated at 37 °C and samples were collected in triplicate at 0 h, 1 h, and 2 h. The viable counts of the free and encapsulated *B. bifidum* BB28 were evaluated as described in Section 1.4.

1.5.2. Bile salt tolerance test. To determine the resistance to bile salts, 1 ml of free and 1 g of encapsulated cells were transferred to 9 ml solution containing 1% bile salts. Triplicate samples were collected after incubation at 37 °C for 0 h, 1 h, and 2 h. Cell counts of the free and encapsulated bacteria were enumerated as described in Section 1.4.

1.5.3. Simulated intestinal fluid test. To evaluate the release time of microencapsulated cells in simulated intestinal fluid, microcapsules (1 g) were added to test tubes containing 9 ml pre-warmed 37 °C simulated intestinal fluid (1.38% potassium dihydrogen phosphate, 0.4% sodium hydroxide, and 1% pancreatic enzymes), and samples in triplicate were taken after incubation at 37 °C for 0 h, 1 h, and 2 h. The viable counts were enumerated as described in Section 1.4.

1.6. Evaluation of the stability of microencapsulated B. bifidum *BB28 in juice, nutrition Express, and pure milk*

One g of microcapsulated *B. bifidum* BB28 was added to 9 ml of orange juice, nutrition Express, and pure milk, and then the viable count, pH, and acidity were measured every 7 days while storage at 4 ºC and room temperature. Also, the control group experiments were performed by adding *B. bifidum* BB28 free cell suspension to orange juice, nutrition Express, and pure milk.

1.7. Determination of pH

The acidity meter PHs-3c was used to determine the pH at room temperature (HE et al., 2011; SHU et al., 2012).

1.8. Determination of acidity

The determination of acidity was performed according to DENKOVA and co-workers (2012).

2. Results and discussion

2.1. Survival of B. bifidum *BB28 in simulated gastrointestinal conditions*

2.1.1. Gastric juice tolerance test. As seen from Table 1, there was a significant decrease in the viability of free *B. bifidum* BB28 cells and monolayer microcapsules compared to double layer and triple layer microcapsules under simulated acidic conditions. The viable counts of free *B. bifidum* BB28 and monolayer microcapsules in 2 h decreased to 0. For the other two samples the protection provided by the chitosan was due to strong bonding between chitosan and alginate by electrostatic interactions, leading to formation of a membrane on the surface of the granules. Similar results were obtained by Annan and co-workers (2008), which illustrated double and triple layer *Bifidobacterium* microcapsules had a better stability and stronger resistance to acid. On the other hand, Sun and Griffiths (2000) found that the viable count of free *Bifidobacterium* decreased from 1.233×10^9 CFU ml^{-1} to an undetectable level in 30 min, however, the viable count of immobilized cells in gellan–xanthan beads decreased by only 0.67 log cycle in the same time interval, and 6.3×10^5 CFU ml^{-1} remained after 120 min, which suggested that immobilization protected further *Bifidobacterium* from the extreme acid environment in the human stomach. The microencapsulation efficiency for monolayer, double layer, and triple layer microcapsules were 88.24%, 89.24%, and 100% in present experiment, respectively.

Table 1. Viability of microencapsulated and non-microencapsulated *B. bifidum* BB28 under simulated acidic conditions (CFU g^{-1})

Samples	0	1 h	2 h
FS	4.41×10^9	1.09×10^3	0
MM	2.14×10^9	6.23×10^4	0
DM	1.97×10^9	3.92×10^7	5.79×10^5
TM	1.97×10^9	1.02×10^9	5.36×10^8

FS: free cell suspension; MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules

2.1.2. Bile salt tolerance test. Table 2 shows that the viable count of free *B. bifidum* BB28 and monolayer microcapsules decreased to 0 after 2 h, but the viable count of double layer and triple layer microcapsules were 6.89×10^5 CFU ml^{-1} and 5.92×10^8 CFU ml^{-1}, respectively. The mall materials used for microencapsulation were chitosan and palm oil, which improved the viability of *B. bifidum* BB28. Similar results were obtained by Chávarri and co-workers (2010).

Prebiotics can promote the growth of probiotics in microcapsules. Previous research indicated that chitosan coating could protect microcapsules in bile salt solution due to an ion exchange reaction (Shi et al., 2013a; b). In addition, Sohail and co-workers (2011) found that the survival rate of probiotics was highly dependent on the species microencapsulated. Castro-Cislaghi and co-workers (2012) found that microencapsulation with whey failed to protect probiotic cells, but in the current study, when using sodium alginate, chitosan, and palm oil to protect probiotic cells, the results showed that the survival rate of encapsulated *B. bifidum* BB28 was satisfactory in bile.

Table 2. Viability of microencapsulated and non-microencapsulated *B. bifidum* BB28 under simulated bile conditions (CFU g^{-1})

Samples	0	1 h	2 h
FS	4.41×10^{9}	2.01×10^{3}	0
MM	2.14×10^{9}	0.93×10^{5}	0
DM	1.97×10^{9}	3.78×10^{7}	6.89×10^{5}
TM	1.97×10^{9}	1.12×10^{9}	5.92×10^{8}

FS: free cell suspension; MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules

2.1.3. In vitro enteric test. Table 3 shows the viability of microencapsulated *B. bifidum* BB28 released under simulated enteric tract conditions. It can be seen that the viable count reached the maximum when the microcapsules were treated for 40 min, however, the number of living bacteria showed a downward trend after that. The enteric test showed the microorganism cells were released from the monolayer, double layer, and triple layer microcapsules completely in 40 min, so the method by microencapsulating to protect probiotics was effective. Moreover, Martoni and co-workers (2007) verified an increase in viability of 0.9–1.0 log CFU ml^{-1} when *L. plantarum* 80 BSH+ strain was exposed to simulated intestinal conditions for a 10 h incubation time.

Table 3. Viability of microencapsulated *B. bifidum* BB28 released under simulated enteric tract conditions (CFU g^{-1})

Samples	20 min	40 min	60 min
MM	1.90×10^{7}	2.14×10^{9}	1.90×10^{9}
DM	3.83×10^{6}	1.97×10^{9}	1.09×10^{9}
TM	1.12×10^{6}	1.97×10^{9}	1.81×10^{9}

MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules

2.2. The stability for the microcapsules of B. bifidum *BB28 in orange juice, nutrition Express, and pure milk.* Figures 1 to 2 show the stability of free *B. bifidum* BB28, monolayer microcapsules, double layer microcapsules, and triple layer microcapsules in orange juice and nutrition Express. It can be seen from the figures that the viable counts of *B. bifidum* BB28 under 4 ºC and room temperature decreased in the following order: free cells, monolayer microcapsules, double layer microcapsules, and triple layer microcapsules. The main reason for the decrease in the number of viable bacteria was the acidic environment in orange juice and nutrition Express, which could inhibit or even kill some of the bacteria, but the viable count was maintained at 1×10^{6} CFU g^{-1} or more, for double layer and triple layer microcapsules after 28 days. Figure 3 shows that the viable counts of free *B. bifidum* BB28, monolayer microcapsules, double layer microcapsules, and triple layer microcapsules remained 10^{10} CFU g^{-1} in pure milk at 4 ºC, but the viable count increased to 10^{12} CFU g^{-1} at room temperature, the reason for this phenomenon were that the temperature promoted the growth and reproduction of the bacteria, what's more, the pH value of pure milk was close to neutral,

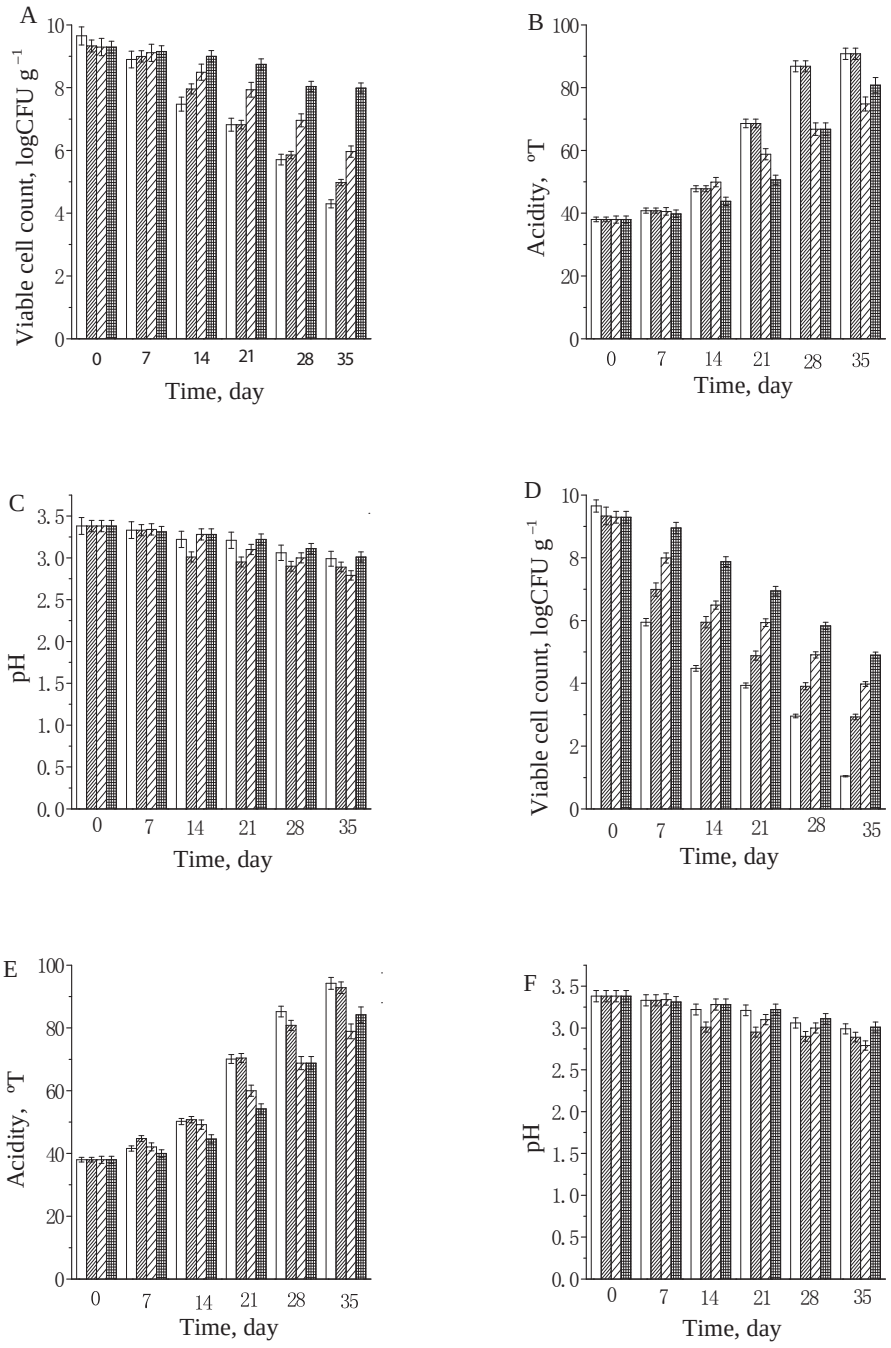

Fig. 1. The viable cell count, acidity, and pH of *B. bifidum* BB28 in fruit orange stored at 4 °C (A, B, C) and room temperature (D, E, F) (FS: free cell suspension; MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules)

□: FS; ▨: MM; ▨: DM; ▨: TM

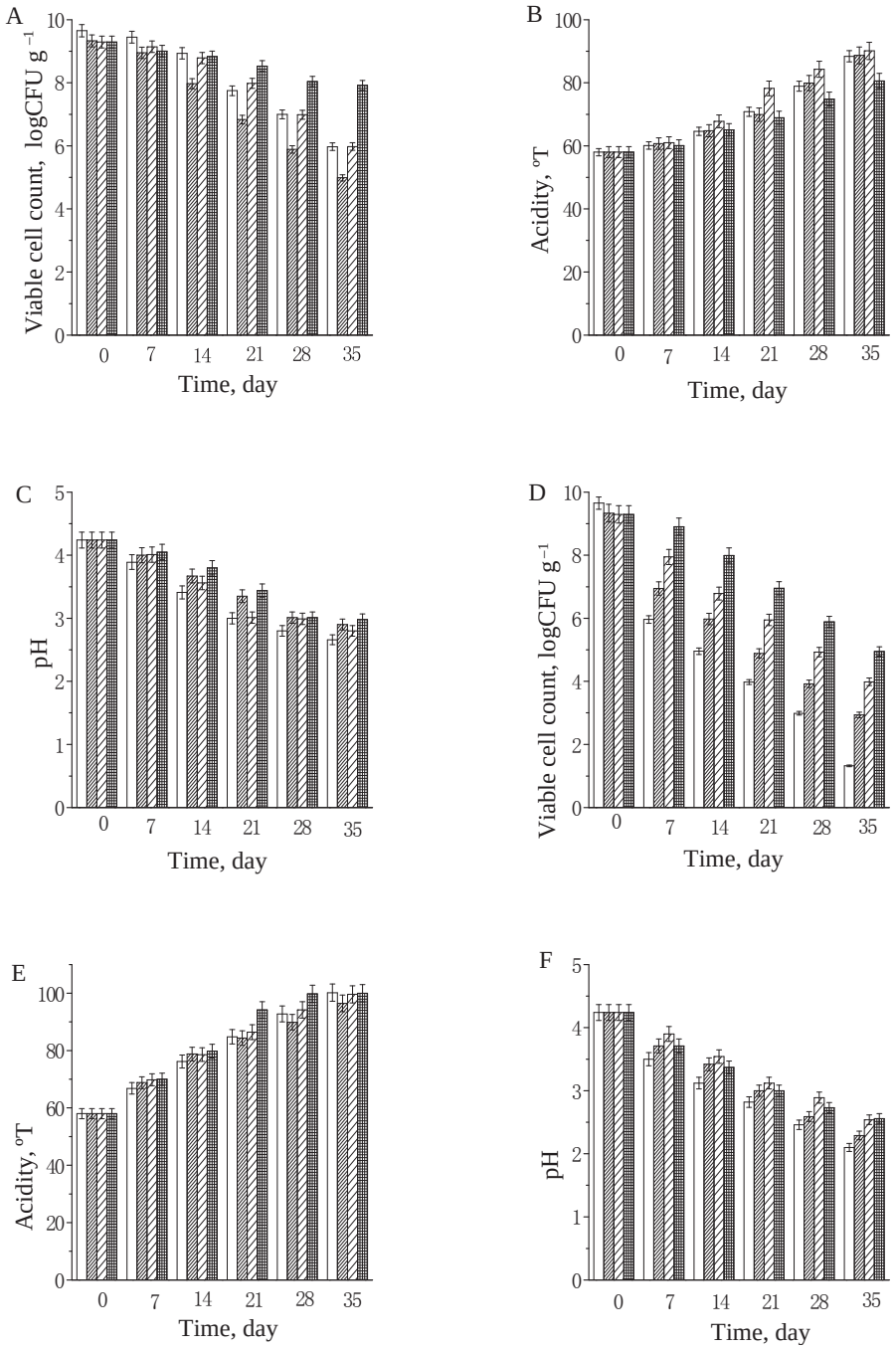

Fig. 2. The viable cell count, acidity, and pH of *B. bifidum* BB28 in nutrition Express stored at 4 °C (A, B, C) and room temperature (D, E, F) (FS: free cell suspension; MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules)
□: FS; ▨: MM; ▨: DM; ▩: TM

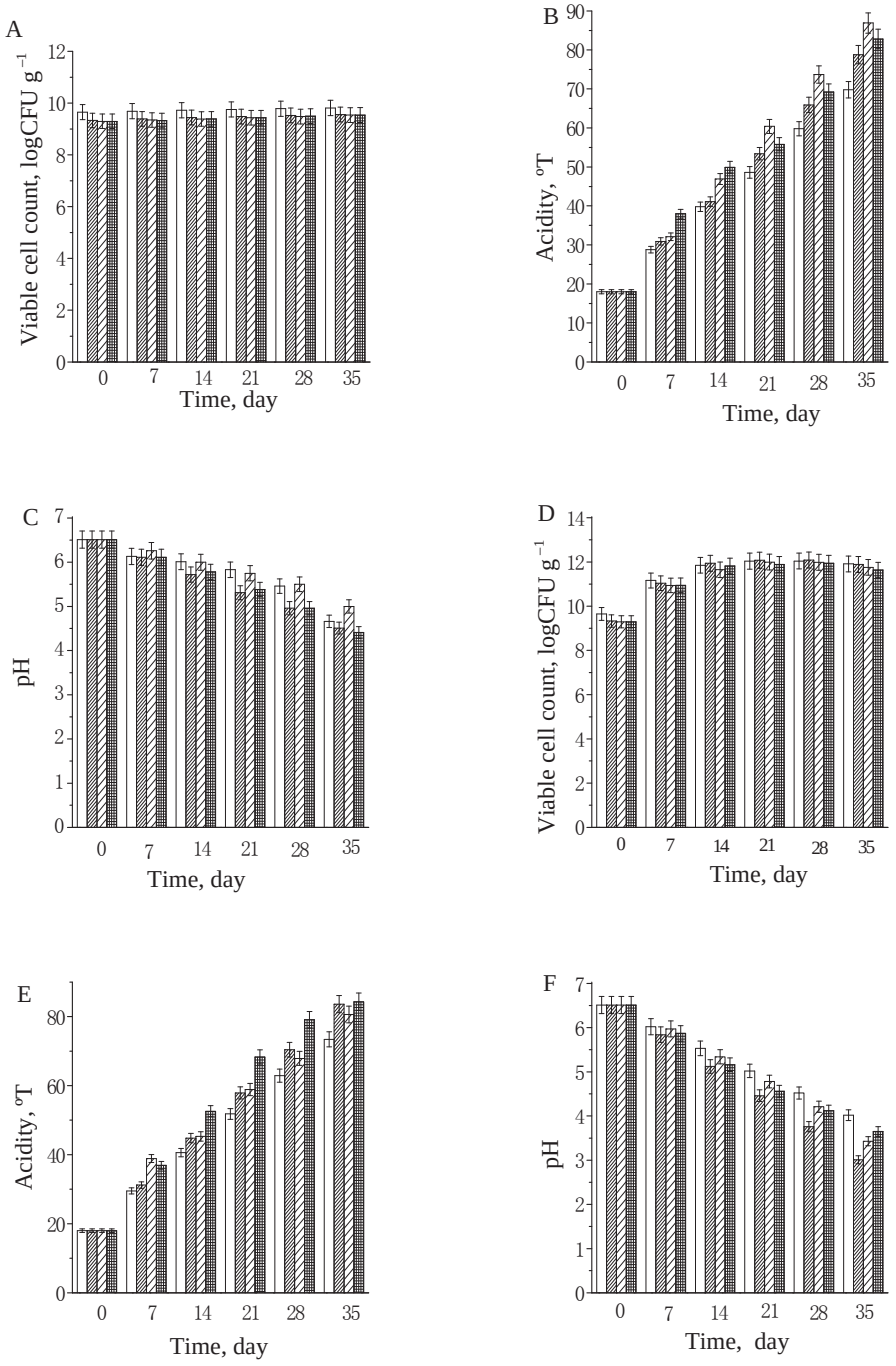

Fig. 3. The viable cell count, acidity, and pH of *B. bifidum* BB28 in pure milk stored at 4 °C (A, B, C) and room temperature (D, E, F) (FS: free cell suspension; MM: monolayer microcapsules; DM: double layer microcapsules; TM: triple layer microcapsules)
□: FS; ▨: MM; ▨: DM; ▨: TM

which was suitable for the preservation of *B. bifidum* BB28. It can be concluded from the figures that the optimum storage times of free *B. bifidum* BB28, monolayer microcapsules, double microcapsules, and triple layer microcapsules were 21 days, 21 days, 28 days, more than 35 days; and 7 days, 14 days, 21 days and 28 days at 4 ºC and room temperature, respectively. Nevertheless, Gross and Fávaro-Trindade (2004) found the number of viable cells of immobilized *B. lactis* in yoghurt presented a gradual decline during the whole storage period, passing from 10^8 CFU ml^{-1} to no count after 28 days of storage, the reasons for this phenomenon were inhibitory substances produced by the yoghurt culture or an excess of dissolved oxygen. But Matias and co-workers (2016) found the populations of *Bifidobacterium* Bb-12 were slightly exceeding 6 log CFU g^{-1} in ice cream with oligofructose at 90 days of storage; meanwhile the viability of *B. animalis* Bb-12 in all synbiotic apple ice cream formulations tested was satisfactory until the 84th day of frozen storage, with populations of around 7.5 to 8.5 log CFU g^{-1}.

3. Conclusions

In this study, acid and bile salt resistance results showed that the viable count of free *B. bifidum* BB28 decreased to 0 after 2 hours in simulated bile salt and gastric juice, and the viable counts of monolayer, double layer, and triple layer microcapsules decreased by nine magnitudes, four magnitudes and one magnitude, respectively. The enteric test showed that the microorganism cells were released completely from the microcapsules under simulated intestinal fluid in 40 min, which demonstrated the applicability of microcapsules of *B. bifidum* BB28 in food. In addition, the optimum storage times, maintaining 1×10^6 CFU g^{-1} or more, of free *B. bifidum* BB28, monolayer microcapsules, double layer microcapsules, and triple layer microcapsules were 21 days, 21 days, 28 days, and more than 35 days, respectively, at 4 °C; and 7 days, 14 days, 21 days, and more than 28 days, respectively, at room temperature overall in orange juice, pure milk, and nutrition Express. The experimental results showed that the double layer and the triple layer microcapsules of *B. bifidum* BB28 had great application possibilities in food.

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