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Improvement of Probiotic Survival in Fruit juice and under Gastrointestinal conditions using Pectin-Nanochitin-Nanolignocellulose as a Novel Prebiotic Gastrointestinal-Resistant Matrix

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Abstract

Background and Objective: Increasing survivability of probiotics in low pH juices and in gastrointestinal conditions is important for probiotic food industry. Nanofibers can reinforce the structure of entrapment matrices protecting probiotics in harsh conditions. This study investigated pectin-based bionanocomposites improved with nanochitin, nanolignocellulose and bacterial nanocellulose to introduce a prebiotic gastrointestinal-resistant matrix for enhancing the survival of *Bacillus coagulans* as a probiotic.

Material and Methods: The bionanocomposites with various compositions were designed using mixture design method. These were fabricated based on cross-linking of calcium ions with pectin for entrapment of *Bacillus coagulans*. The survivability of probiotic was evaluated at 4°C or 25°C over a 5-week storage in peach juice and under simulated gastrointestinal conditions.

Results and Conclusion: The prebiotic score of the pectin-nanochitin-nanolignocellulose (50:25:25% w w^{-1}) was determined as 1.36. The survivability of *Bacillus coagulans* entrapped within the pectin-nanochitin-nanolignocellulose matrix was ~65% under gastrointestinal treatment. The surface structure of the matrix was relatively smooth coherent, compact and wrinkled due to the three-dimensional arrangement of the nanofibers of chitin and lignocellulose incorporated within pectin. The highest survivability of the entrapped bacteria was ~68% compared to the survivability of the free cell (~53%) at the end of 5-week storage period. After 21 day storage in the juice, the survivability of the entrapped bacteria treated under sequential digestion was ~58% as compared to that of the free cell (~43%). The present findings proposed a promising prebiotic matrix to protect probiotics in low pH fruit juice and the gastrointestinal tract.

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1. Introduction

Probiotic foods are a wide variety of traditional functional foods, which have increased considerably in the recent years. Probiotic beverages can be fruit juices and are considered as healthy products containing minerals, vitamins and antioxidants. They are pleasing to all age groups and beneficial medium for probiotics, providing probiotic cultures to consumers [1,2].

Low pH environment of some fruit juices (≤ pH 4.0) is a challenge to maintain a sufficient number of viable probiotics [3]. Furthermore, probiotics must survive after passage through gastrointestinal tract. The minimum recommended level required to achieve health benefits is 10^6 CFU ml^{-1} at the time of consumption [4,5]. Encapsulation has frequently been used for protection against the damages caused by the stressing environmental factors. It can provide a particularly suitable protection for probiotics to survive in fruit juices and gastrointestinal conditions [6]. Among the encapsulating materials, various polysaccharides such as pectin, alginate, resistant starch, chitin, chitosan, cellulose, carrageenan and vegetable gums have been studied to provide protection for probiotics [7,8]. Experimental evidence has demonstrated that microencaps-

ulation of *Lactobacillus (L.) rhamnosus* GG using mixtures of resistant starch and whey protein isolate protected the probiotic in pH 3.5 for apple juice over 5 week storage at 25°C [9]. In addition, encapsulation of *L. rhamnosus* GG within chitosan-alginate bead increased bacterial survivability during 90 days storage of apple juices at both 4°C and 25°C, and exhibited a higher survival rate during gastrointestinal treatments compared to the free bacteria [10]. As evaluation of the potential use of encapsulated *L. paracasei* L26 in fruit juices, alginate-based microcapsules resulted in good viability in both orange and peach juices over 50 days of storage at 5°C despite the low pH values of both juices [11]. The viability of *L. plantarum* microencap-sulated in calcium alginate, in the natural orange juice throughout six months of storage, and after incubation for 120 min in simulated gastrointestinal juices, revealed a significant resistance in these environments [12]. *L. rhamnosus* GG was encapsulated within carrageenan, alginate and pectin coated micro-beads which provided the best cell protection during storage in cranberry and pomegranate juices (pH 2.4; 28 days; 4 and 25°C) and illust-rated high survival after gastrointestinal incubation [13].

Among the polysaccharides used for encapsulation, pectin as a prebiotic is one of the most preferred encapsulation agents due to its non-toxic, biocompatible and inexpensive properties. Chitin as a biocompatible and non-toxic polymer has been considered to fabricate delivery systems passing through the gastrointestinal tract. Furthermore, cellulose as an important biopolymer and the most abundant renewable resource has been studied for protection of probiotics against gastrointestinal conditions by incorpor-ated in a pectin matrix. Lignocellulose mainly including cellulose, hemicellulose and lignin can be assessed as a prebiotic for encapsulation of probiotics [14-16]. Polysacch-aride nanofibers due to their nano-scale diameters reinforce the structure and compact the surface of entrapment matrices, thus promoting the residence time for probiotic cells in harsh conditions such as low pH juices and gastrointestinal conditions. In our previous research, pectin-non-starch nanofibers were investigated as new entrapment matrices [17] but combination of the nanofibers was not studied to enhance the survival of probiotics under gastro-intestinal conditions and low pH juices.

The objective of the present study was to evaluate the effect of microencapsulation of the probiotic *Bacillus* (*B.*) *coagulans* using composites of pectin with nanochitin, nanolignocellulose and bacterial nanocellulose on the probiotic survival under simulated gastrointestinal conditions and in peach juice during 5 week storage at 4°C and 25°C. Few studies have been reported on probiotic encapsulation efficacy during fruit juice storage. To the best of our knowledge, no one has studied pectin-based microencapsulation matrix modified with the nanofibers as a prebiotic protective agent for probiotics in fruit juices.

2. Materials and Methods

2.1 Materials

Probiotic strain *B. coagulans* IBRC-M 10807 was obtained from Iranian Biological Research Center (IBRC). Pectin (pec) was extracted from citrus peel and dried using previously established method [18]. Bacterial nanocellulose (BNC) with the average fibril diameter of 50 nm, nanochitin (NC) with the average fibril diameter of 30 nm, and nanolignocellulose (NLC) with the average fibril diameter of 65 nm were purchased from Nano Novin Polymer Co. (Sari, Iran). Pepsin from porcine gastric mucosa (0.7 FIP-U mg^{-1}), pancreatin from porcine pancreas (350 FIP-U g^{-1} Protease, 6000 FIP-U g^{-1} Lipase, 7500 FIP-U g^{-1} Amylase), and calcium chloride were acquired from Merck (Darms-tadt, Germany). Bile salts were supplied from Sigma–Aldrich (St. Louis, MO, USA). TSB was purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). Commercially available peach juice (pH 3.6) was purchased from a local supermarket (Takdaneh Agro-industrial Co., Tehran, Iran). All chemicals were used as received without any further purification.

2.2 Preparation of microencapsulated probiotics

In order to fabricate microencapsulation matrices, the various formulations of the polysaccharide mixtures are designed using mixture design method, given in Table 1. Based on the concentrations showed in Table 1, the required amount of the constituents were suspended in deionized water and mixed together at 60°C to produce homogenous mixtures with the final concentration of 1% (w v^{-1}). The mixtures were agitated at 500 rpm for 60 min, then autoclaved at 121°C for 20 min.

B. coagulans was grown overnight in TSB medium at 37°C. The cells were centrifuged at 4000 ×g for 10 min, then washed with sterile saline solution (0.5% w v^{-1}) and re-suspended in the same solution. Sterilized mixtures prepared earlier were mixed with the cell suspension at a ratio of 1:1 (v v^{-1}). To obtain the microencapsulated probiotic, the synbiotic (cell + polymers mixture) suspension was added drop-wise to 5% w v^{-1} $CaCl_2$ solution (crosslink-ing agent), then kept at 4°C for 1 h to form and harden the matrix. The synbiotic matrices were centrifuged at 16000 ×g for 10 min, then dried at 50°C for 12 h.

Table 1. Mixture compositions for fabrication of the microencapsulation matrices, their prebiotic scores and potential to survive *B. coagulans*.

Mixture	Pectin (g g^{-1} matrix)	Bacterial nanocellulose (g g^{-1} matrix)	Nanochitin (g g^{-1} matrix)	Nanolignocellulose (g g^{-1} matrix)	Prebiotic score	Viable probiotic (log CFU g^{-1} matrix)	Viable probiotic reduction (log CFU g^{-1} matrix)	Survivability (%)
1	0.50	0.50	0.00	0.00	0.83 ± 0.01	7.88 ± 0.03	0.89 ± 0.03	89.87
2	0.50	0.25	0.25	0.00	0.94 ± 0.02	7.14 ± 0.03	1.63 ± 0.01	81.43
3	0.50	0.25	0.00	0.25	1.24 ± 0.04	5.22 ± 0.03	3.55 ± 0.01	59.49
4	0.50	0.00	0.50	0.00	1.12 ± 0.04	6.92 ± 0.04	1.85 ± 0.01	78.90
5	0.50	0.00	0.25	0.25	1.36 ± 0.02	5.73 ± 0.02	3.04 ± 0.02	65.40
6	0.50	0.00	0.00	0.50	1.60 ± 0.02	5.07 ± 0.01	3.70 ± 0.02	57.80
7	0.50	0.34	0.08	0.08	0.96 ± 0.01	5.88 ± 0.01	2.89 ± 0.01	67.01
8	0.50	0.08	0.34	0.08	1.02 ± 0.01	5.80 ± 0.03	2.97 ± 0.03	66.16
9	0.50	0.08	0.08	0.34	1.2 ± 0.01	4.62 ± 0.01	4.15 ± 0.03	52.74
10	0.50	0.17	0.17	0.17	1.14 ± 0.01	5.37 ± 0.01	3.40 ± 0.01	61.25
11	0.50	0.50	0.00	0.00	0.83 ± 0.03	7.54 ± 0.01	1.23 ± 0.04	88.07
12	0.50	0.00	0.50	0.00	1.06 ± 0.03	6.64 ± 0.02	2.13 ± 0.02	79.80
13	0.50	0.00	0.00	0.50	1.58 ± 0.02	5.00 ± 0.04	3.77 ± 0.01	58.30
14	0.50	0.25	0.25	0.00	0.91 ± 0.01	7.00 ± 0.04	1.77 ± 0.01	83.14
Free cell	-	-	-	-	-	3.95 ± 0.01	4.82 ± 0.03	45.07
Initial cell	-	-	-	-	-	8.62 ± 0.02	-	-

3.2. Preparation of simulated gastrointestinal (SGI) juices

SGI juices were simulated as described previously [19]. In brief, the simulated gastric juice was prepared by suspending pepsin in sterile saline (0.5% w v^{-1}) to a final concentration of 3 g l^{-1} and pH was adjusted to 2.00 using 1 N HCl. The simulated intestinal juice was prepared by suspending pancreatin in sterile saline to a final concentration of 1 g l^{-1} and adding bile salts (4.5% w v^{-1}) and pH was adjusted to 8.00 using sterile 1 N NaOH.

4.2. Prebiotic score of polysaccharide mixtures

Prebiotic score (PS) of each mixture was evaluated by the procedure previously established (20) with some modific-ations. In order to determine PS of the prepared blends (Table 1), the assay was carried out by adding 1% v v^{-1} of an overnight culture of the probiotic to separate tubes containing 1 ml TSB with 0.1% w v^{-1} glucose or polysacch-aride blend prepared, then incubated at 37°C under aerobic condition. After 24 h of incubation, the sample was enumer-ated on TSA. Each assay was repeated three times. The prebiotic score was calculated using the Eq. 1 [20].

5.2. Survivability of microencapsulated probiotic under gastrointestinal conditions

The survivability of the probiotic in the simulated gastric and intestinal juices was determined by the procedure reported [19,21] with some modifications.

Under gastric/intestinal condition, 1.0 ml of the simul-ated gastric/intestinal fluid was transferred into the tube containing the synbiotic matrix (or free cell), and then mixed well. Then, it was incubated at 37°C in an incubator.

Under gastrointestinal conditions, the synbiotic matrix (or free cell) was examined under sequential digestion. The sample was first examined under gastric digestion as described. Then, the sample was centrifuged at 16000 ×g for 15 min and decanted. The remainder was last examined under intestinal digestion as described.

After incubation under gastric/intestinal/gastrointestinal condition, the sample was centrifuged at 16000 ×g for 15 min and decanted. The reminder was serially diluted with 0.1% w v^{-1} sterilized peptone water for cell counts. The diluted sample was transferred onto TSA plate and incubated at 37°C for 48 h under aerobic condition. Individual colonies appeared were counted as colony forming units per gram of the synbiotic matrix (CFU g^{-1}) or per milliliter of the juice (CFU ml^{-1}), and the results were reported as Log_{10} values. The probiotic survival under gastric/intestinal/gastrointestinal condition was determined using the Eq. 2 (22).

6.2. Incorporation of microencapsulated probiotic in peach juice

Microencapsulated *B. coagulans* or free cell was incorporated into peach juice (pH 3.6). The juice was sterilized in 50 ml falcon (20 min at 121°C) before use. One percent dried synbiotic matrix (1% w v^{-1}) was added into the juice and dispersed. The pH of the juice and synbiotic juice were measured in the beginning and at week 1, 2, 3, 4 and 5 during storage at 4 and 25°C. The survivability of microencapsulated *B. coagulans* incorporated into peach juice was also measured at the same time. To avoid sample contamination, subsamples for viability testing were removed prior to measurement of pH. *B. coagulans* were enumerated as the procedure explained earlier in previous section.

7.2. Scanning electron microscopy

The surface morphology of the samples was identified using the scanning electron microscopy (XL30, Philips, Netherlands). The samples were coated with gold using the sputtering technique to improve the conductivity of the samples. The dried samples were fractured and observed at beam energy of 20.0 kv.

8.2. Optical microscopy

The integrity and morphological changes of the matrix during storage in peach juice were observed by optic microscope (Lotus MCX51, Micros, Austria). The samples were placed on glass microscope slides and then observed at ×100 and ×400 magnifications. Images were photographed using a digital camera.

9.2. Statistical analysis

All experiments were carried out in triplicate and the obtained data were analyzed using Design-Expert (Version 7 trial, Stat-Ease Inc., Minneapolis, USA) and expressed as mean standard error. The statistical significance of the results was evaluated using analysis of variance (ANOVA) at the 95% confidence level.

$$\text{PS}= \frac{[(\text{probiotic log (CFU ml}^{-1}\text{) on the prebiotic at 24 h}) - (\text{probiotic log (CFU ml}^{-1}\text{) on the prebiotic at 0 h})]}{[(\text{probiotic log (CFU ml}^{-1}\text{) on glucose at 24 h}) - (\text{probiotic log (CFU ml}^{-1}\text{) on glucose at 0 h})]} \quad \text{Eq. 1}$$

$$\text{Survivability} = \frac{[\text{bacteria log (CFU g}^{-1}\text{) after exposure to gastric/intestinal/gastrointestinal condition}]}{[\text{bacteria log (CFU g}^{-1}\text{) before exposure to gastric/intestinal/gastrointestinal condition}]} \times 10 \quad \text{Eq. 2}$$

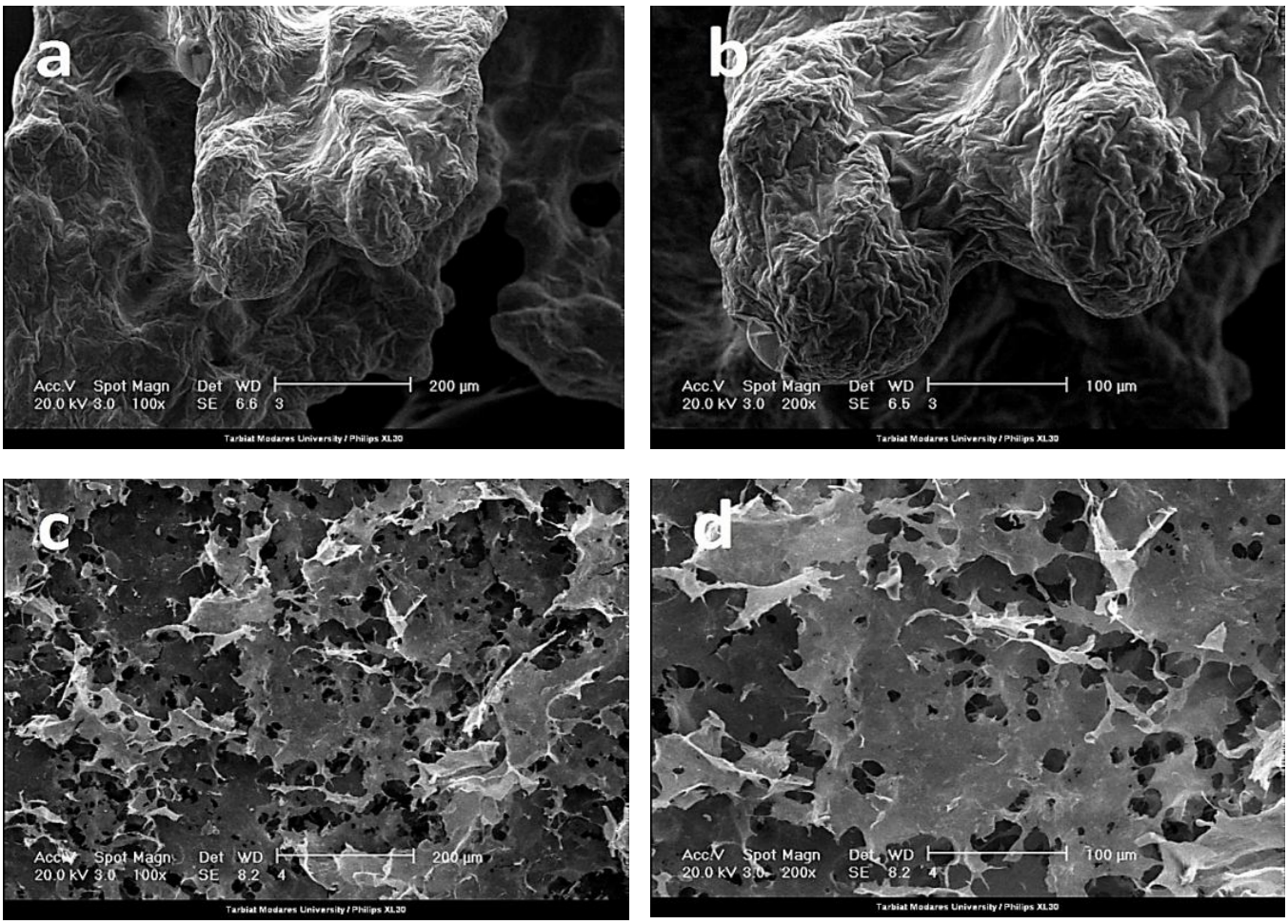

Figure 1. Scanning electron microscopy images of (a, b) the microencapsulation matrix and (c, d) microencapsulation matrix treated under gastrointestinal juices.

3. Results and Discussion

3.1. Microencapsulation matrix

Ten different compositions were designed for fabrication of microencapsulation matrices (Table 1). Prebiotic score of the compositions was determined based on the growth of *B. coagulans* on the composition compared with glucose. Among the mixtures, pectin incorporated with NLC resulted the highest PS (1.59 ± 0.03) and the lowest (0.83 ± 0.01) with BNC. Consequently, NLC indicated good potential for improving the prebiotic score compared to NC and BNC whereas BNC showed the least effect on the prebiotic score.

Properties of a prebiotic determine its fermentability to probiotic. Water solubility and available surface area are the most important properties which can enhance the prebiotic score. Lignocellulosic materials composing of lignin, water soluble and insoluble polysaccharides, can be produced using alkaline and acidic hydrolysis treatment. In the production process, various prebiotic carbohydrates such as sugars and oligosaccharides can also be released. These carbohydrates can be efficiently fermented by the probiotic compared to pectin [23,24]. Furthermore, used NLC fibril diameter of 65 nm creates a high surface area susceptible to the probiotic. Thus, it is expected that NLC could act as a prebiotic for *B. coagulans* more effectively when compared with other polysaccharides used. In comparison to lignocellulose, chitin is poor water-soluble while NC with positively charged group of amine is more soluble than BNC. In addition, used NC with the average fibril diameter of 30 nm has a larger surface area exposed to the probiotic compared to BNC with 50 nm, thereby NC is more effective than BNC on enhancing PS [25,26].

Microencapsulation matrices, containing the probiotic cells, were evaluated under 8 h sequential digestion (4 h intestinal digestion following 4 h gastric digestion). The highest survivability of the probiotic was obtained by the microencapsulation within the pec-BNC matrix. It was approximately 89% ± 0.1 compared with the lowest (52.7% ± 0.1) obtained by the pec-BNC-NC-NLC. However, all compositions were successfully introduced for protection of *B. coagulans* since the survivability of the free cell was approximately 45% ± 0.3 which was 8% less than the lowest survivability associated with the protected cell (Table 1). To select the most appropriate formulation of the matrix, we should consider both survivability and prebiotic score. Among the compositions, pec-NC-NLC composition (mixture 5 in Table 1) was determined as the best prebiotic protective formulation for fabrication of the microencaps-ulation matrix. This formulation resulted PS of 1.36 ± 0.02 and survivability of 65.4% ± 0.2 was selected to protect *B. coagulans* for the next examinations in this study. It is noticed that four different compositions of the microencaps-ulation matrix were compared for the next examinations. Pectin, pec-NC (67:33), pec-NLC (67:33) were the microen-capsulation matrices which compared with pec-NC-NLC (50:25:25) as the best composition in this study.

The pec-NC-NLC matrix after fabrication is exhibited in Figure. 1a and b. The surface structure of the matrix is relatively smooth coherent, compact and wrinkled. This is due to the formation of hydrogen and ionic bonds which connected polysaccharides of the pec-nanofibers together. Moreover, homogenous distribution of the nanofibrillar network structure created a uniform morphology.

3.2. pH on storage of microencapsulated probiotic in peach juice

Figure 2 illustrated the pH changes of the probiotic peach juices when stored at 4 and 25^{o}C for 5 weeks. The variation of pH in the juice containing the free cell and in the other juices was different. In the peach juice and synbiotic peach juices, no statistical significant variation of pH ($p>0.05$) was observed over the 5 weeks of the storage but the pH of the juice containing the free probiotic cell was significantly changed ($p\leq0.05$). Similar observation was reported in a study on chitosan-alginate encapsulation of *L. rhamnosus* GG for use in apple juice [10].

pH variation is naturally related to the presence of sugars or oligofructoses in the juice which can be metabolized to organic acids by *B. coagulans* [11]. Since the microencaps-ulation matrix probably limited the diffusion of sugars into the matrix [27], pH variation was found to be statistically significant ($p\leq0.05$) in the peach juice containing the free cell. The maximum variation of pH occurred in the peach juice with the free cell during 5 weeks was approximately 0.2 (Figure 2). Nevertheless, it is noticed that the synbiotic juices during week 1 revealed a reduction in pH (~ΔpH = 0.5). It is due to the prebiotic microencapsulation matrix found to be metabolized by the probiotic. Although the pH reduction in the synbiotic juices (~ΔpH = 0.5) was observed more than in the other samples, it was not significantly varied during 5 weeks compared to the juice with the free cell. This was in contrast to the results reported as an investigation of storage stability of *L. paracasei* as free cells or encapsulated in alginate-based microcapsules in peach and orange juices since it was dependent on the type of probiotic strains that metabolize substrates based on different metabolic pathways [11]. Furthermore, the type of prebiotics used in a synbiotic formulation is an important factor to reduce pH in fruit juices when it undergoes some decomposition at low pH. In acidic environments of the juices, oligosaccharides such as oligofructoses may be hydrolyzed and metabolized by probiotics [28]. Conseq-uently, use of the prebiotics in synbiotic compositions, which are not easily metabolized, such as pectin, chitin and lignocellulose aids to maintain physicochemical properties of the fruit juices.

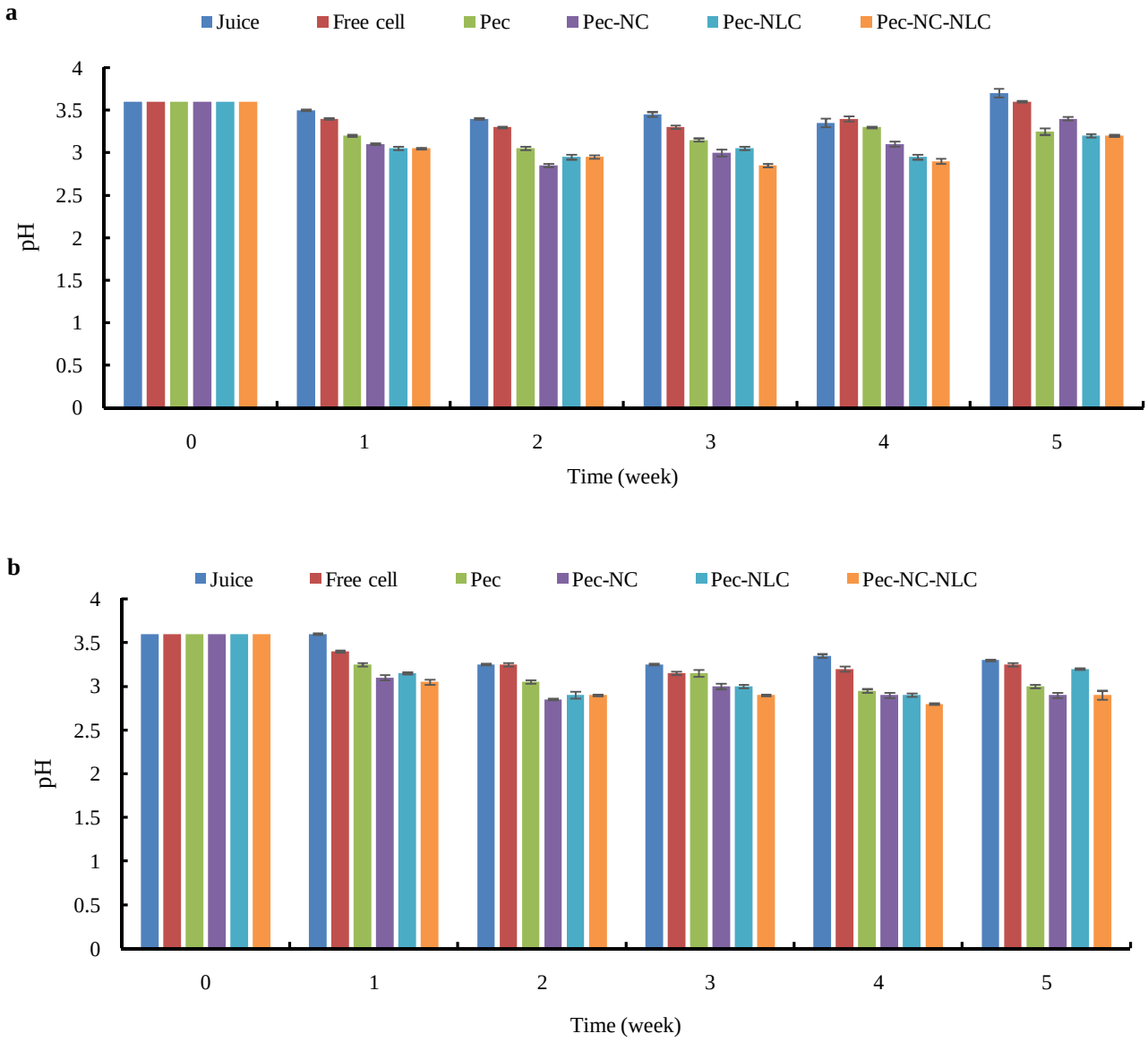

Figure 2. pH of synbiotic peach juice over 5 week storage at (a) 4°C and (b) 25°C

As shown in Figure 2a and b, the pH changes of the samples stored at 25°C were similar to those at 4°C. It was expected that the reduction of pH in the juices stored at 25°C was more than in those stored at 4°C since the bacteria could be active at 25°C to ferment the sugars in the juice to reduce the pH, but it was not occurred through the storage period. It could be due to the effect of the juice on limitation of physiological activity of the probiotic, and also the low temperature of the storage (25°C) when compared with the optimal growth temperature (37°C) [29]. Similar results were observed during storage of synbiotic apple juice at 4°C and 25°C [10].

One of the most important challenges for commercial probiotic products is probiotic fermentation that may lead to important product losses [30]. The results of this study addressed the prebiotic bionanocomposite isolating the probiotic from the juice environment to inhibit the fermentation of sugars. Thus, the food quality of peach juices containing the microencapsulated probiotic can remained constant.

3.3. Survivability of microencapsulated probiotic in peach juice during storage

Figure 3 shows the survivability and cell density of *B. coagulans* as free or microencapsulated cells which stored during 5 weeks at 4°C and 25°C in the peach juices. Survivability variations of the all samples were statistically non-significant ($p>0.05$) through the 5 week storage period.

When the probiotic peach juice stored through the first 3 weeks, there was a decrease of the survivability for the

systems containing the microencapsulated or free probiotic while it increased through the last 2 weeks (Figure 3). The most reduction during the storage usually occurred within the first day of storage because of the sudden exposure of the cells to the harsh condition of the fruit juice, especially the low pH (3-4) [10]. The reduction in cell viability 3 weeks after incorporation of the microencapsulated *B. coagulans* into the juice at 25°C was 3.4-4.4 log ± 0.04 (CFU 100 ml^{-1} juice) but the reduction of the free cell was 5.3 (Figure 3c). The results of the reduction at 4°C were different. The reduction in the microencapsulated probiotic viability was 3.1-3.7 ± 0.01 log (CFU 100 ml^{-1} juice) but it was 4.1 ± 0.01 for the free cell at 4°C (Figure 3a). This trend of decreasing the probiotic viability corresponded to increasing tempera-ture of the storage, irrespective of whether the probiotic was microencapsulated or free. This was in agreement with the results showed that there was a reduction in the number of viable cells of *L. rhamnosus* GG encapsulated in whey protein and resistant starch matrices during 4°C storage in apple juice [9]. Nevertheless, it was in contrast with the results showed that an increase in the number of viable cells of *L. acidophilus* encapsulated in an alginate–xanthan gum mixture during 4°C storage in carrot juice [31]. This difference could be related to the type of probiotic strains, juice compositions and the initial pH of the juices. However, the results obtained during week 4-5 revealed an increase in the cell counts. This could be due to adaptation of the probiotic with the juice condition, thus growing more than before.

There was no empirical relationship between viability of the probiotic and pH of the juices (Figure. 2), with the decrease in the *B. coagulans* viability being proportional to the change in pH of the juice. This was in contrast to the study that indicated a relationship between viability of *L. rhamnosus* GG encapsulated and pH of apple juice during 35 day storage [9].

It should be emphasized that the juice formulation and the type of probiotic strains as the most important variables strongly play role in the viability of probiotics during storage since natural additives in the juices can affect the probiotic growth through the storage period [32]. According to the reports, some of strains of Lactobacillus such as *L. rhamnosus*, *L. plantarum*, *L. reuteri* and *L. fermentum* were able to survive in a commercial fruit juices with pH of 4.2 at 4°C for up to 80 days whereas *L. rhamnosus* and *L. paracasei* L26 did not survive well in the other fruit juices environment [33,34].

The decrease in the survivability was most pronounced in the case of microencapsulated *B. coagulans* formulated with pectin alone and more gradual for microencapsulated *B. coagulans* formulated with pec-NLC or pec–NC-NLC mixtures. The results demonstrated that three pectin-based composites could efficiently maintain the viability of *B. coagulans* in the peach juice during storage. Pec-NC-NLC matrix, which was evaluated as the best prebiotic digestion-resistant composite, performed a new procedure for increasing efficiency in shelf life of the probiotic in the peach juice. When the probiotic cells were microen-capsulated using pec-NC-NLC matrix and incorporated into the peach juice during 5 weeks, there was a decrease of ~2.98 ± 0.02 log (CFU 100 ml^{-1} juice) for the juice stored at 25°C (Figure 3c) and ~2.66 ± 0.04 log (CFU 100 ml^{-1} juice) for the juice stored at 4°C (Figure 3a). Consequently, the highest survivability obtained by this matrix was ~68% ± 0.1 in comparison to the survivability of the free cell that was obtained ~53% ± 0.3 at the end of the 5 week storage period at both of 4°C and 25°C (Figure. 3b and d).

According to Figure 4, pec-NC-NLC composite had the ability to form a gel structure in the juice during storage as shown in optical microscopy images of the microencaps-ulation matrix at 4°C. However, the images were similar at 4 and 25°C storage. The most of *B. coagulans* was kept microencapsulated within the swelled pec-NC-NLC composite. Figure 4a shows perfect synbiotic matrix with a thin layer of pec-NC-NLC as a wall material encapsulating the probiotic cells clearly observed in Figure 4b. Although some deformation of the matrices could occur throughout storage (Figure 4c), no evidence of rupture was ever observed (Figure 4d). This suggests that physical isolation within the matrix is sufficient to protect the live probiotic cells from the surrounding low pH environment of the peach juice. However, it was reported that isolation alone within the whey protein and resistant starch matrices was not sufficient to protect the *L. rhamnosus* GG from apple juice (pH 3.5) [9].

As shown in Figure 4a, the average size of the matrix could vary in micro scale. Such small dimensions cannot be detected in the mouth, which is of a major importance for sensorial acceptance.

Depending on the microencapsulation matrix formul-ation, the local chemical environment near the microencaps-ulated cells may be different since the gel matrix is likely to be influenced by the buffering capacity of the biopolymer used in the composition. Thus, local pH within the matrix may have a role in protecting the probiotic against the external environment since the matrix can retain much of their particulate nature.

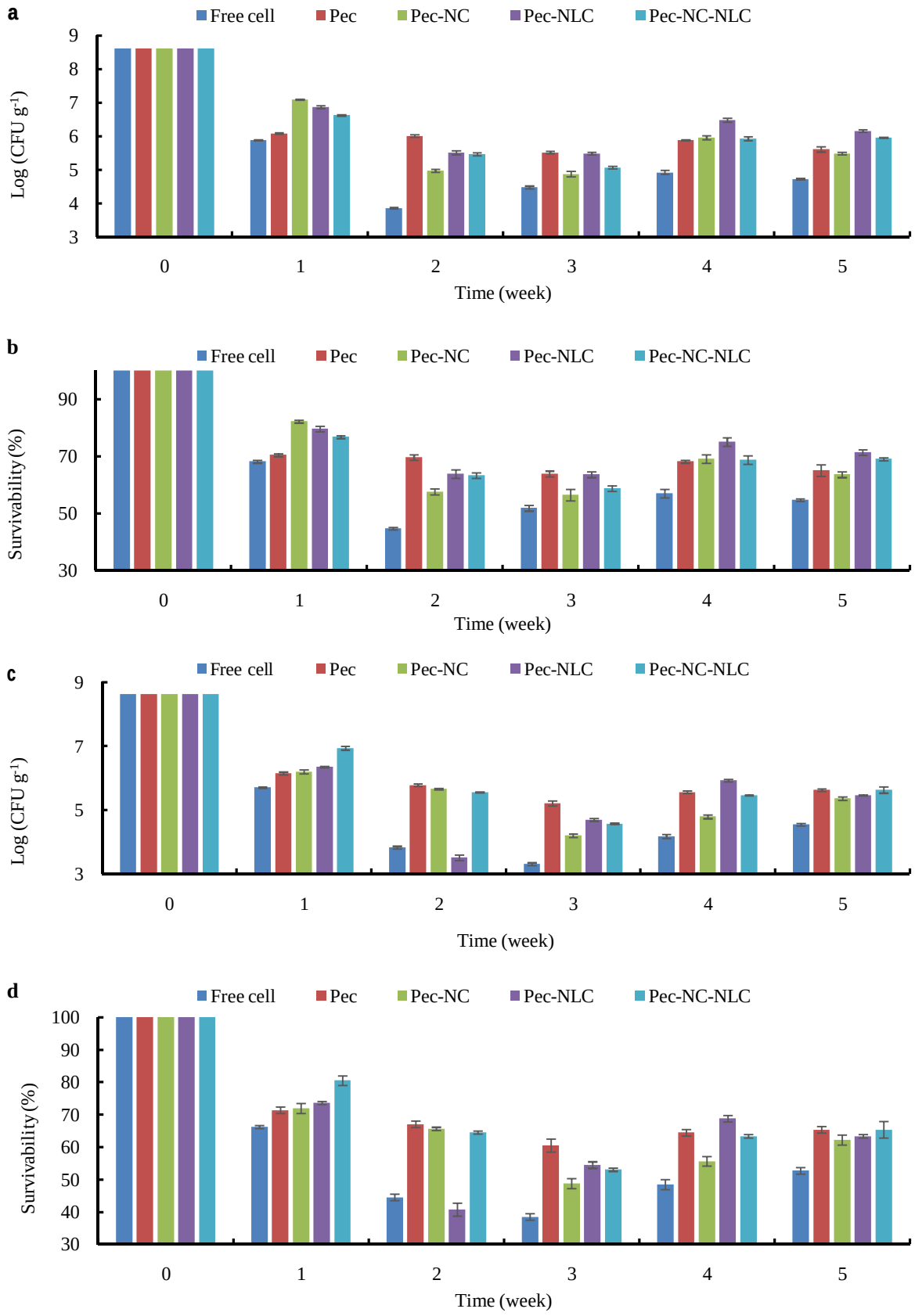

Figure 3. Viable probiotic (a, c) and survivability of probiotic (b, d) over 5-week storage at 4°C (a, b) and 25°C (c, d).

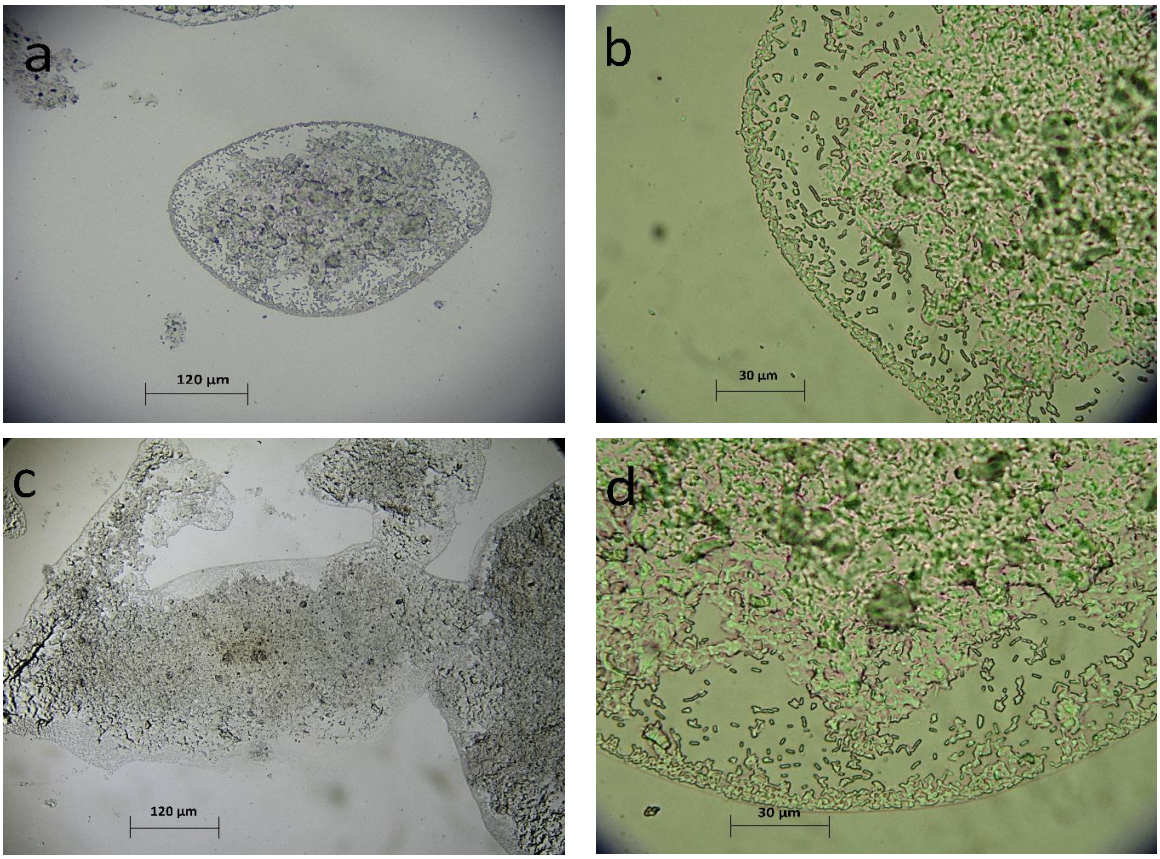

Figure 4. Optical microscopy images of the microencapsulation matrix in peach juice at 4°C after (a, b) 0 day and (c, d) 35 day storage.

3.4. Survivability of microencapsulated probiotic under SGI conditions

After 3 week storage at 4°C, the synbiotic matrices (or free cell) harvested from the stored probiotic juices by the centrifugation at 16000 ×g for 15 min, were examined under SGI conditions. Hence, evaluation of the survivability was carried out with regard to the cell counts obtained after the storage as initial cells before the GI treatment. As indicated in Figure 3a, the cell densities of *B. coagulans* after 3 week storage were determined as 4.48 ± 0.02 (free cell), 5.51 ± 0.03 (pec), 5.07 ± 0.01 (pec-NC-NLC), 4.87 ± 0.01 (pec-NC) and 5.49 ± 0.03 (pec-NLC) log (CFU 100 ml^{-1} juice) based on the used microencapsulation matrices. Concerning the survivability of *B. coagulans*, it reduced during exposure to the simulated gastric and intestinal juices for the all samples, as shown in Figure 5. The reduction variations in the survivability of the all samples through the treatment period were statistically insignificant ($p > 0.05$). The free cell count showed a reduction of ~0.7 ± 0.03 and ~1 ± 0.03 log (CFU 100 ml^{-1} juice) after exposure to the simulated gastric juice (pH 2.0) and simulated intestinal juice (pH 8.0), respectively (Figure 5a and c). Among the microencapsulated probiotic under gastric condition, the highest survivability (~98% ± 0.4) was obtained when *B. coagulans* was protected using the pec-NC-NLC matrix (Figure 5b). However, pec-NC and pec-NLC resulted the survivability of ~97% ± 0.3 and ~96% ± 0.4, respectively, when compared with the survivability of the free cell (~84% ± 0.4). Under intestinal condition (Figure 5d), pec-NC-NLC resulted the survivability of ~98% ± 0.4 that was 20% more than that of the free cell (~78% ± 0.4). As obtained results, the survivability under intestinal condition was more reduced when compared with under gastric condition. This was related to the mechanism of pH-sensitive swelling of the pectin-based matrix that involves the deprotonation of carboxyl groups of pectin at high pH. When pectin with a pK_a of 3-4 was exposure to the gastric fluid (pH 2), its carboxyl groups was protonated and thereby, the pec-nanofibers deswelled. On the other hand, when pH further increased to 7.4, the carboxyl groups on the pectin become progressively ionized. At pH 8 (intestinal fluid), the deprotonated carboxyl groups of pectin increased negatively charged in the pectin-based matrices and then electrostatic repulsion created between the groups caused swelling of the pectin-based matrices. Hence, the matrices under intestinal condition were more swollen , leading to the fast release of the probiotic and exposure to the enzymatic digestion [35].

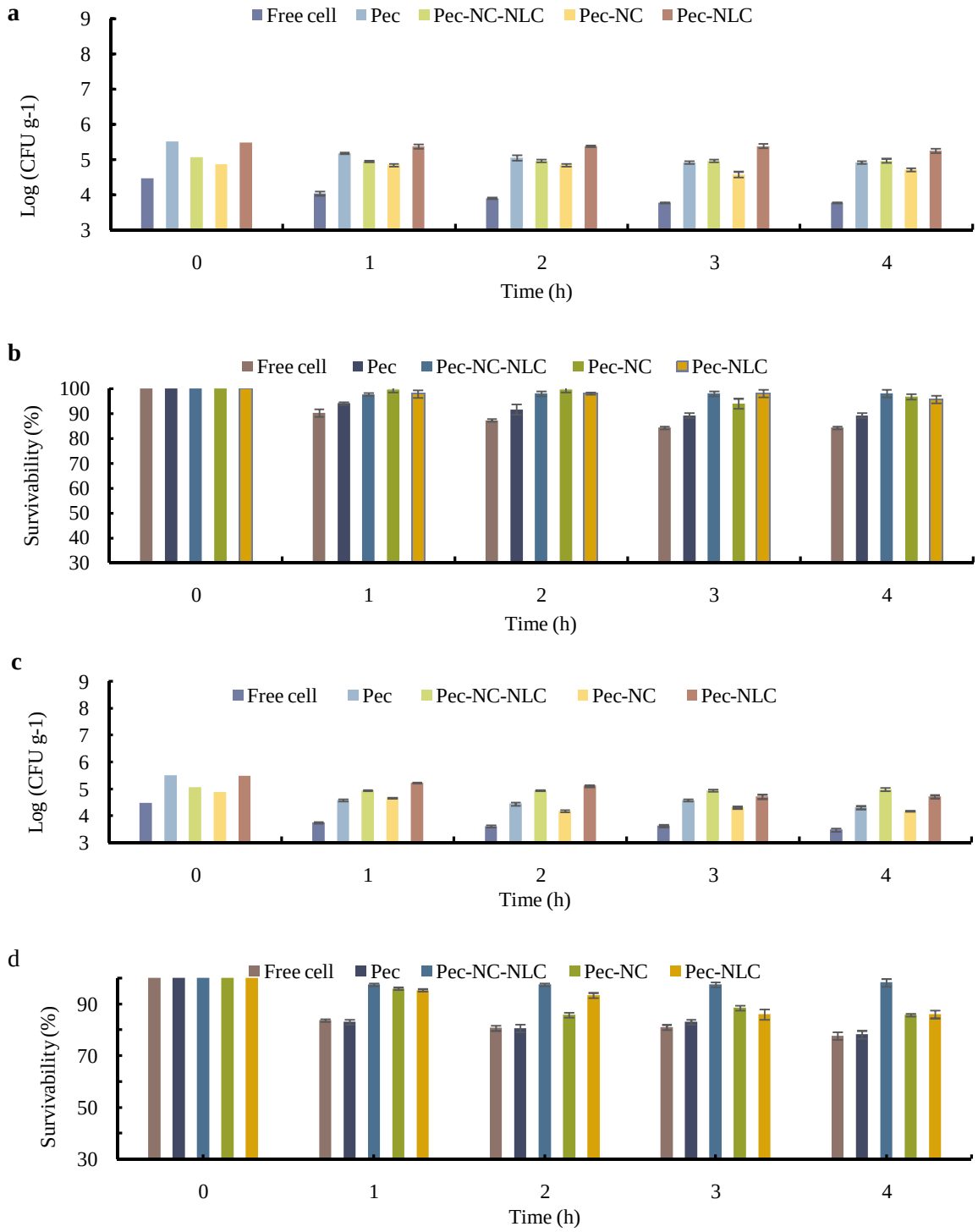

Figure 5. Viable probiotic (a, c) and survivability of probiotic (b, d) under 4 h gastric treatment (a, b) and 4 h intestinal treatment (c, d).

The positively charged amino groups on chitin electrostatically bonded to the negatively charged carboxyl groups on pectin. These ionic interactions could stabilize the microencapsulation matrix, thus improving the sustainab-ility of the matrix in the aqueous conditions of the GI tract. Furthermore, fiber-fiber interactions in the polysaccharide matrix caused to adhesion of NC fibers to pectin and NLC. This created a compact surface for the matrix via drying since pore shrinkage occurred during freeze-drying [36,37]. The compact surface hindered the uptake of the GI juices into the matrix, increasing the viability of probiotics microencapsulated.

To evaluate the survivability of *B. coagulans* under sequential digestion, the harvested synbiotic matrices were examined during 4 h intestinal digestion following 2 h gastric (Figure 6).

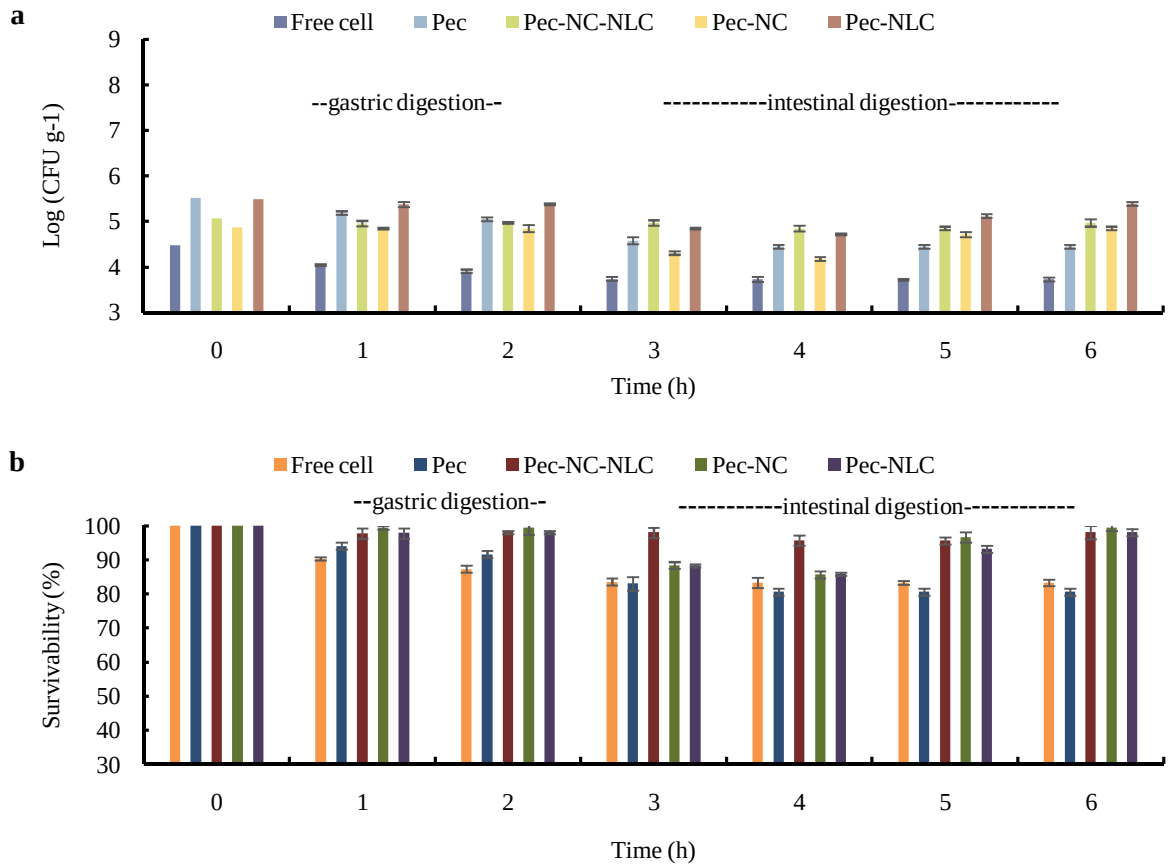

Figure 6. (a) Viable probiotic and (b) survivability of probiotic under 2 h gastric and 4 h intestinal treatments.

The results obtained for the gastric treatment were similar to those represented in Figure. 5a and b. Under intestinal digestion, the cell densities of the all samples reduced after gastric treatment during the first 2 h of the intestinal treatment (Figure 6a). Although the survival of the probiotic associated with the free cell and pectin matrix was similar (Figure 6b), it was lower than that related to the other matrices. It was attributed to the pectin matrix destruction related to the open structure, high dissolution and swelling of the matrix in intestinal condition when compared to the matrices improved by the NC and NLC [35,38]. Although the cell densities related to the free cell and pectin matrix were constant during the intestinal treatment, the cell densities related to the other matrices increased during the last 2 h of the treatment. The main reason explaining this increase under intestinal condition is stability of the pec-nanofiber matrix in comparison with the pectin matrix. Although inhibition of bile salts and degradation of pancreatin enzymes decreased the viable cells of the all samples, construction of the pec-nanofiber limited penetration of the bile salts and digestive enzymes into the matrix. In addition, intestinal pH (7-8) could provide a suitable condition for growth of the probiotic in comparison to the gastric pH (1.5-2). In such condition, the probiotic consumed the matrix as a prebiotic to grow. Consequently, the survivability obtained at the end of the GI treatment was ~58% ± 0.2 associated with the pec-NC-NLC matrix with regard to the initial cell microencapsulated before storage whereas the survivability of the free cell was obtained ~43% ± 0.3. Although gastrointestinal-resistance of the pec-NC-NLC matrix was demonstrated, the peach juice (pH 3.6) as a harsh environment lowered the efficiency of the matrix to protect the cells against GI digestion when the synbiotic matrix delivered to the GI tract.

Stability of the structure of pec-NC-NLC can be seen in Figure 1c and d. No evidence of collapse was ever observed; however, a highly porous and unwrinkled surface of the matrix were displayed after digestion under simulated gastrointestinal juices. This was attributed to immersion of the matrix for 6 h and its degradation by the acidic juice and digestion enzymes.

The results demonstrated that the microencapsulation matrix used in this study preserved the probiotic in the peach juice during the storage, making them more stable against the harsh GI conditions.

4. Conclusion

The pectin-based bionanocomposite formulated with 50% pectin, 25% NC and 25% NLC exhibited a promising

microencapsulation matrix with good prebiotic and gastrointestinal-resistant properties. Furthermore, the microencapsulation of probiotic *B. coagulans* within the pec-NC-NLC matrix protected the probiotic cells in the low pH environment (pH 3.6 peach juice) over 5-week storage at both 4°C and 25°C. A three-dimensional arrangement of the nanofibers of chitin and lignocellulose incorporated within pectin created a uniform, relatively smooth coherent, compact and wrinkled morphology without pores and cracks. Moreover, water-insolubility of the nanofibers caused resistance in the microencapsulation matrix to degradation under gastrointestinal digestion and during storage in peach juice. Therefore, it is concluded that the pec-NC-NLC matrix with the enhanced prebiotic and gastrointestinal resistance properties has the potential to deliver live probiotics in low pH fruit juice to the consumers.

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6. Conflict of Interest

The authors declare no conflict of interest.

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بهبود زنده‌مانی پروبیوتیک در آبمیوه و شرایط معده ای- روده‌ای با استفاده از پکتین-نانوکیتین-نانولیگنوسلولز به عنوان یک شبکه نوین پری‌بیوتیکی مقاوم به شرایط معده و روده

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چکیده

سابقه و هدف: افزایش زنده‌مانی پروبیوتیک‌ها در آبمیوه‌های با pH پایین و شرایط معده ای – روده ای در صنعت غذاهای پروبیوتیک موضوع مهمی است. نانوالیاف می‌توانند ساختار شبکه‌های حفاظت‌کننده از پروبیوتیک‌ها را در مقابل محیط‌های نامناسب تقویت کنند. این مطالعه به بررسی زیست‌نانوکامپوزیت‌های برپایه پکتین و بهبودیافته با نانوکیتین، نانولیگنوسلولز و نانوسلولز باکتریایی برای معرفی یک شبکه پری-بیوتیکی مقاوم در برابر محیط گوارشی برای افزایش زنده‌مانی پروبیوتیک *باسیلوس کوآگولانس* پرداخته است.

مواد و روش‌ها: با استفاده از روش طراحی ترکیب، زیست‌نانوکامپوزیت‌هایی با ترکیب‌های مختلف طراحی شدند. این زیست‌نانوکامپوزیت‌ها براساس اتصالات عرضی یون‌های کلسیم با پکتین و برای به دام انداختن *باسیلوس کوآگولانس* تهیه شدند. زنده‌مانی پروبیوتیک در دوره نگه‌داری 5 هفته‌ای در آب هلو در دماهای 4 و 25 درجه سلسیوس، و محیط‌های شبیه‌سازی شده معده‌ای -روده‌ای ارزیابی شد.

یافته‌ها و نتیجه‌گیری: امتیاز پری‌بیوتیکیپکتین-نانوکیتین-نانولیگنوسلولز (50-25-25، درصد وزنی/ وزنی)، 1/36 تعیین شد. زنده‌مانی *باسیلوس کوآگولانس* به دام افتاده در شبکه پکتین-نانوکیتین-نانولیگنوسلولز تحت تیمار معده‌ای-روده‌ای، حدود 65% بود. ساختار سطح این شبکه به دلیل نظم سه بعدی ایجاد شده بین پکتین و نانوالیاف کیتین و لیگنوسلولز نسبتا صاف و منسجم، فشرده و بدون روزنه بود. در پایان دوره نگه‌داری 5 هفته‌ای، بیشترین زنده‌مانی باکتری‌های به دام افتاده حدود 68% بود، در مقایسه با زنده‌مانی سلول‌های آزاد (حدود 53%). پس از 21 روز نگه‌داری در آب هلو، زنده مانی باکتری به دام افتاده حدود 58% به‌دست آمد، در حالی‌که زنده‌مانی آن درحالی که زنده‌مانی سلول آزاد حدود 43% بود. یافته‌های حاضر، یک شبکه پری‌بیوتیکی مناسب را برای حفاظت پروبیوتیک‌ها در آبمیوه با pH پایین و دستگاه گوارش پیشنهاد می‌کند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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- *باسیلوس کوآگولانس*
- نانوکیتین
- نانولیگنوسلولز
- پکتین
- آبمیوه سین‌بیوتیک

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