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Biopolymer immobilization enhanced the survival of lactobacillus casei ATCC334 in mangosteen probiotic beverage

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Abstract: This study aimed to enhance the viability of Lactobacillus casei ATCC334 in highly acidic fruit juice via an immobilization process using coconut meat insoluble fibres. Immobilized probiotics were introduced to mangosteen juice, and the probiotics' viability was evaluated for 4 weeks at 4°C. The immobilized probiotics were studied for their effects on the beverage's physicochemical and rheological properties and metabolites. Mangosteen with immobilized probiotics showed increased probiotic viability, lower glucose, fructose, and sucrose reduction, lower production of lactic acid, and higher pH than the control. The cell count of the immobilized probiotics was log10 9.25 CFU mL-1 and the free cell probiotics was log10 7.89 CFU mL-1 after 4 weeks at 4°C. The results illustrated that coconut meat insoluble fibres can be used to immobilise probiotics with enhanced viability and improved metabolites that enhance consumer acceptability and product quality.

Keywords: *Encapsulation, Lactic acid bacteria, Mangosteen, Metabolites, Probiotics beverage.*

1. Introduction

Dairy products are the most common carrier to deliver probiotics. However, increased demand is observed for non-dairy probiotic food and beverages [1]. Plant-based eating was the top topic at the International Food Technologist (IFT) SHIFT20, showing a staggering growth of over 30% in the last two years. The market growth of health-promoting beverages including plant-based probiotic beverages heavily depends on the region and product type. Developing fruit probiotic beverages is significantly challenging due to the low survival rate of the probiotic strains due to the acidic conditions. However, encapsulation techniques are employed for the protection of probiotics by forming a barrier between the internal phase and its surroundings [2]. Encapsulation is a physicochemical or mechanical process in which the probiotics cells are entrapped within coatings of hydrocolloidal materials, and that will protect them from high acidity, bile salts and cold shock [3]. Encapsulation has been applied as an efficient method for improving the viability of probiotics leading to increased bioactive metabolite production [4]. Common techniques applied for probiotic encapsulation are emulsion, extrusion, spray-drying, and freeze-drying [5]. On the other hand, the probiotics are encapsulated using different coating materials such as alginate [6], chitosan [7], and k-Carrageena [8]. The encapsulation can enhance the survival rate of the probiotic cells throughout the shelf life of the product. Nevertheless, the coating materials are highly soluble in aqueous solutions which may limit their application in probiotic beverages [9]. Therefore, immobilization of the probiotics using insoluble plant-based cellulose demonstrated the potential to enhance the survival of probiotics in liquids [10].

Recently, the demand has been increased for fermented foods and beverages containing probiotics due to the COVID-19 pandemic [11]. Sundararaman et al., (2020) suggested that probiotics play an important role in mitigating Covid-19 severity and rapid recovery via the gut-lung axis interaction. According to Nishihira et al., (2018), the molecular mechanism of the probiotics to enhance the immune system response is via the interaction with epithelial cells, dendritic cells, and T-cells that lead to the

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induction of several cytokines including TGF-β, IL-6, IL-10 [12]. The immune system was observed to positively respond to the bioactive compounds generated by the probiotics such as extracellular polysaccharides, bacteriocins, butyric acid and phenolic compounds [13-15]. However, the probiotics must be delivered via a suitable carrier and introduced to the intestine at adequate concentration [16]. Mangosteen (*Garcinia mangostana* L.) is a delicious tropical fruit that can be found abundantly in many countries in Southeast Asia [17]. The fruit contains dietary fibres (0.90 g 100g-1, fresh weight), polyphenols (190.3 mg of GAE 100g-1, fresh weight) and flavonoids (54.1 mg of CE 100 g-1, fresh weight) [18]. Mangosteen has high carbohydrate content (18.4 g 100g-1) and it is a rich source of several vitamins such as thiamine (B1), riboflavin (B2), niacin and vitamin C [19]. In addition, mangosteen is a rich source of secondary bioactive compounds such as mangosteen, xanthones, tannin, garcinone, chrysanthemin and gartanin [20]. The fruit has several biological activities including antimicrobial [21], antioxidant [22], anti-inflammatory [23], and anti-cancer [24]. No previous study was conducted to determine the potential for developing probiotic mangosteen beverages. Therefore, this study aimed to develop a plant-based probiotic beverage utilizing mangosteen juice as a substrate. The probiotics are immobilized using coconut meat fibres and their survival was determined during cold storage at 4 °C for 4 weeks.

2. Materials and Methods

2.1. Materials

The coconut meat was obtained fresh from the wet market, in Serdang, Selangor. Mangosteen (20 kg) was purchased from TESCO, Putrajaya. De Man, Rogosa and Sharpe (MRS) broth (Himedia, India). Deuterated methanol, (99.8%), Deuterium oxide (99.9%), and 2,2,3,3-tetradeuteropropionic acid (TSP) (98%) and sodium 3-(trimethylsilyl)propionate were purchased from Cambridge Isotopes, UK.

2.2. Preparation of Probiotic Inoculum

The probiotic strain *Lactobacillus casei* ATCC334 was growing in De Man, Rogosa and Sharpe (MRS) broth for 48 h at 37 °C. The cells were subjected to centrifugation at 3,000 × g for 4 minutes (Eppendorf centrifuge, Hamburg, Germany), the cells were suspended in 0.1% peptone water and vortexed. The washing protocol was repeated three times.

2.3. Preparation of Coconut Meat Insoluble Fibres

The coconut meat was freeze-dried and ground using a kitchen grinder (BL-3075, Tefal, Malaysia). The powder was sieved using different sizes 1.6 mm mesh, 0.7 mm mesh and 0.4 mm mesh, the powder resulting from the 0.4 mm sieve was used in this study. The powder was defatted following the method described by Patil, & Benjakul, (2017). Briefly, coconut meat powder was mixed with hexane 1:10 (w/v) twice. The defatted powder was subjected to freeze drying using a Labconco freeze dryer (New Jersey, USA) [25]. The dry powder was kept at -20 °C for further application.

2.4. Preparation of Immobilized Probiotics

The probiotic strain was immobilized using the defatted coconut meat insoluble fibres (CMIF) following the method described by He *et al.*, (2021) with modification [26]. The probiotic (10^8 CFU mL^{-1}) was inoculated in MRS broth (100 mL) and supplemented with 1 gram of the defatted coconut meat powder. The samples were incubated at 37 °C for 48- hours in a shaking incubator at 150 rpm for homogenised distribution for the probiotic cells. The samples were centrifuged at 3,000 × g for 4 minutes after the 48 hours incubation and washed two times using 0.1% peptone water. The pellets were collected to determine the cell counts.

2.5. Probiotics Cell Count

The cell count of the immobilized probiotic cells was carried out following the method described by da Cruz Rodrigues *et al.*, (2019). The collected pellets (10 grams) were diluted with 90 mL of 0.1% peptone water and followed by serial dilution. A total of 0.1 mL was inoculated on an MMRS agar plate and incubated at 37 °C for 48 hours in the anaerobic jar. The cell count was determined via manual

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counting using a colony counter with an electronic register (MRC, CLC-570, Essex, UK). The cell count was expressed as Log10 CFU mL-1. The cell count was done in triplicate [27].

2.6. Preparation of Lacto-Fermented Mangosteen

Fresh mangosteen was free of mechanical damages and physical microbial growth was purchased. The mangosteen fruits were washed with tap water, peeled off and the seeds removed to obtain the pulp. The pulp (200 grams) was mixed with sterile water (800 mL) and blended using a kitchen blender. The mixture was sterilized at 90 °C for 30 min using a double jacket pot. Mangosteen juice was cooled down to 40 °C for the inoculation of the probiotic strain. The immobilized and cell-free probiotics were inoculated in the juice at 5% w/v and incubated at 37 °C for 24 hours. The juice samples were stored at 4 °C to determine the survival of the probiotics and the effects on the physiochemical properties of the beverage.

2.7. Metabolomics Profiling

The ^{1}H-NMR metabolomics profiling was carried out to determine the effect of immobilized probiotics on the chemical profile of the Mangosteen beverage in comparison to the cell-free probiotics. The ^{1}H-NMR analysis was performed following the method described by Muhialdin *et al.*, (2020). The fermented juice samples were freeze-dried. The freeze-dry samples (10 milligrams) were dissolved in 400 mL potassium phosphate buffer in deuterium oxide (pH 6.0) and 400 mL deuterated methanol with 0.1% trimethylsilylpropanoic acid (TSP). The samples were sonicated for 10 minutes at 25 °C and centrifuged at 5,000 g for 5 minutes. The metabolites profiling was carried out using a Varian INOVA 500 MHz NMR spectrometer (Varian Inc., California, USA). The spectra analysis was performed using 1D and 2D J-resolved (JRES) NMR experiments and the spectra were analyzed using MestReNova (Mestrelab, Santiago de Compostela, Spain). The identification of the metabolites was performed using Chenomx software version 8.5 (Chenomx, Edmonton, Alberta, Canada). The concentration of the metabolites was calculated as the average of the 6 replications for the sample and compared to the internal standard concentration (TSP) [28].

2.8. Measuring the pH, Titratable Acidity, and Brix

The pH was measured at room temperature 28 °C using a digital pH meter (JENWAY 3505, London, UK). The titratable acidity was measured following the standard method (No. 947.05) of the Association of Official Analytical Chemists Method (AOAC, 2000), and expressed as % lactic acid [29]. The total soluble solid (TSS) content was measured using an optical Brix refractometer (Atago, Tokyo, Japan). The measurement was carried out in duplicate for 4 weeks.

2.9. Rheological Property Determination

The flow behaviour of the mangosteen beverage prepared using immobilized and cell-free probiotics was determined following the method described by Ludena Urquizo *et al.*, (2017). The rheological property was determined using an AR-G2 rheometer (TA Instruments, Sussex, UK), equipped with 60 mm cone-plate geometry (1° angle). The juice samples were placed in the plate and temperate was adjusted at 4 °C. The flow behaviour was tested via linear gradient shear rates (10 – 200s-1)[30]. The flow behaviour of the juice samples was determined using the following equation:

$\tau = k(\gamma)^{n}$

τ (shear stress, Pa), γ (shear rate, s^{-1}), and k (consistency factor, Pa s^{n}).

2.10. Survival of Probiotics During Cold Storage

Mangosteen juice was stored at 4 °C for 4 months to determine the survival of the immobilized and cell-free probiotics during cold storage. The cell count was carried out according to the protocol in section 2.5. The cell count was done in triplicate.

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2.11. Statistical Analysis

The triplicate readings were analyzed by one-way ANOVA using Minitab software version 18 (Minitab, Pennsylvania, United States of America). The results were expressed as the mean readings ± standard deviation (STDVE), and the $p < 0.05$ value refers to a statistically significant value.

3. Results and Discussion

3.1. Cell Count for Immobilized Probiotics

The cell count for the immobilized probiotic *L. casei* was $\log_{10}$ 9.186±49 CFU mL^{-1} after 48 hours of incubation in the MRS broth. Thus, the immobilized probiotics were washed twice, but the number of cells was high. The results indicated that the probiotics were adhered on the surface and in the porous of the coconut meat insoluble fibers (CMIF) at high concentration. In a previous study, Agri by-products' insoluble fibers were reported to efficiently improve the viability of several probiotics that were subjected to heat shock and digestive fluid [26]. In another study, the probiotics immobilized using rinds of durian and jackfruits demonstrated enhanced survival rate, high acid production, and lower pH compared to the cell-free probiotics in soy milk [31]. Several studies reported that immobilized probiotics have a high viability percentage and suggested the application of different Agri materials such as chocolate [32], gum gel beads [33], and sugarcane bagasse [34]. Probiotic immobilization has been applied to obtain a high density of cells and metabolite production in solid and liquid fermentation processes [35]. According to Lacroix *et al.*, (2005), immobilized probiotics have several advantages over cell-free probiotics including the production of high cell density, enhanced resistance to contamination, enhanced plasmid stability, and physical and chemical protection for the cells [36].

3.2. Metabolomics Profiling of Lacto-Fermented Mangosteen Juice

The NMR analysis demonstrated a significant difference in the concentration of the primary and secondary metabolites in the Mangosteen beverage prepared using free cells and immobilized probiotics (Table 1). The organic acids including lactic and acetic showed significantly ($p < 0.05$) high content in the cell-free sample compared to the immobilized (Figure 1 A, B and C). Moreover, the ethanol content was very high in beverages prepared using cell-free probiotics. On the other hand, the sugar content was higher in the beverage with the immobilized probiotics. Several secondary metabolites such as the free amino acids and vitamins were found at higher concentrations in the beverage sample with the immobilized probiotics. The results demonstrated that the free-cell probiotics utilized a large amount of the carbon source (carbohydrates) and produced organic acids as the main secondary metabolites. However, the immobilized probiotics utilized lower amounts of carbohydrates, and the main secondary metabolites produced were amino acids (proline and isoleucine) and vitamins (riboflavin). The results indicated the impact of the immobilization on the metabolic pathways of the probiotics. Teusink, & Molenaar, (2017) reviewed the effects of the complex substrates and the rapid adaptation of lactic acid bacteria (LAB) on the metabolic pathways and the secondary metabolites [37]. In another important review, the dietary fibers applied for the encapsulation of probiotics demonstrated effects on the microbial communities including the shifting of the metabolites' activity towards producing health benefits metabolites [38]. The encapsulated Bifidobacteria showed a significant reduction of organic acid production in yogurt and improved the acceptability of the product [39]. Kakimov et al., (2017) reported that the encapsulated probiotics produced high amounts of free amino acids in yogurt [40]. The result of this study agrees with the previous studies regarding the effects of encapsulation on the secondary metabolites of the probiotics during the fermentation process. These changes play an important role in increasing consumer acceptability and to extend the shelf life of the foods.

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Table 1.
The metabolites identified in fermented and non-fermented Mangosteen with the relative concentrations (mmol/mL).

No	Metabolites	^{1}H-NMR characteristic signals	Cell-free probiotics	Immobilized probiotics
1	Alloisoleucine	δ 0.96 (m)	0.020	0.001
2	Valine	δ 1.02 (d)	0.005	0.004
3	Ethanol	δ 1.16 (t)	0.184	0.021
4	Lactic acid	δ 1.32 (d)	0.088	0.001
5	Alanine	δ 1.46 (d)	1.010	1.126
6	Arginine	δ 1.65 (m)	0.098	0.003
7	Acetic acid	δ 1.88 (s)	0.021	0.005
8	Gamma-aminobutyric acid	δ 1.92 (m)	0.009	0.100
9	Acetone	δ 2.20 (s)	0.075	0.002
10	Acetoacetate	δ 2.27 (s)	0.014	0.006
11	Riboflavin	δ 2.35 (s)	0.004	0.026
12	Succinic acid	δ 2.38 (s)	0.008	0.006
13	Pyruvate	δ 2.46 (s)	0.041	0.009
14	Creatinine	δ 3.00 (s)	0.018	0.005
15	Choline	δ 3.19 (s)	0.161	0.105
16	Betaine	δ 3.30 (s)	0.606	0.870
17	Proline	δ 3.34 (m)	3.487	4.909
18	Isoleucine	δ 3.62 (d)	1.908	2.340
19	Leucine	δ 3.72 (m)	5.164	4.426
20	Caffeine	δ 3.96 (s)	0.120	0.084
21	Fructose	δ 4.02 (dd)	11.003	8.663
22	Xylose	δ 4.56 (d)	28.620	19.566
23	Glucose	δ 5.16 (d)	6.1269	8.834
24	Sucrose	δ 5.4 (d)	0.213	0.0551

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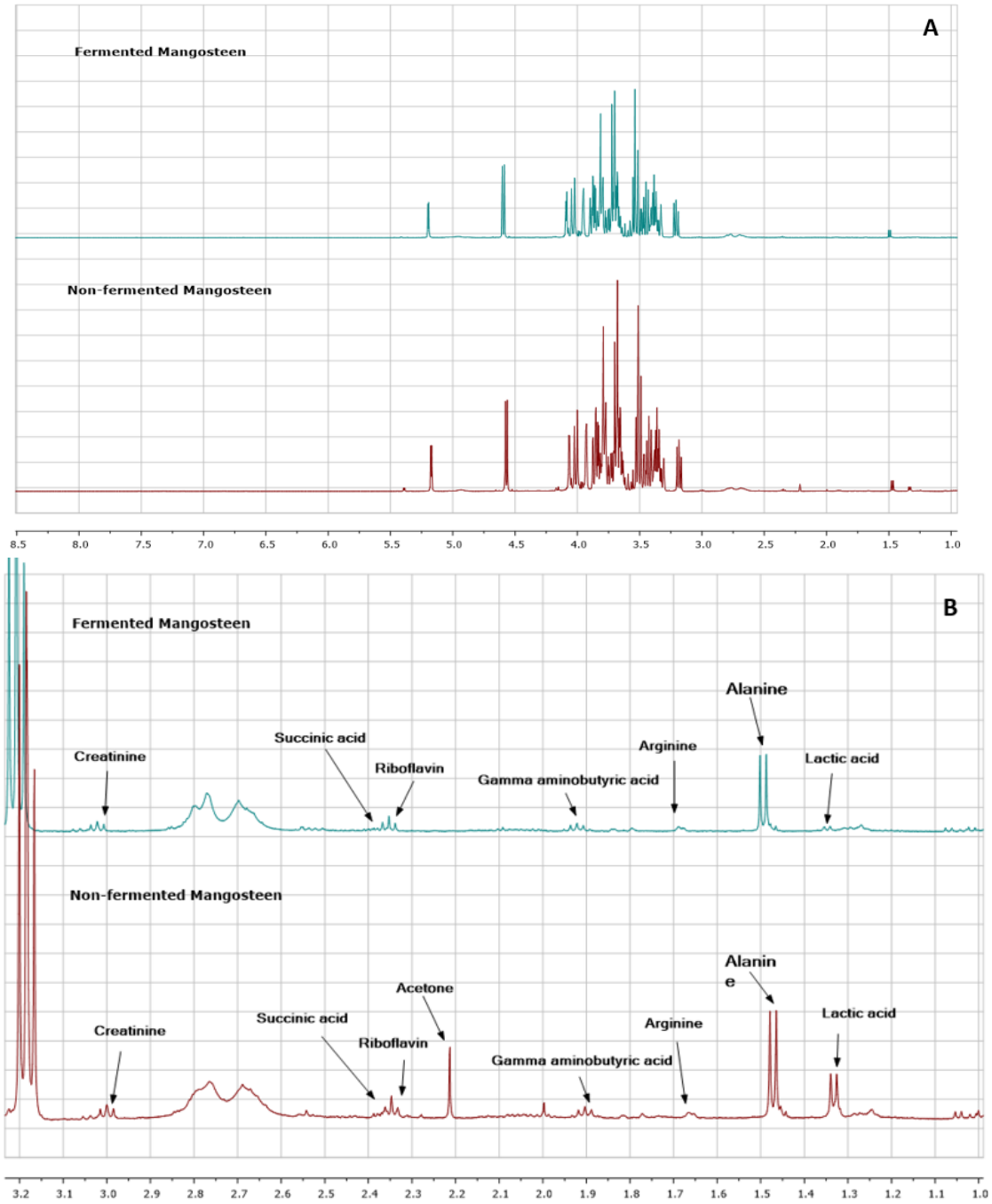

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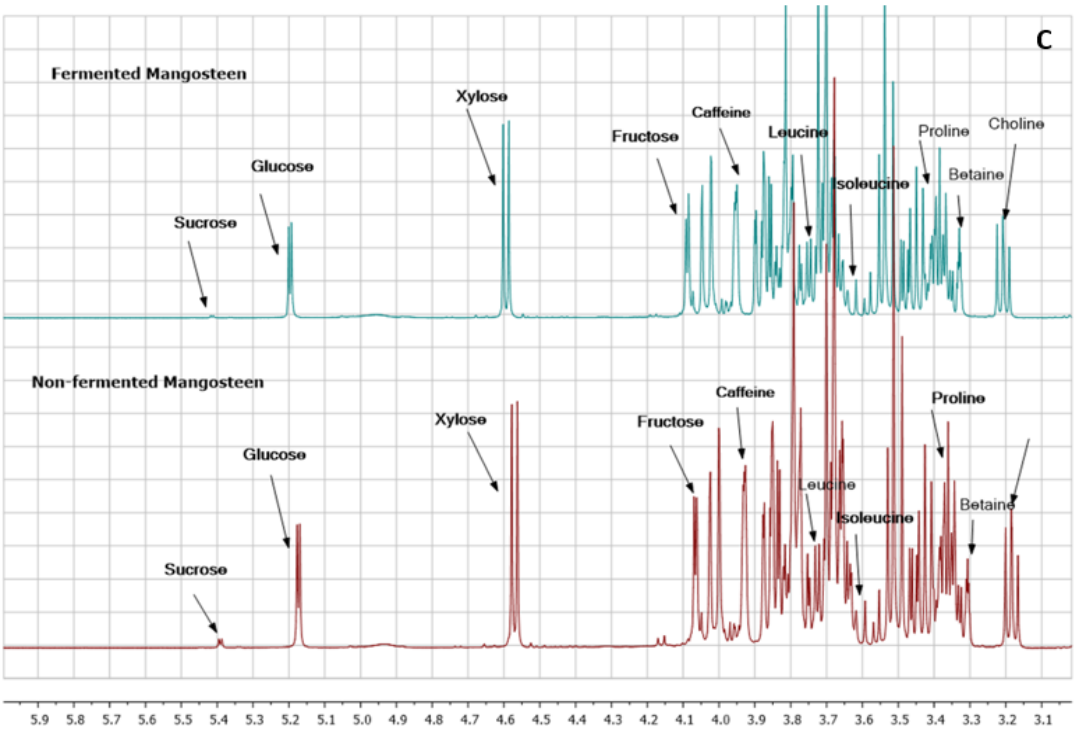

Figure 1.
The representative of ^{1}H-NMR spectra A full spectrum, B (1 to 3ppm), and C (3 to 6ppm) of the fermented and non- fermented Mangosteen.

3.3. Changes in pH, Titratable Acidity and Brix Values in Lacto-Fermented Mangosteen Juice

The immobilized and cell-free probiotics were inoculated in mangosteen juice to determine the effects on the chemical properties of the beverage. The results showed significantly ($p < 0.05$) lower acid production and higher pH for the immobilized probiotics compared to the cell-free (Figure 2 A and B). However, the pH values showed a sharp decline after week 1 from 4.625 to 3.55 for immobilized probiotics and from 3.995 to 3.055 for the cell-free probiotics. In the following weeks of storage, the pH was consistent with a very slight decline. The pH value is a very important indicator to ensure the safety of food and beverage products due to the prevention of pathogenic microorganisms at a pH below 3.8 [41]. Moreover, the pH value correlates with the acid produced by the probiotics, and both have a strong influence on the sensory of the beverage [42]. In this study, the titratable acidity (TA) of the free-cell probiotics was 0.34 and the immobilized probiotics were 0.14 after the fermentation. The TA value significantly increased to week 4 at 4 °C, but it was higher in the cell-free probiotics at all the weeks. The TA values are comparable to the TA values reported for other fermented beverages such as soy-based beverages (0.42) [43], and dairy-based beverages (0.75) (Gomes et al., 2013). The results of this study indicated that mangosteen juice is suitable for the growth of probiotics and it contains the sugars required to produce lactic acid. In addition, the low pH and high acidity of the cell-free probiotics can cause changes in the physical properties of the beverage. On the other hand, the total soluble solids (TSS) expressed as Brix° was lower in the cell-free mangosteen juice (Figure 2 C). This can be explained by the production of higher acid content and reduction of sugars that are utilized by the cell-free probiotics at a high rate compared to the immobilized probiotics. The TSS in stored fruit juice were reported to be increased at low temperatures due to the enzymatic hydrolysis [44]. However, the probiotics (live culture) will utilize the soluble sugars and consistently reduce the TSS value of the juice [45]. Nevertheless, the chemical changes will have a strong influence on the quality and shelf life of the beverages. For example, functional beverages with probiotics should have more than 106 CFU mL-1 to exert their health benefits [46].

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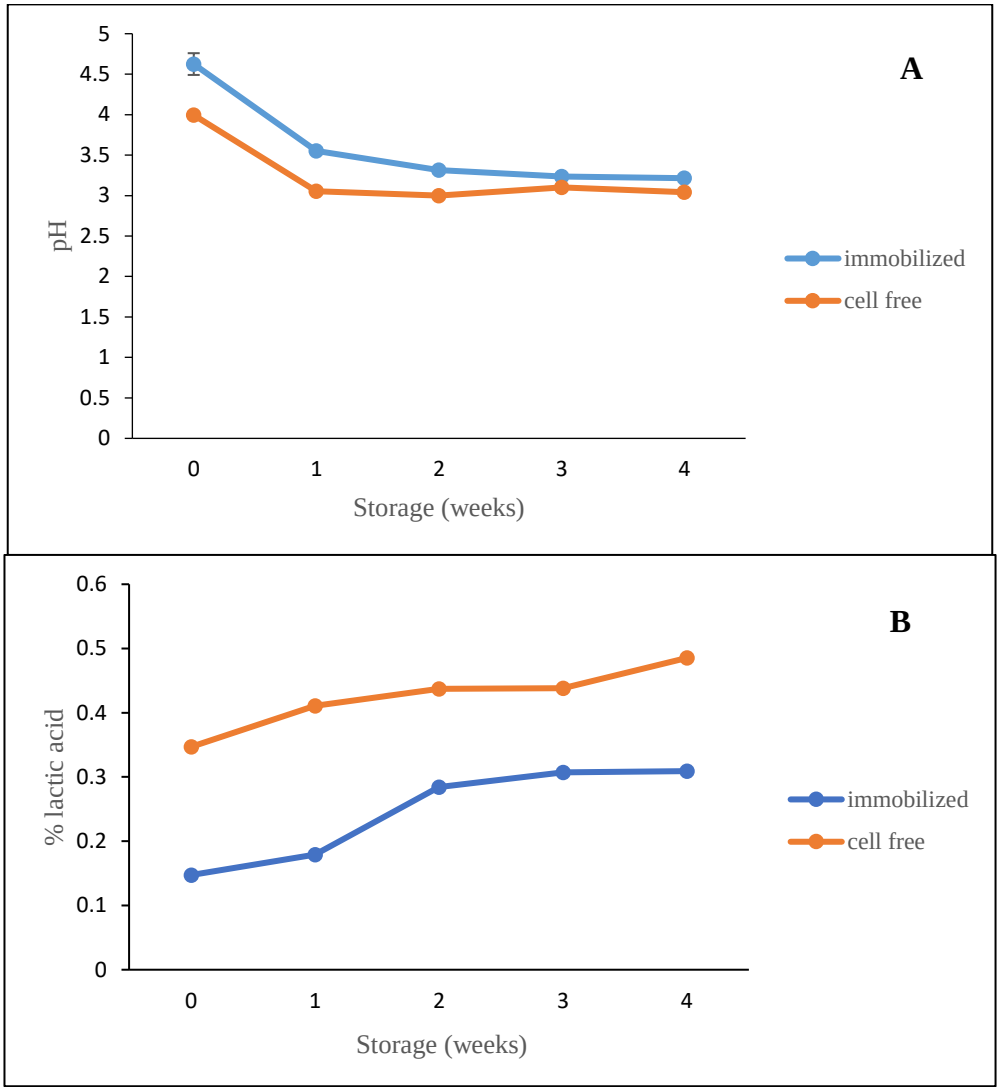

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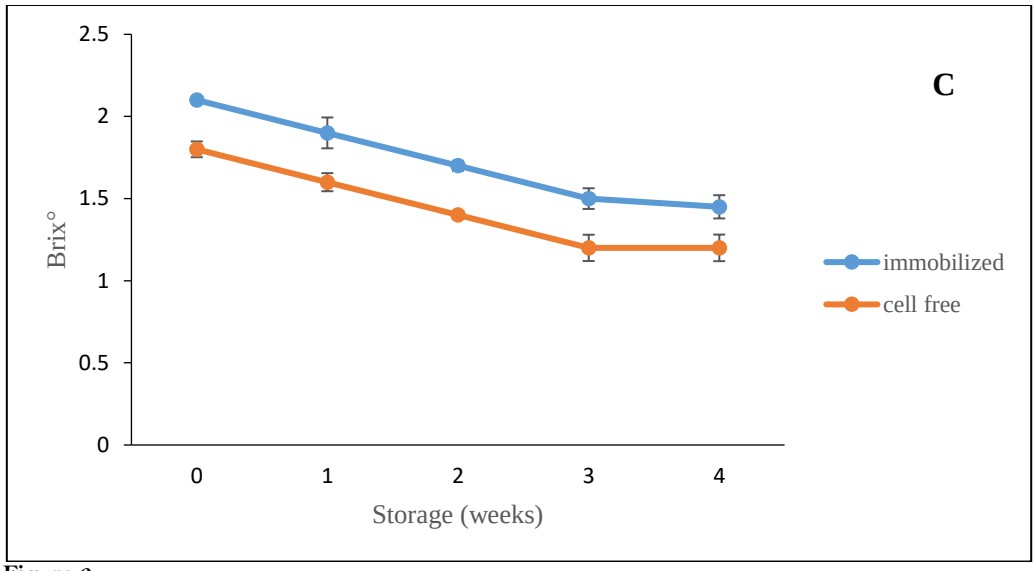

Figure 2.
The physiochemical properties of the mangosteen juice with immobilized and cell free probiotics during the storage for 4 weeks at 4 °C, (A), pH values, (B), titratable acidity, (C) total soluble solid (TSS).

3.4. Effects of Immobilized and Free-Cells on Rheological Properties of Mangosteen Beverage

The viscosity of the mangosteen juice inoculated with the immobilized probiotics was significantly ($p < 0.05$) higher compared to the control and during the 4 weeks of storage (Figure 3). The viscosity of the juice with the immobilized and cell-free probiotics was consistent with minor changes during the storage for 4 weeks. The rheological properties of functional beverages are very critical for the acceptability of the consumers. The viscosity of fermented soy milk was reported to be highly increased after 1 week at cold storage due to the production of polysaccharides [47]. However, the results of this study showed consistent viscosity for the beverage samples during the 4 weeks of storage. The higher viscosity of the immobilized probiotic may be due to the proteins from the CMIF. The CMIF was reported for it is high albumin and globulin contents and contributed to the rheological properties of the coconut milk [25].

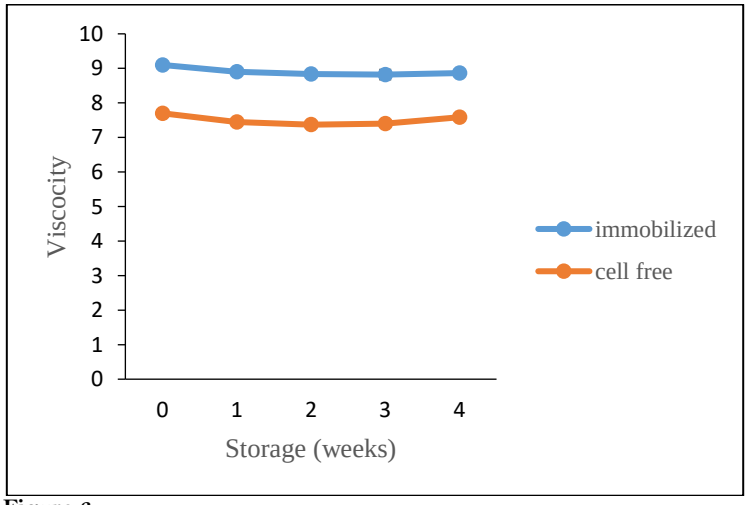

Figure 3.
The viscosity of the mangosteen juice with immobilized and cell free probiotics during the storage for 4 weeks at 4 °C

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3.5. Survival of the Immobilized and Cell-Free Probiotics During Cold Storage

The results of this experiment showed significant ($p < 0.05$) differences in the viability rate between the immobilized and cell-free probiotics after 2 weeks of cold storage (Figure 4). After the lacto-fermentation for 24 hours which is considered week 0 of storage, the cell-free counts were slightly higher than the immobilized. After 1-week storage, the cell counts for the control were slightly declined compared to the immobilized probiotics that was increased compared to the initial cell count. After 2 weeks of storage, the cell counts of the control were in consistent decline, while the cells of immobilized probiotics maintained their concentration till week 4. The results showed that the selected probiotics were able to survive for 4 weeks at low pH. However, the survival rate was significantly increased due to the immobilization process using the CMIF. The environmental conditions have a strong influence on the viability of the probiotics. In this study, the pH of the juice with immobilized probiotics was higher than the cell-free sample and this could be a reason for the high cell counts for the immobilized probiotics. In addition, the results showed the adhesion of high concentrations of the probiotics cells to the CMIF as indicated by the cell count of immobilized probiotics. Hence, CMIF played a very important role as a solid support for the immobilized probiotics. This mechanical support of the CMIF can lead to enhance the survival of the probiotics in harsh conditions such as high acid, low temperature and prolonged storage. Kemsawasd et al., (2016) [32] reported the advantage of the immobilization of probiotics in chocolate and observed the high concentration of viable cells after 60 days at 4 °C. The Agri by-products containing insoluble fibres have a high potential to immobilize the probiotics on their surfaces and increase their viability during storage in unfavourable conditions [31]. Even in yogurt which has an optimal growth condition for the probiotics, it was observed that using biopolymers for the immobilization of probiotics can enhance their viability by 10% compared to free cells [48]. The concept of immobilization has been proven in fruit juice where the survival of cells probiotics is very limited [49]. Therefore, the results of this study were very important to evaluate the potential of using the by-products of the coconut industry that has highly insoluble fibres.

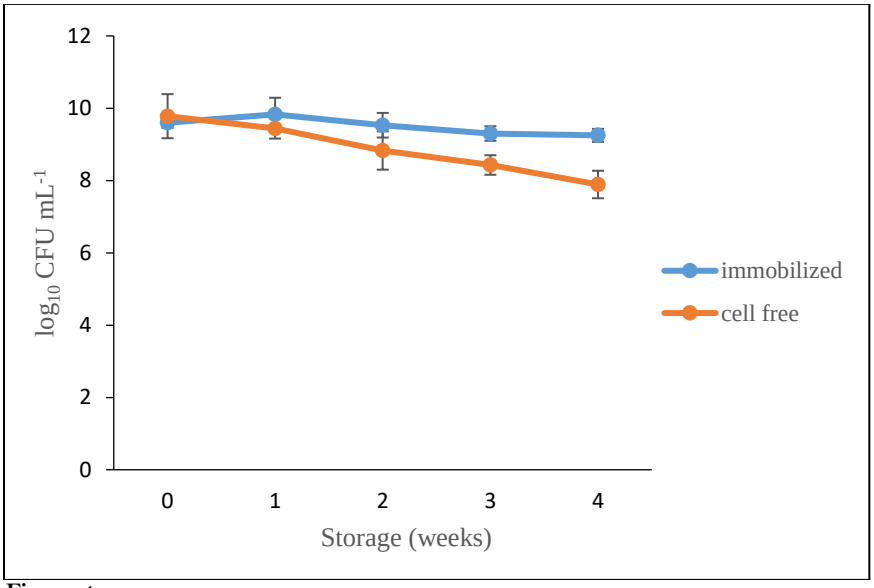

Figure 4.

4. Conclusions

The coconut meat insoluble fibre was found to be a suitable material for the immobilization of probiotics and protecting their cells from unfavourable environmental conditions. The selected probiotic strain in both forms cell-free and immobilized demonstrated good adaption and high growth in the mangosteen juice. The effect of the cell-free and immobilized probiotics on the pH value, acid production and TSS was significantly different with preference for the immobilized probiotics. The effects were also

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observed on the secondary metabolites of the probiotics including the organic acids, amino acids, and vitamins. Moreover, the immobilized probiotics showed higher cell counts during prolonged storage. Based on those results, immobilized probiotics have good potential to be used for the development of plant-based functional beverages looking at the fact that they can survive the storage conditions. The findings of this study are important for the development of probiotic beverages with extended shelf life and high consumer acceptability.

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