

Survival of *Lactobacillus casei* 01 in probiotic-supplemented Mamao (*Antidesma bunius*) juice powder during storage

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Abstract

This research aimed to quantify the survival rates of *Lactobacillus casei* 01 cells in probiotic-supplemented Mamao juice powder with different packaging conditions during storage at 4 and 37°C for 12 weeks. Physicochemical qualities (moisture content, water activity, bulk density and solubility) and bioactive compounds (total phenolics, ascorbic acid and total anthocyanins) of each powder were also assessed. The results showed that the highest survival rate of *L. casei* 01 was found in the vacuum-sealed package stored at 4°C. Also, the significant changes of moisture content, water activity, bulk density and solubility were detected throughout the storing period. The refrigerated storage could allow the powdered samples, especially that in the vacuum-sealed package, to have the best characteristics. The high levels of total phenolics, ascorbic acid and total anthocyanins were observed at this condition. To claim as probiotic food, the shelf-life of this product was 6 weeks (viable cells > 10⁶ CFU/g). This could be concluded that the appropriate package conditions and storage temperatures were necessary for effective preservation of the probiotic-supplemented Mamao juice powder.

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Introduction

The products that are supplemented with probiotics, particularly in the genera *Lactobacillus* and *Bifidobacterium*, are playing an important role in the healthy food industry. This may be because the probiotics have been proved to provide health benefits for the host when administered alive and in the appropriate quantities (Chaikham, 2015a; Huang *et al.*, 2017). The minimum recommended dose for live probiotics was between 6 and 7 log CFUs per gram or milliliter of the products at the time of consumption (Nualkaekul *et al.*, 2013). In recent years, the manufacturing of such probiotic-supplemented food products essentially requires the production of cell cultures for direct inoculation and the dehydration of probiotic cells (Salar-Behzadi *et al.*, 2013). In addition, spray drying is an important approach for the production of probiotic powders. This method offers the benefit of minimal time- and cost-consuming production of the cultures in large-scales as well as the easy and inexpensive shipping and storage of the dried cultures (Silva *et al.*, 2011; Heidebachm *et al.*, 2012). Moreover, this immobilization method can be successfully applied for protecting the probiotic cells during processing and moving through the harsh

environments of digestive system (Chaikham *et al.*, 2017). However, application of high temperature during the process of spray drying can cause critical damage to the bacterial cells. Cell injury and loss of viability have been reported as common negative side effects due to drying process (Silva *et al.*, 2011). In addition, Salar-Behzadi *et al.* (2013) revealed that the production yield of probiotic powder depends on the microbial species and strains, the protective carriers and the processing temperatures. Silva *et al.* (2011) reported that heat stress during spray drying may also impact the stability and the survival rate of probiotics during storage.

Currently, there are only few studies on the fruit juices that containing probiotic bacteria and being powdered by spray drying. Indeed, probiotic-fruit juice powders can retain most of the natural bioactivity found in the original fresh fruit with the additional benefit of a longer storage life (Barbosa and Teixeira, 2017; Huang *et al.*, 2017). They also offer a great benefit to the consumers who are lactose intolerant or following vegetarian diet (Chaikham *et al.*, 2017). In Thailand, a novel, high-quality functional drink from Mamao or Maoluang fruits (*Antidesma bunius* (Linn) Spreng) that are rich in phytochemical antioxidants is currently becoming

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popular among the consumers of all ages (Chaikham, 2015b; Chaikham and Baipong, 2016). Jorjong *et al.* (2015) reported that Mamao fruits contained large amounts of anthocyanins (69g/100g DW), flavonoids (715 mg/100g DW), phenolic acids (398 mg/100g DW), and also high levels of antioxidant capacity as shown by FRAP, TEAC (ABTS^{•+} radical cation assay) and VCEAC (DPPH assay) values at approximately 24 mmol Fe(II)/g DW, 30 mmol TE/g DW and 68 mmol VCEAC/g DW, respectively. The other parts of Mamao have also been evaluated. Choi and Hwang (2005) reported that *A. buniis* leaf extract could inhibit NO release in RAW264.7 cells and exhibit high level of antioxidant activity. Also, Butkhup and Samappito (2011) found that Mamao residues contained significant amounts of phenolic compounds and also antimicrobial activities against some pathogenic bacteria. Therefore, it is interesting to supplement Mamao juice with potential probiotics for production of the functional beverage.

The objective of this research was to monitor the viability of *Lactobacillus casei* 01 cells in probiotic-supplemented Mamao juice powder with different packaging conditions during storage at 4 and 37°C for 12 weeks. Changes of physicochemical qualities and some bioactive compounds in the product were also evaluated.

Materials and Methods

Probiotic strain and cultivation

Freeze dried *L. casei* 01 culture was purchased from Chr. Hansen (Hørsholm, Denmark). Dried cells (0.5g) was rehydrated in MRS broth (50ml) (Hi-Media, Mumbai, India), shaken for 5 mins, and incubated at 37°C for 20hr in an anaerobic jar. After that, the bacterial culture was activated by mixing with MRS broth at 1% (v/v) and incubating anaerobically at 37°C for 14 hr. For separation of cell pellet, the activated culture was centrifuged at 4000 rpm for 20 mins and washed twice with 0.85% (w/v) sterile saline water (Hi-Media, Mumbai, India) before being diluted using sterile saline water to be approximately 10¹² CFU/ml (Chaikham, 2015a).

Preparation of Mamao juice

Mamao fruits were harvested from the Mamao germplasm collection in Rajamangala University of Technology Isan, Sakon Nakhon province, Thailand, then washed twice and extracted using a fruit juice extractor. Subsequently, the juice was pasteurized using thermostatic water bath at 90°C for 1 mins, then cooled down to around 20°C, and finally stored in a refrigerator until required (Chaikham and

Baipong, 2016). This thermal pasteurization was found to effectively eliminate the indicator microbes in Mamao juice such as total aerobic bacteria, *Escherichia coli*, yeasts and moulds (undetectable).

Spray drying of probiotic-supplemented Mamao juice

To produce the powder, pasteurized Mamao juice was blended with 15% (w/v) maltodextrin (10 DE; VR Bioscience, Bangkok, Thailand) and 1% (v/v) probiotic culture (of which cell counts were approximately 10¹⁰ CFU/ml). The mixture was then fed into a spray dryer (JCM Engineering concept, Bangkok, Thailand) which was equipped with a fluid atomizer that had internal diameter of 5 mm. The drying conditions were 30°C feeding temperature, 1 L/h feeding rate, 15 psi atomizing pressure and 160°C hot-air-inlet temperature to generate 80°C outlet temperature (Chaikham *et al.*, 2017).

Storage conditions

Spray dried sample powder was packed into laminated bags with or without vacuum condition, and then stored at 4 and 37°C for 12 weeks. The powders were sampled every 2 weeks for evaluating the number of viable cells and the changes of both physicochemical qualities and bioactive compound contents.

Quantification of viable probiotic cells during storage

To release the probiotic cells, 1 g of sample powder was mixed with 9 ml of 0.1 M sterile phosphate buffer (pH 7) (Merck, Munich, Germany) for 10-15 mins using a stomacher (IUL Instruments, Barcelona, Spain). After that, the mixture was 10-fold diluted using 0.1% (w/v) sterile peptone water (Hi-Media, Mumbai, India) before spreading onto MRS agar (Hi-Media, Mumbai, India) and incubating at 37°C for 48 hr in anaerobic jar. The colonies were counted and calculated as CFU/g sample.

Determinations of bulk density and solubility

Bulk density of the samples was determined according to the modified method of Goula and Adamopoulos (2005). In brief, 5 g of sample powder were added to a 10 ml cylinder and shaken on a vortex vibrator for 2 mins. The ratio between the mass of the powder and the volume occupied in the cylinder was indicated as the bulk density value (g/ml).

To determine the solubility, 1g of sample powder was mixed with 100 ml of distilled water using a magnetic stirrer at a medium speed for 10 mins. Then, the mixture was centrifuged at 4000 rpm for 5 mins, and 25 ml of supernatant were transferred to each Petri dish, which was dried at 100°C for 12 hr using a

hot air oven. The solubility (%) was calculated as the weight difference (Cano-Chaucab *et al.*, 2005).

Measurements of moisture content and water activity

The measurement of moisture content was carried out by placing 5 g of sample powder in a hot air oven at 100°C until reaching a constant weight (AOAC, 2000). Water activity (a_w) of the powder was measured using a water activity meter (AquaLab Series 3, Decagon Devices, Inc., Pullman, WA, USA).

Determination of total phenolic contents

According to the modified method of Chaikham and Baipong (2016), 5 g of sample powder were stirred with 25 ml deionized water for 30 mins using a magnetic stirrer. After that, 5 ml- aliquot of the mixture were well-mixed with 15 ml of absolute ethanol for 10 mins before centrifugation at 4,000 rpm for 10 mins. One ml of the supernatant was added to 5ml of 10% Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA), mixed with 4 ml of saturated sodium carbonate solution (Ajax, Sydney, Australia), kept in the dark for 2 hr, and then measured for the absorbance at a 765 nm using a Perkin Elmer UV WINLAB spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). Total phenolic contents were expressed as mg GAE (gallic acid equivalent)/g powder.

Determination of ascorbic acid

Briefly, 5 ml- aliquot of the mixture (from above Section) was added to 20 ml of diluted sulphuric acid (pH 2.2; Merck, Munich, Germany) for 10 mins and filtered through 0.20- μ m nylon membrane (Vertical, Bangkok, Thailand). Subsequently, 20 μ l of the filtrate were injected to HPLC system (Shimadzu LC-10AD; Shimadzu, Kyoto, Japan), following the modified protocol of Chaikham and Baipong (2016). The isocratic system used 0.1 M acetic acid (a mobile phase) with a flow rate of 1.5 ml/min at 30°C. The peak area of L-ascorbic acid was identified and expressed as mg/g concentration.

Determination of total anthocyanins

Two ml- aliquot of the mixture (from above Section) were well-mixed with 8 ml of 0.03 M potassium chloride buffer (a mixture of 1.9 g of KCl and 980 ml of deionized water; pH 1.0) or 8 ml of 0.4 M sodium acetate buffer (a mixture of 54.4 g of $\text{CH}_3\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$ and 960 ml of deionized water; pH 4.5). The absorbance values of both mixtures were measured at 520 and 700 nm, respectively. Total anthocyanin contents (mg CE/100g) were calculated

[total anthocyanins = $(A \times \text{MW} \times \text{DF} \times 10^3) / \epsilon \times 1$], where, CE is cyanidin 3-glucoside equivalent, A is the absorbance [$A_{\lambda_{\text{vis-max}} \text{ pH } 1.0} - (A_{\lambda_{\text{vis-max}} \text{ pH } 4.5})$], MW is the molecular weight of CE (449.2 g/mol), DF is the dilution factor and ϵ is an extinction coefficient of CE (26,900 $\text{L} \times \text{cm}^{-1} \times \text{mol}^{-1}$) (Lee *et al.*, 2005).

Statistical analysis

The experiments were performed at least in triplicate and the results were expressed as means \pm standard deviations. Analysis of variance (ANOVA) was carried out using IBM SPSS Version 23 (SPSS Inc., Chicago, IL, USA). Also, Duncan's multiple range tests were used to compare the significant differences among the treatment means at $\alpha = 0.05$.

Results and Discussion

Viable cells of *Lactobacillus casei* 01 in probiotic-supplemented Mamao juice powder

Survival of the probiotic cultures during storage is highly crucial. As shown in Figure 1, after storage at 4°C for 12 weeks, the viable cells of *L. casei* 01 apparently decreased ($P \leq 0.05$) around 5.61 and 4.94 log CFUs for normally and vacuum-sealed powders, respectively. For the samples kept at 37°C, there were no probiotic cells under both sealing conditions after 6 weeks of storage. It was noticed that higher survival rates of *L. casei* 01 could be obtained at lower storage temperature and without the presence of oxygen in packaging. Anekella and Orsat (2014) found that the shelf-life of the probiotics in spray dried raspberry powder was reduced when storage temperature increased. Storage at lower temperature ensured longer shelf-life and higher cell count retention at the end of 30 days. This result was similar to the previous report of Corcoran *et al.* (2004), who examined the spray-dried *Lactobacillus rhamnosus* GG in synbiotic powder products. Jonhson and Etzel (1993) and Chaikham (2015a) suggested that the levels of temperature and oxygen content in the package are critical for probiotics during storage. Oxygen may affect the probiotics by three mechanisms: (i) oxygen may directly be toxic to the cells; certain probiotic cultures are very sensitive to oxygen and die in its presence, (ii) some microbial cultures may produce toxic peroxides in the presence of oxygen and (iii) free radicals produced from the oxidation process may be toxic to the probiotic cells (Korbekandi *et al.*, 2011). During storage, lipid oxidation of the probiotics' cell walls, which resulting in permanent damage, is considered as the major cause of short shelf-life of the spray dried cultures (Meng *et al.*,

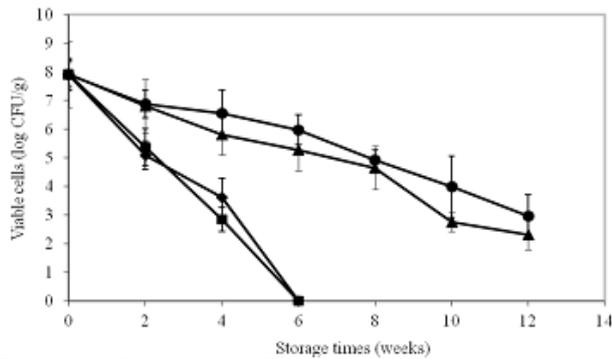


Figure 1. Survival of *L. casei* 01 cells in probiotic-supplemented Mamao juice powder during storage; (▲) normally sealed sample stored at 4°C, (●) vacuum-sealed sample stored at 4°C, (■) normally sealed sample stored at 37°C and (◆) vacuum-sealed sample stored at 37°C

2008). Maness *et al.* (1999) and Shokri *et al.* (2015) demonstrated that one of the causes of the drop in the live probiotic population is lipid oxidation of the plasma membrane constituted by unsaturated phospholipids or fatty acids.

Considering the US FDA recommendation, the minimum probiotic count in a probiotic food should be at least 10^6 CFU/g or CFU/ml at the time of consumption (Tripathi and Giri, 2014). In this study, storage temperature of 4°C and vacuum packaging were recommended to the suitable condition for extension the shelf-life of probiotic-supplemented Mamao juice powder. Therefore, to claim as probiotic food, the shelf-life of this product was 6 weeks.

Changes of moisture content and water activity

Different storage temperatures and sealing conditions had significant effect ($P \leq 0.05$) on moisture content and a_w of probiotic-supplemented Mamao juice powder (Figure 2). The moisture content and a_w of the powder were found to significantly increase ($P \leq 0.05$) throughout the storage period. At the final stage of storage (week 12), the maximal increases of these values were found in the sample packed under normal-sealing condition and stored at 37°C, while vacuum-sealed sample kept at 4°C was found to have the noticeably lower moisture content and water activity than the other treatments ($P \leq 0.05$). Relative humidity (RH) inside the package has been shown to influence the glass transition temperature (T_g) of the dried powders, as there was a report of high RH that induced caking in dried powders and subsequently losing of probiotic cells' viability (Barbosa and Teixeira, 2017). Chavez and Ledebor (2007) reported that increased a_w triggered a faster death rate of probiotics during storage as it stimulated the growth of other microorganisms as well as the occurrence of undesirable chemical reactions, in

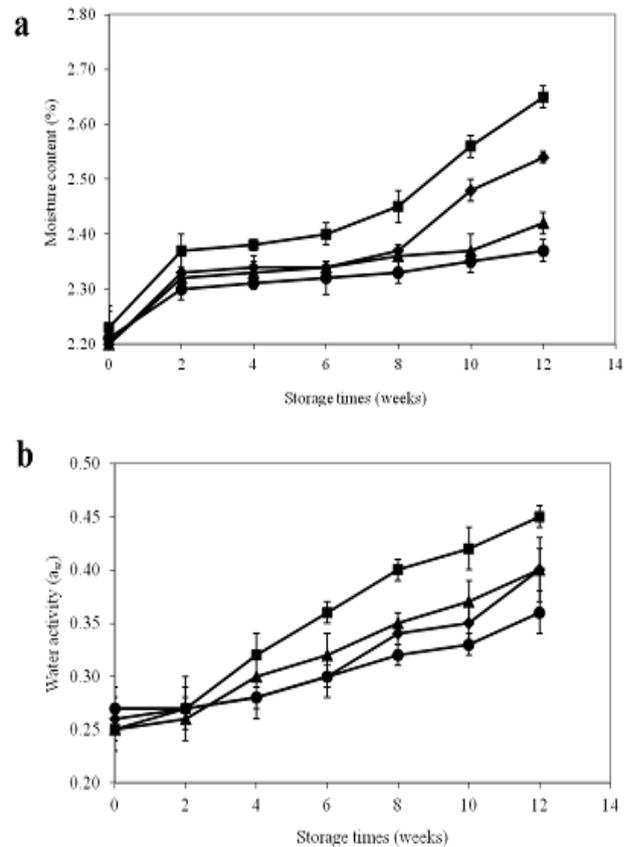


Figure 2. Changes of (a) moisture content and (b) water activity of probiotic-supplemented Mamao juice powder during storage; (▲) normally sealed sample stored at 4°C, (●) vacuum-sealed sample stored at 4°C, (■) normally sealed sample stored at 37°C and (◆) vacuum-sealed sample stored at 37°C

particular at $a_w \geq 0.6$. At high a_w , the formulations of probiotic powder were in a rubbery state (Ying *et al.*, 2012). Additionally, Peighamardoust *et al.* (2011) revealed that maintaining of the residual water was essential for spray dried probiotics to preserve the protein conformations for enzymatic activities, cell wall-lipid membrane structural stability, ribosomal functions, and any other organelles. However, a_w of the spray-dried powder was shown to be dependent on the cell suspension media, carriers, additives, and spray drying conditions (Wang *et al.*, 2004). Furthermore, water activity and presence of oxygen were found to be important factors which affecting the survival of probiotics during storage (Weinbreck *et al.*, 2010). Chavez and Ledebor (2007) and Koc *et al.* (2010) suggested that $a_w < 0.3$ is essential for the survival of the probiotics during storage and the ideal a_w for most of *Lactobacillus* species should be between 0.11 and 0.23. At this ideal a_w , the characteristics of spray dried sample were in a glassy state (Ying *et al.*, 2012).

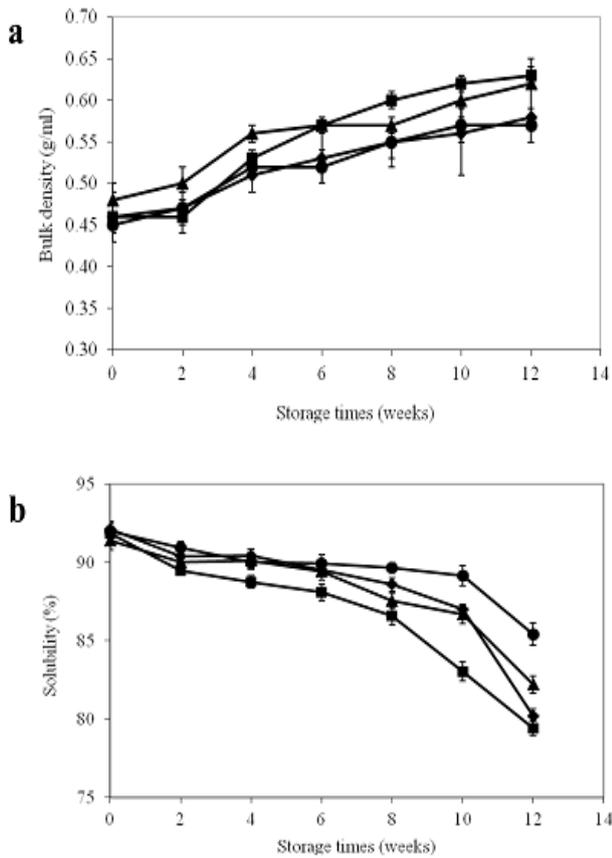


Figure 3. Changes of (a) bulk density and (b) solubility of probiotic-supplemented Mamao juice powder during storage; (▲) normally sealed sample stored at 4°C, (●) vacuum-sealed sample stored at 4°C, (■) normally sealed sample stored at 37°C and (◆) vacuum-sealed sample stored at 37°C

Changes of bulk density and solubility

Figure 3a shows that the bulk density of probiotic-supplemented Mamao juice powder slightly increased during the storage. At the last stage of storage (week 12), the levels of bulk density of powders stored under normal-sealing at 4 and 37°C and vacuum-sealing at 37°C were significantly higher ($P \leq 0.05$) than that of vacuum-sealed powder kept at 4°C. This result could be affected by the increase of moisture content and a_w in the samples (Figure 2a). Moreover, the results also exhibited that storage temperatures and sealing conditions could significantly affect ($P \leq 0.05$) the solubility of the samples (Figure 3b). The highest solubility values were achieved at the initial stage of storage (week 0); however, the solubility tended to decline afterwards, particularly for normally sealed powder stored at 37°C. In this case, moisture contents and a_w of the powders stored at week 12 were significantly higher ($P \leq 0.05$) than that of the other stages of storage (Figure 2), which suggesting that, at the end, the dried powders could have lower solubility. The similar trends of both parameters were reported by Wirjantoro and Phianmongkhol (2009)

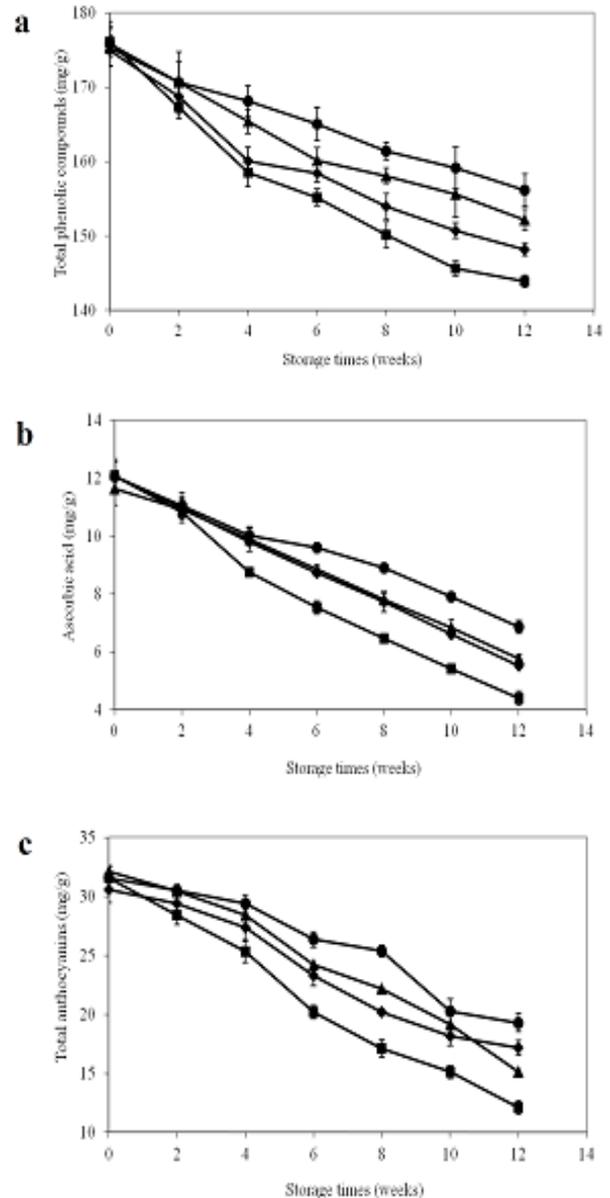


Figure 4. Levels of (a) total phenolic compounds, (b) ascorbic acid and (c) total anthocyanins of probiotic-supplemented Mamao juice powder during storage; (▲) normally sealed sample stored at 4°C, (●) vacuum-sealed sample stored at 4°C, (■) normally sealed sample stored at 37°C and (◆) vacuum-sealed sample stored at 37°C

that investigating *Bifidobacterium bifidum* in yoghurt powder. This may be because that heterogeneous particle distribution could allow the rearrangement of the individual particles and consequently a more compact powder during storage (Fuchs *et al.*, 2006; Flores-Belmont *et al.*, 2015). For this reason, bulk density and solubility for the stored powders for 12 weeks were found to be significant lower ($P \leq 0.05$) than that of the rest. This problem may be resolved using different compositions of coating materials for spray drying probiotic-fruit juice (Barbosa and Teixeira, 2017).

Changes of bioactive compounds

Mamao juice has been known as a great source of total phenolics, ascorbic acid and total anthocyanins (Chaikham, 2015b; Jorjong *et al.*, 2015). As shown in Figure 4, total phenolics, ascorbic acid and total anthocyanins in all powders significantly diminished ($P \leq 0.05$) when the storage time rose. However, most of these bioactive compounds in the vacuum-sealed powders stored at 4°C still remained at high levels as compared to the other samples throughout the storage period. Bakowska-Barczak and Kolodziejczyk (2011) found that the remaining rates of total phenolic acids and anthocyanins in immobilized black currant powders stored at 8 and 25°C for 12 months were approximately 80% and 70%, respectively. The similar results were obtained by Fang and Bhandari (2011) and Ersus and Yurdagel (2007) that evaluating the spray dried bayberry and black carrot powders, respectively. Barbosa *et al.* (2015) reported that the contents of ascorbic acid in probiotic-orange powder were reduced by about 40% after storage 4°C for 180 days. In this study, the levels of total phenolic compounds, ascorbic acid and total anthocyanins in normally sealed samples (21% oxygen content) stored at 37°C were also found to greatly reduced. Zorić *et al.* (2017) reported that sour cherry powder stored at low temperatures (4 and 20°C) were shown to have greater stability of phenolic compounds and antioxidant capacity (DPPH method), whereas the packaging types (PET/PPMET/PE and PET/AL/PE) did not have significant effect on the retention of phenols and antioxidant capacity during the storage for 6 months. Zerdin *et al.* (2003) revealed that oxygen could diffuse into the matrix of the products during storage and oxidize the antioxidant compounds and degrade various bioactive compounds over the period of storage time. Santos and Silva (2008) suggested that ascorbic acid may be extremely degraded when being exposed to high oxygen content, temperature and light.

Conclusion

In this study, the probiotic-supplemented Mamao juice powder in vacuum-sealed package stored at 4°C could maintain high levels of *L. casei* 01 viability and bioactive compounds than those in the other packaging conditions throughout the storage period. Some properties of the powders *viz.* moisture content, a_w , bulk density and solubility were found to change significantly. Refrigerated storage allowed the powder, especially that in the vacuum sealing package, to have the optimal parameters. Thus, this indicates that stability of probiotic, powder properties

and bioactive compounds in spray dried probiotic-supplemented Mamao juice could be preserved for a long time at storage temperature of 4°C under vacuum packaging.

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