

Effect of calcium-alginate bead and *Anoectochilus formosanus* Hayata extract fluid on the viability of *Lactobacillus plantarum* ATCC 8014 and bioactive compounds in fermented apple juice

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Abstract

The aim of this study was to determinate the effect of the encapsulation by calcium-alginate containing (MA sample) or non-containing *Anoectochilus formosanus* Hayata extracted fluid (M sample) on the survival of *Lactobacillus plantarum* ATCC 8014 in fermented apple juice for 60 hours. The antioxidant activity, total polyphenol, polysaccharide, pH values, and the density of *L. plantarum* were determined every 12 hours of fermentation. The fermented apple juice was stored at 4°C in 5 weeks. The pH value and the viable *L. plantarum* were evaluated during storage and in the simulation gastric medium after 4 weeks of storage. The results showed that bioactive compounds increased in the first 24 hours but decreased slowly in subsequent hours of fermentation in which the sample containing encapsulated bead had better results than free cells (F samples). The scavenging activity DPPH, total polyphenol, and polysaccharide of the MA sample were 6.58 mg Vit C/100mL; 304.65 mg GAE/100mL; and 2.98 mg Glu/100 mL, respectively. The viability of *L. plantarum* was maintained over 6 log CFU/mL for the encapsulated samples compared to 4 log CFU/mL for the F samples. The viability of encapsulated *L. plantarum* in A and MA samples was no significant difference during storage, but the survival rate of *L. plantarum* in MA sample was significantly higher than M samples in the SGF (Simulated gastric fluid) medium. The results indicated that adding the *A. formosanus* Hayata extract fluid into the calcium-alginate matrix protected *L. plantarum* cells during fermentation, storage and in the SGF medium.

1. Introduction

Apple was the fruit (*Malus domestica*) used commonly in the whole world and contributed the benefits for human health. Bioactive substances in apple such as phytochemical, triterpenoid and fiber played an important role in defending cells against cancer, cardiovascular therapy, diabetes, asthma, pulmonary edema, aging-resistant and lose weight (Gerhauser 2008; Hyson 2011). Due to these advantages, apples have produced a variety of kind products including candy, apple jam, apple vinegar, and fermented apple juice. In the fermentation process, the activity of available enzymes in materials and bacterial metabolism transformed the nutrition and bioactivity compounds into the valued form for health. Simultaneously, the end products of fermentation such as ethanol, organic acid, and bacteriocin prevented again harmful bacteria in foods (Marco *et al.*, 2017). Besides, using probiotic

bacteria in fermentation not only produced many of advantaged metabolites but also contributed abundant sources of probiotic for customer health. However, the low pH value after fermentation processing, as well as storage time, would affect the viable probiotic. For commercial probiotic products, request for the viability of bacteria is a considered issue. Therefore, the choice of the method to protect probiotic cells is necessary, in which encapsulated technology considered very interesting. Encapsulated preparations contributed an anaerobic environment for sensitive probiotics, simultaneously, were the barrier prevented the impact of acidic environment (Ding and Shah. 2008). Materials often used in encapsulated technology were non-toxic and no effect to bacteria; thus, alginate used commonly in previous studies and protected effectively probiotic (Kailasapathy, 2002; Ding and Shah, 2008; Nualkaekul *et al.*, 2012). Besides many benefits products like apples,

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surveys about herbal medicine realized that *Anoectochilus formosanus* Hayata with many herbal drug activities had the hepatoprotective effect, improved human immunity, and applied in cancer therapy (Tseng et al., 2006). Moreover, compounds in *Anoectochilus formosanus* Hayata have demonstrated a role as prebiotic (Yang et al., 2013). However, studies on the role of *Anoectochilus formosanus* Hayata on the survival of encapsulated probiotic in fermented apple juice, and the storage process was poorly reported. In this study, *A. formosanus* Hayata extracts adding in encapsulated calcium-alginate beads of *Lactobacillus plantarum* ATCC 8014 by extrusion method and adding to apple juice for 60 hours fermentation. Total polyphenol, the scavenging activity DPPH, polysaccharide, and pH changing were evaluated. The viability of probiotic was also determined during storage and simulated digestion environment.

2. Materials and methods

2.1 Preparations for apple juice and *Anoectochilus formosanus* Hayata extracts

Fuji apples were washed with water, cut into small pieces, and seed left. Then, apples were pressed into juice by juicer (Philips-HR1811) and stored at 4°C

Anoectochilus formosanus Hayata (from Institute Tropical of Biology Ho Chi Minh City) were washed by saline then milled. Adding 100mL of distilled water to 20 g of milled plant, this mix was homologized to receive the extracted liquid. The liquid was stored at 4°C until analysis.

2.2 Preparations for microorganism

Lactobacillus plantarum ATCC 8014 strains were incubated in 10 mL of MRS broth at 37°C in 24 hrs, then subculture in 90 mL of MRS broth in the same conditions. Biomass was received by centrifuge and suspended in 10 mL saline for the next encapsulated process.

2.3 Encapsulation

2.3.1 Plasmolysis treatment

The extrusion method using sodium-alginate was carried out according to methods of (Nualkaekul et al., 2012) with some modifies. The biomass of *L. plantarum* (in section 2.2) added into 20 mL of sodium-alginate 2% (w/v) (with or without *A. formosanus* extracted fluid 1% v/v), and homogenized. Then, the mixture was extruded in 0.1M CaCl₂ solution by a piston and kept in 15 mins. The beads were filtered and washed with distilled water.

2.4 Fermentation process

L. plantarum in different forms including free cells (F), encapsulated bead (M), and encapsulated bead contained *A. formosanus* extracted fluid (MA) added into apple juice (100 mL) and fermented at 37°C. The total polyphenol, the scavenging activity DPPH, polysaccharide, pH value, and the viable *L. plantarum* were determined in fermentation processing every 12 hrs. The pH value and the viable *L. plantarum* were evaluated every week of the storage of fermented apple juice for five weeks at 4°C.

2.5 Analytic method

2.5.1 Determination of scavenging activity DPPH

DPPH test was carried out by the description of Mousavi et al. (2013) with some modifications. 1 mL sample diluted with 1 mL twice distilled water was adding into 10 mL of 0.1 mM DPPH methanolic solution. This mixture was stored in dark conditions for 30 mins. Absorbance was measured triplicate by UV-Vis spectrophotometer at 517 nm. Distilled water used as blank. The result was expressed by mg vitamin C/100 mL samples based on the calibration curve of authentic L-ascorbic acid.

2.5.2 Determination of total phenolic

Total phenolic was measured according to methods of Othman et al. (2009) with some changes. Firstly, 0.1 mL fermented juice and 0.9 mL twice distilled water were mixed, then add 5 mL of 10% Folin-Ciocalteu (v/v). This mixture kept in 5 mins and added 4 mL of 7.5% Na₂CO₃ solution. The absorbance at 765 nm was read using a spectrophotometer. Blank treatment was prepared by distilled water. The total phenol value was calculated by mg GAE/100 mL based on the calibration curve of authentic gallic acid.

2.5.3 Determination of polysaccharide

Polysaccharide test was referred to Le et al. (2019) with some modifications. The polysaccharide was recovered after 24 hrs at 4°C from mixture sample: Ethanol (1:5, v/v) by centrifuge 5000 rpm in 10 mins. The polysaccharide was diluted by 10 mL of distilled water (solution B). Then 0.1 mL solution B and 1.9 mL twice distilled water added into 8 mL of Anthrone reagents 2% (w/v). Measurement of absorbance at 630 nm was carried out triplicate. Distilled water was used as blank. The result was displayed by mg Glucose/100 mL on the basic of the calibration curve of glucose.

2.5.4 Simulated gastric and intestinal fluid (SGF and SIF) experiments

SGF and SIF environments were prepared according

to the method of Lieu et al. (2017). SGF contained 9 g/L NaCl, 3 g/L pepsin, and adjusted pH to 2.0 by 5N HCl solution. SIF included 9 g/L NaCl, 3g/L beef bile, and adjusted pH to 6.5 by 5M NaOH solution. The juice was incubated in SGF for 2 hrs and in SIF for 4 hrs. The viable *L. plantarum* was determined indirectly by the plate count method.

2.5.5 Determination of the survival of *Lactobacillus plantarum* during fermentation, storage, and simulated gastric intestinal

A total of 10 mL of sample and 90 mL of pH buffer 7.0 were mixed and carried out by the stomacher in 15 mins. The sample was diluted and determine the viability of *L. plantarum* by the plate count method in MRS agar at 37°C for 24 hrs.

2.6 Statistical analysis

The data analysis was carried out using Statgraphics 15.1 software. The result was recorded as mean \pm standard deviation. Significant differences between means were determined by LSD test of ANOVA procedures ($p < 0.05$). Graphs were described by Microsoft Office Excel 2010.

3. Results and discussion

3.1 Effect of fermentation on the DPPH radical scavenging activity, total polyphenol and polysaccharide contents

The DPPH radical scavenging activity (mg VitC/100mL) of all fermented juices is shown in Figure 1. The scavenging activity trended to enhance maximum at 24 hrs and decrease gradually in the next fermented time for MA (6.58 ± 0.28 mg vitC/100 mL) and M (6.67 ± 0.38 mg vitC/100 mL). In contrast, apple juice with free cells showed a trend to decline DPPH radical-scavenging activity by 50% compared to the initial ($p < 0.05$) (Figure 1). The results showed that all the samples decreased the scavenging activity after the fermentation process (Figure 1). The previous study showed that DPPH radical-scavenging activity enhanced after fermentation (Sabokbar et al., 2016; Le et al., 2019). Le et al. (2019) indicated that lactic fermentation increases the extracted efficiency of the bioactive compounds in *A. formosanus* compared to microwave and ultrasonic treatments. Similarly, the antioxidant activity of fermented pomegranate juice improved regard to the increase of free phenolic content, and products of other metabolic occurred during fermentation (Mousavi et al., 2013). Pyo et al. (2005) indicated that the antioxidant activity enhanced due to the release of aglycone through biologic metabolism during fermentation (Pyo et al., 2005). Li et al. (2012) indicated

that the surface of *L. plantarum* contained protein and polysaccharide that could improve the antioxidant activity. However, modifications could lose these compounds, which caused the decline of DPPH radical-scavenging activity (Li et al., 2012). That would be that metabolisms in the matrix of the material occurred at a particular moment, which improved antioxidant activity but decreased it in the subsequent time.

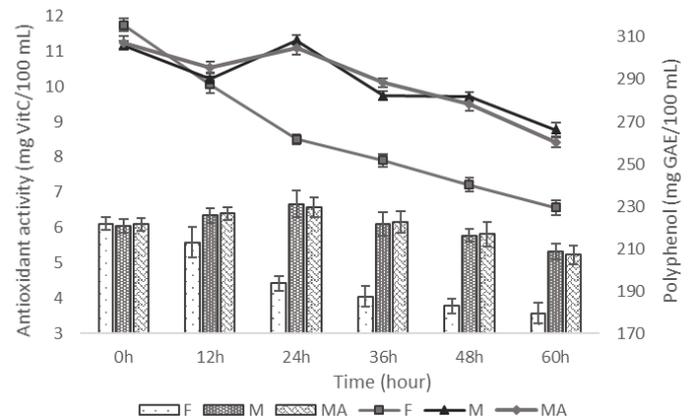


Figure 1. Antioxidant activity (column graph) and total polyphenol (line graph) during fermentation

The changing of total phenolics is indicated in Figure 1. Total phenol content trended decline absolutely in all fermented samples. The samples containing free cells (F samples) decreased completely for 60 hours fermentation (315.65 ± 3.11 mg GAE/mL to 229.45 ± 3.44 mg GAE/100 mL). Total phenol content in M and MA samples decreased from 305.96 ± 2.15 and 307.24 ± 3.05 mg GAE/100 mL to 266.24 ± 3.44 and 260.22 ± 2.45 mg GAE/100 mL, respectively. In M and MA samples, at first 12 hrs, polyphenol content decreased dramatically but enhanced at 24 hours and continuously declined in the next period. In previous studies, the decrease of polyphenol was due to the appearance of biofilms preventing the release of phenolic compounds and affected by phenolics metabolic procedure by *L. plantarum* (Othman et al., 2009; Hashemi et al., 2017). After fermentation processing, the total phenolics level might enhance or decrease the initial value due to the metabolism of probiotic (Đorđević et al., 2010). The loss of polyphenol content during fermentation caused the reduction of antioxidant capacity (Othman et al., 2009). Materials matrix could influence on metabolic activity and biologic converts of cells.

The results of the polysaccharide content test are shown in Figure 2. Although samples adding encapsulated beads reduced polysaccharide content during fermentation, their glucose level was higher than that of free cells. Polysaccharide content at 60 hours was 2.78 ± 0.10 ; 2.65 ± 0.07 ; and 1.56 ± 0.11 g Glu/100 mL for MA; M; and F samples respectively, which reduced 13%

and 52% than initial for encapsulated and non-encapsulated cells. The results showed that free probiotic cells consumed more sugars than encapsulated cells. The sugars level declined significantly during fermentation. In the fermentation procedure, the bacteria cells participated in converting glucose and fructose as carbon energy resources for metabolism in which *L. plantarum* strains consume more glucose than fructose (Mousavi et al., 2013). The carbohydrate converts by *L. plantarum* calculated on Glucose value occurred in the sample with free cells faster than that of encapsulated cells (Hashemi et al., 2017). The results from Figure 1 and Figure 2 showed that most of the biologic metabolism happened in the first 24 hours, and there was no significant change if extending fermentation time. Therefore, 24 hrs of fermentation was an appropriate moment to finish the fermentation procedure.

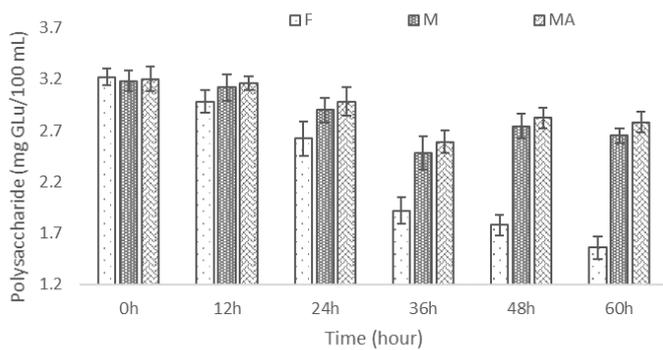


Figure 2. Polysaccharide data during fermentation

3.2 Changes of pH value and the viability of *Lactobacillus plantarum* during fermentation

The pH value of M and MA samples after fermentation increased from the initial pH value of 3.71 to 3.84 and 3.81 respectively (Figure 3). In contrast, the F samples declined to 3.45 compared to the initial pH value. At 24 hrs of fermentation, the pH values of three samples were 3.96 - 4.09 (Figure 3), which in the safe pH value for storage (Costa et al., 2013). Roberts et al. (2018) reported that the pH value of fermented apple juice containing free or encapsulated cells has a significant difference after 72 hours of fermentation, in which the pH value of the free cell sample was lower than encapsulated samples. The results indicated that free probiotic cells produced more acid than encapsulated probiotic. Due to the protective capacity of the matrix, the pH value of fermented juice containing encapsulated bacteria changed insignificantly (Antunes et al., 2013).

At 60 hours of fermentation, the viability of *L. plantarum* in F, M, and MA samples were respectively 6.48 ± 0.22 ; 7.37 ± 0.28 ; and 7.97 ± 0.27 log CFU/mL (Figure 3). The results realized that there was a significant difference through 60 hours fermentation of

apple juice ($p < 0.05$). All the samples had a trend to decrease the viable *L. plantarum* during fermentation, in which the viable *L. plantarum* in F samples was lower than encapsulated samples. In the first 24 hrs of fermentation, the viable *L. plantarum* in F samples was reduced approximately 4 log CFU/mL compared to 2.10 to 2.75 log CFU/mL in the encapsulated samples. The viable *L. plantarum* in all samples was stable to the last of fermentation. In the previous study, the viability of probiotic bacteria in the fermented pomegranate juice was decreased in the first 24 hrs of fermentation (Mousavi et al., 2013). The results due to the difference in the pH value of the fruit juice and the preliminary culture medium in which the low pH of fruit juice caused the vibration for probiotic bacteria leading to a decrease in the survival of probiotic bacteria. Moreover, the appearance of acetic acid, lactic acid, hydroperoxide, oxygen, and low pH decreased the survival ability of probiotic strain (Rokka and Rantamäki, 2010). Though alginate matrix could limit the impact of acid released from juice and maintains the sensory of foods, the alginate matrix did not protect probiotic bacteria against the adverse medium completely (Sohail et al., 2012). Therefore, adding the support component into the gel matrix is necessary to improve the viable probiotic bacteria. Yang et al. (2012) indicated that polysaccharides extracted from *A. formosanus* affected positively on the growth of *Bifidobacterium breve* 2.5 times higher than using inulin in the in vitro test. In the present study, the viable *L. plantarum* in MA samples was slightly higher than that of M samples, but a significant difference was not recorded.

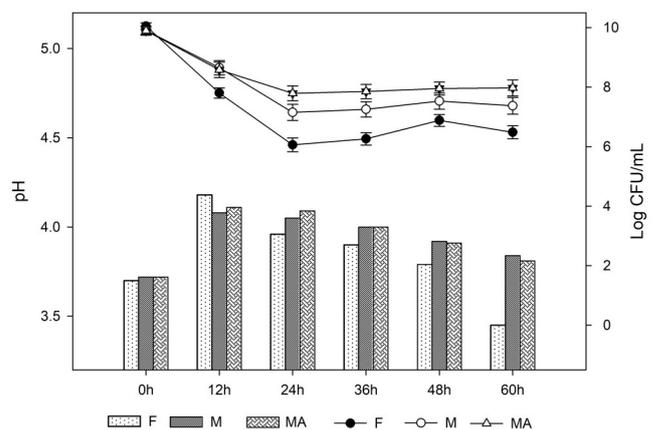


Figure 3. pH value (column graph) and the viability of *L. plantarum* (line graph) during fermentation.

3.3 Effect of microencapsulation on pH value and viability of *Lactobacillus plantarum* during storage

During storage time, the change of pH occurred in all the samples (Figure 4). The pH values of MA and M samples (4.09 and 4.05 initially) were declined to 3.65 and 3.70 respectively. The juice fermented by free cells declined from 3.96 to 3.09. Both encapsulated treatment

and free cells affected pH value in preservation. The previous studies carried out the experiments to control the change of pH during storage weeks (Ding *et al.*, 2008; Sohail *et al.*, 2012; Antunes *et al.*, 2013). Ding *et al.* (2008) indicated that the pH value of fermented fruit juice containing free probiotic cells was reduced significantly compared to encapsulated cell samples during six weeks of storage. Similar, the pH of orange juice containing encapsulated probiotics (alginate 2% w/v) was significantly ($p < 0.05$) higher than that containing free probiotic bacteria (Sohail *et al.*, 2012). This phenomenon could be explained that free cells bacteria were unstable in the low pH environment, and the release of sugars disintegrated enzymes from dead bacteria declined pH value. It's demonstrated that microencapsulation contributed protective ability for bacteria cells during storage and had a higher pH value than free cells (Antunes *et al.*, 2013).

The results of the viable *L. plantarum* showed that free probiotic cells had the lowest survival rate with 3 log CFU/mL of loss after five weeks of storage compared to the encapsulated cells with 3.34 ± 0.25 ; 5.28 ± 0.16 ; and 5.82 ± 0.24 log CFU/mL for F, M and MA samples respectively (Figure 4). After four weeks of storage, the fermented apple juice containing encapsulated probiotics maintained above 6 log CFU/mL that appropriate to the requirements of functional foods. The encapsulation technique contributed to an anaerobic environment suitable for sensitive bacteria, simultaneously, was the barrier against the acidic environment in juice, the low pH and digestive enzyme (Ding and Shah, 2008). Besides, encapsulated beads protect probiotic cells to avoid damages from the adverse medium such as cold storage, and the digestive system (Krasaekoopt *et al.*, 2003). The results from the present study suggested that the viability of *L. plantarum* could stable maintain during four weeks of storage at 4°C, which might meet completely demands to improve the survival rate of probiotic.

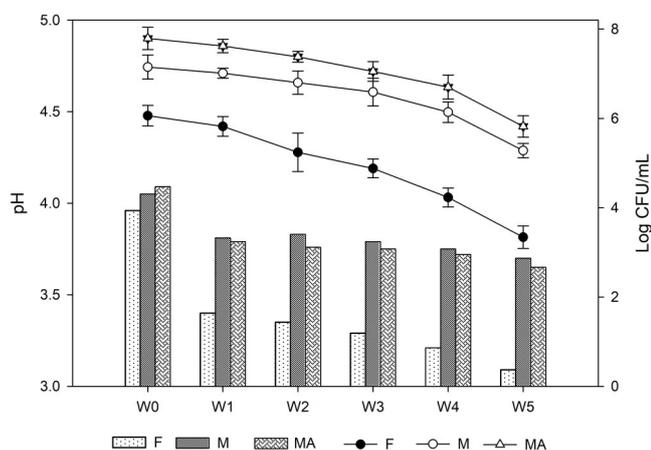


Figure 4. pH value (column graph) and the viability of *L. plantarum* (line graph) during storage.

3.4 Viability of *Lactobacillus plantarum* ATCC 8014 in SGF and SIF

The survival rate of *L. plantarum* in the simulated gastric intestinal after four weeks of storage was presented in Figure 5. In the SGF assay, the viability of *L. plantarum* in the F samples was lost completely, whereas one to two log CFU/mL loss was recorded in the M and MA samples (Figure 5). The previous study demonstrated that the resistance of bacteria decreased significantly in the low pH of SGF (Lieu *et al.*, 2017; Roberts *et al.*, 2018). Therefore, microencapsulation protected bacteria cells from the impacts of low pH in the gastric environment (Nualkaekul *et al.*, 2012). In the SIF assay, the survival rate of *L. plantarum* in F, M, and MA samples were 3.11 ± 0.32 ; 5.66 ± 0.22 , 5.05 ± 0.17 log CFU/mL respectively. A similar result was reported by Nualkaekul *et al.* (2012) showed that probiotic bacteria was not sensitive in the SIF as much as that in the SGF medium.

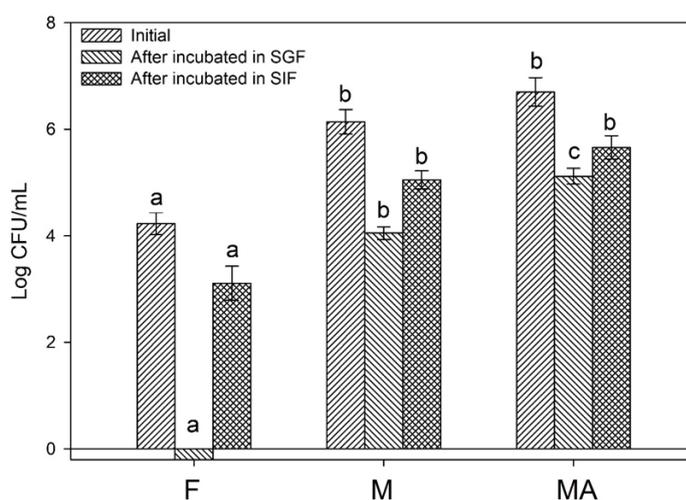


Figure 5. The viability of *L. plantarum* after SGF and SIF. Different letters above the bars indicate significant difference among samples ($p < 0.05$).

The previous studies showed that probiotic improved significantly the viability of probiotic bacteria in the simulated gastric fluid (Kingwatee *et al.*, 2014; Lieu *et al.*, 2017). Kingwatee *et al.* (2014) indicated that inulin 5% (w/v) improved significantly the resistance of *Lactobacillus casei* 01 against the gastrointestinal environment. Besides, the study of Yang *et al.* (2012) demonstrated that *A. formosanus* extract fluid could improve the growth of probiotic bacteria that 2.5 times higher compared to inulin. In the present study, it is interesting to note that the viability of encapsulated *L. plantarum* in M and MA samples were no significant difference ($p > 0.05$) during storage (Figure 4), but the survival rate of *L. plantarum* in MA sample was significantly higher ($p < 0.05$) than M samples after 2 hours incubation in SGF medium (Figure 5). *A. formosanus* extracts improved the viability of probiotic

bacteria due to the adjustment of ATP transportation – a part of nutritional absorbance (Lu *et al.* 2013). This mechanism would help the survival rate of *L. plantarum* in MA samples was improved higher than M samples (Figure 5).

4. Conclusion

Probiotic cells encapsulating by calcium-alginate 2% (w/v) containing *A. formosanus* extracted fluid showed the best protection and preservation capacity. The DPPH scavenging activity, total polyphenol, and polysaccharide decreased trendily during fermentation in all samples, especially juice adding the free cells, and 24 hrs of fermentation process was a suitable moment to finish the fermentation. The resistance of encapsulated probiotics in fermentation and storage was improved effectively. The results of SGF and SIF assay showed that plant extracted fluid from *A. formosanus* in MA samples improved the resistance of *L. plantarum* under the low pH impact and hydrolytic enzymes.

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