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RESEARCH ARTICLE

Quantification of Bioactive Compounds in Freeze-Dried Fermented Bitter Melon (*Momordica charantia* L.) Juice Using *Lactobacillus fermentum* LLB3

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ABSTRACT
Bitter melon (*Momordica charantia* L.) is one of the plant-based functional foods, which has many health benefits potential due to its bioactive compounds. The usage of *Lactobacillus fermentum* LLB3 to ferment bitter melon juice has been studied to increase its functional activities. However, the amount of bioactive compounds contained in the fermented juice have not been studied. Therefore, this research provides the quantification of bioactive compounds, including total phenolic and total flavonoid contained in fermented bitter melon juice. Bitter melon fruit was juiced, filtered, and pasteurized at 80˚C for 5 minutes. Fermentation was done by using Lactobacillus fermentum LLB3 with incubation times of 24, 48, and 72 hours. The samples were freeze-dried, and the bioactive components were quantified using the spectrophotometry (UV-Vis) method. All data obtained were analyzed statistically to determine the significance of the difference. Both total phenolic and total flavonoid contents were found to be increased by the pasteurization proses due to the thermal treatment applied. However, the fermentation process did not perform any increase either in total phenolics or total flavonoids. Furthermore, the freeze-drying process did not give a significant effect on flavonoid contents, but higher values of phenolic content were obtained.

KEYWORDS: *Bioactive compounds, Bitter melon, Fermentation, Freeze-dried*

Introduction

Nowadays, people have changed toward a healthy lifestyle, where they became to care about their food and how it would affect them, especially in this post-pandemic era of COVID-19 (Murphy *et al.*, 2021). Unhealthy diets could lead to chronic inflammation and impaired host defense against viruses (Butler & Barrientos, 2020). Adequate intake of specific nutrients and the combination with the essential one may provide a positive effect on the immune system by way of cell activation and alteration of both gene expressions and signalling systems. Moreover, the effects of dietary ingredients on gut microbiota activities can also influence the body's immune responses (Lange, 2021).

Bitter melon (*Momordica charantia*) is one of the most nutrient-dense plant-based food, which rich in bioactive compounds. It is known that bitter melon has phenolics, flavonoids, terpenoids, carotenoids, and phytosterols, which are responsible for their extensive range of beneficial health effects, including immunomodulatory, anti-inflammatory, anti-diabetic, antiviral, anticancer, analgesic, hypocholesterolemic, and hypolipidemic activities (Tan *et al.*, 2016; Villarreal-La Torre *et al.*, 2020).

Lactic acid fermentation on bitter melon has proven to cause several positive biotransformations and results in higher quality along with the enhancement of its benefits potential. Furthermore, several studies have linked the consumption of fermented bitter melon with its potential to treat diabetes (Hartajanie *et al.*, 2020; Hartajanie *et al.*, 2018;

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Mazlan *et al.*, 2015). Research by Hartajanie *et al.* (2018) and Mazlan *et al.* (2015) reported that fermented bitter melon by lactic acid bacteria has performed a reduction in bitterness and lesser sugar content, as well as the increment of its antioxidant activities, which is presumed to be related with the increase in bioactive components. Further, an *in-vivo* study by Hartajanie *et al.* (2020) found that fermentation on bitter melon juice by the use of *Lactobacillus fermentum* (LLB3) strain results in a better α-glucosidase inhibitory activities compared to the unfermented juice, and has a potential in treating type-2 diabetes. However, the increase in the number of bioactive compounds contained in fermented bitter melon has not been studied further.

Drying is the most common preservation method of food products that implies a decrease in the product's volume and weight, which makes it easier to carry and handle. However, drying method and conditions are highly affect the biomaterials' quality attributes, including physical and sensorial changes, also nutritional and functional transformations (Karam *et al.*, 2016). Freeze-drying is a non-thermal dehydrating technique which involves the sublimation of water in a product, thus able to maintain the food qualities like flavor, color, and appearance while also limiting the breakdown of thermolabile chemicals (Silva-Espinoza *et al.*, 2020).

This research is a follow-up study from a previous work carried out by Hartajanie *et al.* (2018), which used bitter melon juice and further be pasteurized and be fermented by the addition of probiotics culture (*Lactobacillus fermentum* LLB3) with varied incubation times. The products obtained have been tested quantitatively to determine the amount of bioactive components contained in fermented bitter melon juice. The results will be freeze-dried for preservation, and the bioactive components were re-quantified. The amount of bioactive components measured including total phenolic content (gallic acid equivalent / GAE) and total flavonoid content (quercetin equivalent / QE). Each bioactive components have been quantified in fresh juice, pasteurized sample (15 minutes pasteurization at 80˚C), fermented samples (24, 48, and 72 hours of fermentation time), and freeze-dried samples. All data obtained were analyzed statistically to determine the significance of the difference.

Materials and Method

Preparation of Bitter Melon Juice

Fresh unripe bitter melon fruits with the age of 40 days after planting were purchased from Pasar Bandungan, Ambarawa. The fruits were washed and split in half, to separate the seeds from the flesh. The flesh was cut into smaller pieces, and was juiced by using food processor (Philips). The juice obtained was pasteurized in a light-resistant glass bottle for 5 minutes at 80˚C. In order to prevent spoilage, the pasteurized juice was stored in the freezer until next use (Modified from Hartajanie *et al.*, 2020, 2018).

Preparation of *Lactobacillus fermentum* LLB3

About 1 g of freeze-dried pure culture was obtained from previous study (Hartajanie *et al.*, 2018). The strain was reactivated by using 10 ml of MRS broth (Merck) and was incubated at 37˚C for 48 hours. The inoculum was then be cultured in 30 ml of MRS broth at 37˚C for another 48 hours. The 40 ml inoculum obtained was separated into sterilized 4 centrifuge tube (10 ml each) and was centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and the precipitated bacterial cells were rinsed by adding and homogenizing 10 ml of saline water (0.9%) in each tube, then were re-centrifuged at 3000 rpm for another 15 minutes. The pellets were then be homogenized with 40 ml mixture of skim milk (IndoPrima) and glycerol in a 1:1 ratio. The system was then be divided into 40 microtubes (1 ml each tube) and stored as frozen stocks for further use. All processes were carried out aseptically.

Figure 1. Fresh Bitter Melon Fruit (source: personal documentation).

Freeze Drying Process of Fermented Bitter Melon Juice

The samples were frozen to −22˚C before being put into the freeze-dryer chamber (Thermo Scientific Heto PowerDry LL1500). The freeze-drying process was run for 48 hours at the temperature of −100˚C. The freeze-dried samples were rehydrated to its original volume for further assays.

Juice Preparation

The method was modified from Ghasemzadeh *et al.* (2010), in which 1 gram of each fermented juice sample or 0.1 gram of each freeze-dried sample was taken and juice continuously with 10 ml of analytical-grade methanol. The solution was sonicated (Branson Sonicator) for 15 minutes to ensure its homogenization, and further will be stored at refrigerator temperature for further assay.

Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Total phenolic content of the fermented bitter melon juice was determined using the method modified from Ghasemzadeh *et al.* (2010). Analytical gallic acid standard (Sigma Aldrich) was diluted into a concentration of 0, 6.25, 12.5, 25, 50, 75, and 100 ppm. Juiced samples were then added into 7.5 mL of deionized water and then added with 0.75 mL of Folin-Ciocalteu reagents (Merck). The samples were then vortexed and were reacted to for 5 minutes. Following short, 1.75 mL of 20% sodium carbonate (Na_2CO_3) solution was added and the solutions were again vortexed to ensure even reaction. The samples were then kept in total darkness for 1 hour before the absorbance was measured at 750 nm using a UV-Vis spectrophotometer (SHIMADZU UV-1780). Results were then expressed as gallic acid equivalents (GAE) in ppm.

Determination of flavonoid content in fermented bitter melon juice was done by modifying Ghasemzadeh *et al.* (2010) method. Analytical quercetin standard (Sigma Aldrich) was used diluted into a concentration of 0, 10, 20, 40, 60, 80, and 100 ppm. Standard solutions' dilution series were then reacted using the same following total flavonoid assay to obtain a standard curve. Juiced sample containing flavonoids of 0.4 mL was mixed with 5% (w/w) sodium nitrite ($NaNO_2$) of 0.28 mL and 30% (v/v) ethanol of 4 mL. After a wait of 5 minutes, 10% aluminium chloride ($AlCl_3$) of 0.28 mL was added. The solution that has been reacted for 6 minutes was then mixed with 1 M sodium hydroxide (NaOH) of 5 mL before diluting to 10 mL using 30% (v/v) ethanol. The sample was then

reacted for 10 minutes, and the absorbance was measured at 430 nm using a UV-Vis spectrophotometer (SHIMADZU UV-1780). The results obtained were expressed as quercetin equivalent (QE) in ppm.

Statistical Analysis

The data analysis was performed using adapted method from Bryman & Cramen (2005). All samples from each treatment were assessed with three repetitions. Data from the test results were analyzed statistically using the *Statistical Package for the Social Sciences* (SPSS) 13.0 program. Paired T-test has been used for statistical analysis to demonstrate the effect of pasteurization and the significance of differences in the levels of bioactive contents tested, as well as before and after fermentation. Similar method has also been used to demonstrate the significance of differences in the levels of tested bioactive contents before and after freeze-drying process. In order to determine the differences in pH values, also the differences between bioactive contents analyzed in the samples with different fermentation time, the One-Way ANOVA test has been used. The value is considered statistically significant if the p value <0.05 (Modified from Mahindrakar & Rathod, 2020).

Results and Discussion

Pasteurization is the most common technique to inactivates harmful microorganism and enzymes (Deshaware *et al.*, 2019). This step became especially important in the making of fermented bitter melon juice to ensure undisturbed fermentation process (Santhirasegaram, Razali, George, & Somasundram, 2015). **Error! Reference source not found.** shows a decreased pH value of the pasteurized sample compared to the fresh sample. The pH value of the pasteurized sample then decreased with the length of the fermentation period due to the production of organic acids. Each sample fermented for 24, 48, and 72 hours had significantly different pH values ($p < 0.05$). Based on the results, pasteurized bitter melon juice has a significantly lower pH compared to the fresh bitter melon juice. According to Le Chatelier's principle, when the temperature of the bitter melon juice is increased, it will cause a molecular vibration, which leads to ionization and formation of H+ ions, causing the solution to be more acidic (Dmitriev, 2007).

Table 1. Experimental pH value test results on fresh, pasteurized, and fermented bitter juice melon juice.

Fresh	Pasteurized	Fermented		
		24 hours	48 hours	72 hours
5.5 ± 0.15^{a}	$4.76 \pm 0.03^{b,1}$	4.45 ± 0.02^{2}	4.33 ± 0.04^{2}	4.26 ± 0.02^{2}

* Note:
- pH values of fermented samples are represented by mean ± standard deviation of 3 measurement repetitions.
- Values with different letter superscripts in one line indicate a significant difference between treatments at a 95% confidence level ($p < 0.05$) by using:
  - ab = *Paired T-Test* for fresh and pasteurized samples.
  - 12 = *Paired T-Test* for pasteurized and fermented samples.

The result of the total phenolic, flavonoid contents, and antioxidant activity of the fresh and pasteurized samples can be seen in Table 2. The result of each bioactive component measurement on fresh and pasteurized samples were all significantly different when analyzed using paired T-Test analysis. Both total phenolic (GAE) and total flavonoid (QE) contents have significantly increased due to the pasteurization process applied on fresh bitter melon juice. The highest values of total phenolic, flavonoid content, and antioxidant

activity were all obtained in pasteurized samples with the values of 339,27 ppm GAE, 24.38 ppm QE, and 82.34% respectively.

Table 2. Total phenolic (GAE), total flavonoid (QE) contents, and antioxidant activity in fresh and pasteurized bitter melon juice.

Component	Fermented	
	Fresh	Pasteurized
Total Phenolic (GAE)* (ppm)	220.63 ± 8.38[a]	339.27 ± 43.76[b]
Total Flavonoid (QE)** (ppm)	5.09 ± 2.29[a]	24.38 ± 2.68[b]
Antioxidant Activity (%)	20.08 ± 0.73[a]	82.34 ± 1.52[b]

Note:
- *GAE: gallic acid equivalent.
- **QE: quercetin equivalent.
- Total phenolic content, flavonoid contents, and antioxidant activity in both fresh and pasteurized samples are represented by mean ± standard deviation of 3 measurement repetitions.
- Values with different letter superscripts [(a, b)] in one row indicate a significant difference between treatments at a 95% confidence level ($p < 0.05$) by using paired *T-Test* for fresh and pasteurized samples.

Antioxidants are beneficial compounds that eliminates harmful free radicals. Because of its importance, antioxidant activity of bitter melon juice posed beneficial function for the body (Islam *et al.*, 2011). As shown on Table 2, it can be seen that pasteurized bitter melon juice has a higher phenolic and flavonoid content compared to fresh bitter melon juice. It was known that bioactive compounds in food are bound to insoluble polymers by covalent bonds, heating thus released the bound phenolics. This resulted in higher antioxidant properties indicated by significantly increased total phenolic, flavonoid content, and antioxidant activity (Table 2). Lou *et al.* (2014) and Choi *et al.* (2011) also reported the same result, in which longer heat treatment on citrus fruits result in higher phenolics and flavonoids content due to liberation of the bioactive compounds. Furthermore, increase of antioxidant activity due to heat treatment has also been observed at quince fruit due to loss of some water during heating, in which heat modifies the lignocellulosic and protein structure, thus resulting in increased presence of phytochemicals in bitter melon juice matrix (Maghsoudlou *et al.*, 2019).

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Gallic acid liberation was caused by heating of phenolic precursors such as tannin into gallic acid and pyrogallol, hence increasing the antioxidant activity of bitter melon juice after pasteurization (Kim *et al.*, 2011). This was also confirmed by Alavi Rafiee *et al.* (2018), in which heat degradation of tannin produced gallic acid and pyrogallol which has more antioxidant activity due to the carbocyclic group. Conversion of rutin glycoside in bitter melon into quercetin through thermal process and hydrolysis may also be the reason to

the increase of antioxidant activity as observed in Yang *et al.* (2019) research. Recent findings by Hsieh *et al.* (2021), confirmed that there is a possibility of increasing antioxidant activity through thermal processing.

Consistent to Hartajanie *et al.* (2018), the result obtained from this work also performed a significant decrease in pH value of the samples over the fermentation periods (Table 1.). This lowered pH value refers to the production of organic acids and this indicates the occurrence of fermentation process in bitter melon juice. Common acids produced during fermentations includes lactic acid and acetic acids (Degrain, Manhivi, Remize, Garcia, & Sivakumar, 2020), however other acids such as alcoholic acids, succinic, pyruvic, oxalic, and other acids may also be produced depending on the fermentation substrates (Chidi, Bauer, & Rossouw, 2018). The declining pH on fermented bitter melon juice may bring about benefits such as reducing the spoilage of the bitter melon juice by inhibiting pathogenic microorganism, therefore prolonging the shelf life of the produce (Heredia-Castro *et al.*, 2021; Torres *et al.*, 2020; Hartajanie *et al.*, 2018).

Normally, during fermentation process, the phenolic content of the food matter would be increased, as the microorganism, in this case *Lactobacillus fermentum* LLB3 produces the β-glucosidase enzyme, which is able to breakdown glycosides into its aglycone that is biologically more active (Liu *et al.*, 2021). This enzyme produces aglycone and carbohydrate through catalysing the hydrolysis of *O*-glycosidic bonds between two or more carbohydrates or between aglycones and carbohydrates (Rokni *et al.*, 2021). Aglycones are more biologically active as it is easier to be absorbed to the intestines compared to glycosides, since glycosides has greater molecular weight and hydrophilicity (Myagmardorj *et al.*, 2018). Myagmardorj *et al.* (2018) reported that the highest enzyme activity of β-glucosidase in *Lactobacillus fermentum* was during the first 20 hours of fermentation (substrate used was soymilk). Conversion of gallic acid through tannin catalysed by tannase has also been observed in some *Lactobacillus fermentum* species (Seveline *et al.,* 2021; de las Rivas *et al.*, 2019).

Table 3. Total phenolic (GAE), total flavonoid (QE) contents, and antioxidant activity in pasteurized and fermented bitter melon juice.

Component	Pasteurized	Fermented		
		24 hours	48 hours	72 hours
Total Phenolic (GAE)* (ppm)	339.27±43.76^{a}	231.38±18.76^{b,1}	214.00±7.70^{b,1}	199.03±25.11^{b,1}
Total Flavonoid (QE)** (ppm)	24.38±2.68^{a}	18.08±5.29^{a,1}	6.70±1.65^{b,2}	3.19±0.13^{b,2}
Antioxidant Activity (%)	82.34±1.52^{a}	65.44±1.26^{b,1}	31.70±1.34^{b,2}	27.47±1.34^{b,3}

* Note:
- GAE: gallic acid equivalent.
- QE: quercetin equivalent.
- Total phenolic content, flavonoid contents, and antioxidant activity in both pasteurized and fermented samples are represented by mean ± standard deviation of 3 measurement repetitions.
- Values with different letter $^{(a, b)}$ and number $^{(1, 2, 3)}$ superscripts in one row indicate a significant difference between treatments at a 95% confidence level ($p < 0.05$) by using:
  - abc = *Paired T-Test before and after fermentation.*
  - 123 = *One Way ANOVA Duncan in each treatment with a different fermentation period.*

This research however, presents a decline of phenolic content as the fermentation time prolongs (Table 3). It can be seen that between pasteurized and fermented samples, there is a significant difference regarding total phenolic, flavonoid content, and antioxidant activity. The results also show that longer fermentation time result in a declining amount of all the bioactive components measured, with only the total flavonoid content and antioxidant activity showing a significance difference due to different fermentation time.

This reduction is possible because during fermentation, the phenolic content in bitter melon juice may undergo decrease due to self-polymerization of the phenolic structures. This self-polymerization happens at acidic pH, and in this case, the fermentation substrate that has been converted in organic acid will lower the pH of the fermented bitter melon juice. Thus, the self-polymerization could occur since the pH were lowered, and this reaction will reduce the juice-ability of the phenolic content (Adebiyi *et al.*, 2017). James *et al.* (2020), also added a possibility where some lipophilic polyphenols may decrease juice-ability, in which this statement is consistent with Sánchez-Maldonado *et al.* (2011), that the decrease in pH value could cause an increase in lipophilic phenolic acid. This change occurs due to the substitution of hydroxyl groups with methoxy groups in phenolic acids, which also results in lower antioxidant activity.

The *Lactobacillus* has been studied to have better tolerance to phenolic acids through their ability to metabolize phenolic acids. Sánchez-Maldonado *et al.* (2011) has observed the metabolism of phenolic acid was by *Lactobacillus fermentum* FUA3165, and found that the probiotic exhibits decarboxylases and reductases activity. The species also shows esterases activity by hydrolyzing chlorogenic acid into caffeic acid though not as significant. Degrain *et al.* (2020) called this metabolism the bacterial decarboxylation, which is a method for the LAB to cope with the phenolics in fermentation substrate. In detail, *Lb. fermentum* decarboxylated caffeic acid into vinylcatechol, reduced caffeic acid into dihydrocaffeic acid, decarboxylated *p*-coumaric acid into *p*-vinylphenol and phloretic acid, and reduced ferulic acid into dihydroferulic acid (Sánchez-Maldonado *et al.*, 2011). Filannino *et al.* (2015), also shares the same findings, that phenolic acid metabolism by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree result in the degradation of the mentioned compounds as a method of detoxification by the lactic acid bacteria. Alavi Rafiee *et al.* (2018) also confirmed that the antioxidant activity of phenolics comes from its carboxyl group, thus decarboxylating the group causes reduction of the antioxidant activity. This was also confirmed through Folin Ciocalteu radical scavenging activity by Terpinc *et al.* (2011), in which decarboxylation significantly decrease the activity of the phenolics.

Although Sánchez-Maldonado *et al.* (2011) reported that *Lactobacillus* are more tolerant to phenolic acid, phenolic compounds was also linked to have bactericidal effect to lactic acid bacteria. Rodríguez *et al.* (2009), exclaimed that 24 hours incubation of *Lactobacillus plantarum* in phenolic compounds shows partial destruction of bacterial surface and longer incubation denounce the disappearance of the cell wall. Rodríguez *et al.* (2009) suggested that phenolic compound may disrupt the peptidoglycan of the cell wall which may results to cell death.

Lactic acid bacteria metabolism on phenolic includes the substitution of hydroxyl groups into methoxy groups. This result in the loss of +R (electron donating) ability of the phenolic content in fermented bitter melon juice throughout fermentation process, leading to lower phenolic content detected during total phenolic content assay. Total phenolic content was quantified using *Folin-Ciocalteu* reagent, in which this essay is a colorimetric essay that measures phenolic content in terms of the absorbency measured using UV-Vis spectrophotometer by the availability of chromophore groups. The phenolic content in the fermented bitter melon juice will reduce the *Folin-Ciocalteu* reagent in a redox reaction, producing a blue colored complex measured at 750 nm by UV-Vis spectrophotometer (Blainski *et al.*, 2013). By losing its +R ability, the phenolic content in fermented bitter melon juice also loses its ability to reduce the *Folin-Ciocalteu* reagent. On Table 3, it can also be seen that the antioxidant activity significantly decreased throughout the fermentation because phenolic also plays a role as one of the main antioxidants in plants. Adetuyi & Ibrahim (2014), also obtained a similar result with this research, by which the antioxidant activity and total phenolic content of the sample is also lowered as the fermentation time prolongs. Hence, the longer the fermentation time, the more phenolics are being

metabolised by the *Lb. fermentum* LLB3, resulting in lower total phenolic content and antioxidant activity.

According to the result obtained, the fermentation time and total flavonoid content of the bitter melon juice are inversely related (Table 3). This research found that the longer the fermentation process, less flavonoid are found in the samples. Changes of flavonoids during fermentation is possible through glycosylation, deglycosylation, ring cleavage, glucuronidation, and others, which can produce products such as quercetin glycosylates, 3-(3,4-dihydroxyphenyl) propionic acid, quercetin-3-*O*-glucoside, and 3'-*O*-methylquercetin (Huynh *et al.*, 2014).

According to Tan *et al.* (2016), flavonoids in bitter melon are dominated by rutin glycoside and quercetin aglycone. Quercetin, as the more biologically active flavonoid may undergo biotransformation during fermentation into its other degraded metabolite forms, such as quercetin glycosylates and 3-(3,4-dihydroxyphenyl) propionic acid (Qi *et al.*, 2021). Hence, this biotransformation could lead to the decline of total flavonoid content at longer fermentation time because of the degraded flavonoids. Though detailed mechanism on how flavonoids are metabolised by lactic acid bacteria, other possibilities of total flavonoid content decrease may be through the interactions of polyphenols with proteins, fibres, or lipids. In which these interactions may limit their juice-ability, thus causing changes in molecular weight, solubility, and even structure of the flavonoids. Flavonoids are also susceptible to degradation by pH and enzymes (Zieliński *et al.*, 2021).

Zhao & Shah (2016), finds that glycosides forms of flavonoid is more resistant towards degradation during fermentation because of the sugar moieties. This means that some glycosides of flavonoid are still available in the samples. Previously, Yang *et al.* (2019) mentioned, the glycoside form of flavonoids has lower antioxidant activity, hence this explained the lowered antioxidant activity detected during the assay. The more-susceptible-to-degradation aglycone form is possibly degraded during the fermentation process, which leads to decrease of total flavonoid content as fermentation time prolongs (Zhao & Shah, 2016). Even though some biotransformation of flavonoids may result in lowered antioxidant activity, some conjugated metabolites of flavonoids produced through fermentation may exert other function than antioxidant activity, such as physiological functions. These physiological functions enables the conjugated metabolites to serve as enzymatic inhibitors and ligands for receptors (Murota *et al.*, 2018). 3-*O*-methylquercetin is also an example, in which this is a methylated form of quercetin which has lower antioxidant activity compared to the original quercetin, but this compound still has the same anti-inflammatory activity as the precursor (Lotito *et al.*, 2011).

Table 4. Total phenolic (GAE), total flavonoid (QE) contents, and antioxidant activity in pasteurized and fermented bitter melon juice.

Component	Fermented			Fermented & Freeze-Dried		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Total Phenolic (GAE in ppm)	231.38 ±18.76^{x}	214.00 ±7.70^{x}	199.03 ±25.11^{x}	306.99 ±29.54^{x}	295.08 ±22.34^{y}	261.92 ±6.74^{x}
Total Flavonoid (QE in ppm)	18.08 ±5.29^{x}	6.70 ±1.65^{x}	3.19 ±0.13^{x}	9.95 ±0.45^{x}	6.58 ±0.47^{x}	4.24 ±0.92^{x}

* Note:

- GAE: gallic acid equivalent.
- QE: quercetin equivalent.
- Total phenolic and flavonoid contents in both fresh and pasteurized samples are represented by mean ± standard deviation of 3 measurement repetitions.
- Values with different letter superscripts in one line indicate a significant difference between treatments at a 95% confidence level ($p < 0.05$) by using *Paired T-Test* for fermented and freeze-dried samples.

Based on research by Karam *et al.* (2016), it can be concluded that freeze-drying did not provide a significant decrease in the bioactive contents, due to the low temperature used during the entire drying process. This theory is supported by several other studies that reported no significant effect on the number of bioactive compounds in freeze-dried samples (Chumroenphat *et al.*, 2021; Kidoń & Grabowska, 2021; Ghelichkhani *et al.*, 2019; González *et al.*, 2020; Silva-Espinoza *et al.*, 2021). Thus, dehydration by freeze-drying method is more preferred for products with valuable thermally sensitive components, including bioactive compounds. The results of this study satisfy the theory that the implementation of freeze-drying does not have a significant effect on the flavonoid content of the sample (Table 4). However, higher values of total phenolic content were obtained in this work. This was probably due to the liberation of gallic acid from the solution matrix throughout the freeze-drying process, thus the juice of the gallic acid component becomes easier (Pérez-Gregorio *et al.*, 2011).

Similar results also reported by Ghelichkhani *et al.* (2019), whereby the freeze-dried samples performed a higher phenolic content, and the author clearly mentioned the difference between the juice of bioactive compounds from aqueous and from powdered samples. This can support the findings of this study because the fermented samples juiced were liquid samples, while the freeze-dried samples were in the form of powder samples. Hewavitharana *et al.*, (2020) has previously stated that the differing water content in each sample may affect the number of detectable bioactive compounds, especially of those more polar. The phenolic compound tested in this study refers to gallic acid, where this compound itself has the polar structure and has water-soluble properties. Thus, juice using an organic solvent such as ethanol could affect the end results of such compounds that can be detected in the aqueous sample, since there is a possibility that the gallic acid content was strongly bound to the water fraction (Hewavitharana *et al.*, 2020).

Conclusion

Based on this research, it can be concluded that pasteurization is able to increase both of total phenolic and total flavonoid contents of fresh bitter melon juice. However, this research also found that lactic acid fermentation of bitter melon juice performed a decrease in both total phenolics and total flavonoids of the samples. This probably due to several degradation mechanism related to the metabolism of *Lactobacillus fermentum* LLB3 through decarboxylation, reduction, and hydrolysis of phenolic compounds. Dehydration by freeze-drying method could be an adequate method to preserve the fermented bitter melon juice, since the process did not perform any significant decrease on its bioactive content. In addition, it is necessary to evaluate the number of viable cells in the fermented bitter melon juice in further research. It is also important to study the freeze-dried bitter melon juice to maintain the bacteria's viability.

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Conflict of Interest

The authors have no conflicts of interest to declare. This work is a form of learning outcomes, only for university educational purposes. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is an original work and is not under review at any other publication.

Author Contributions

Michael Sean and Sherlina Audrey Marcellino conducted the experiment, calculations, and wrote the manuscript. Lindayani and Laksmi Hartajanie provided the research funding, developed the research method and revised the manuscript. All authors agreed to the final version of this manuscript.

Ethical Statement

Ethics approval was not required for this study. This article does not contain any studies involving animals performed by any of the authors.

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