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Effects of fermentation conditions using *Lactobacillus plantarum* on antioxidant properties and bitterness of bitter gourd (*Momordica charantia* L.) juice

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Abstract
The bitter gourd is bringing health benefits to human; however bitterness of the fruit limits its therapeutic effects. Fermentation processes have been reported to be able to reduce the bitterness of the bitter gourd. In this study, effects of fermentation factors including time (0, 12, 24, 36, 48, 60 and 72h), temperature (20, 25, 30, 35 and 40℃) and inoculum volume (v/w) (0, 1, 5, 10, 15 and 20%) of *Lactobacillus plantarum* on pH, total soluble solids (TSS), total phenolic content (TPC), antioxidant capacity (AC) and bitterness evaluation of the bitter gourd juice were studied. In general, TPC and AC values of the fermented samples increased significantly ($p < 0.05$) compared to those of the control ones. In the first experiment, the TPC value of 24h-fermented sample reached a peak, meanwhile the highest AC value obtained after 72h fermentation. In the second experiment, the highest TPC and AC values were recorded at 40℃. For the last experiment, with 20% inoculum volume, the highest TPC and AC values were recorded. The fermentation with 10% of *L. plantarum* for 24 h, at 30℃ resulted in a higher total phenolic content. Changing fermentation conditions significantly changed bitterness of the juice. Through sensory evaluation test, significant differences ($p<0.05$) in the bitterness among unfermented and fermented samples were recorded. Most of the panelists recognized there was reduction in bitterness of fermented sample compared to the control one.

Keywords
Fermentation duration, Fermentation temperature, Inoculum volume, Antioxidant capacity, Juice fermentation

Introduction
Bitter gourd (*Momordia charantia* L.) also known as bitter melon is a type of fruit that belongs to the family of Cucurbitaceae growing abundantly in tropical countries (Nirupama et al., 2018). Unlike other cucurbitaceous vegetables, bitter gourd has been attracted increasingly attention due to owning several bioactive compounds (Harinantenaina et al., 2006; Nguyen and Nguyen, 2020) that have been linked to various therapeutic effects including anti-cancer, anti-inflammatory, antivirus, and especially lowering blood glucose level, that can assist in treating diabetes (Nirupama et al., 2018). A pilot study of Selvakumar et al. (2017) clearly exhibited the positive effect of bitter gourd on type 2 diabetes mellitus patients. Interestingly, the recent research of Yan et al. (2021) also revealed the promising effect of polysaccharides obtained from fresh bitter gourd that can assist in lowering the cholesterol with great potential for treating hyperglycemia.

However, despite bringing remarkable health benefits, bitterness of the bitter gourd prevents people from having it and therefore, limits its therapeutic effects (Rashima et al., 2017). To overcome this disadvantage, many treatments including fermentation have been studied on the bitter gourd and its products.

Recently, the focus on *Lactobacillus plantarum* fermentation has been increased. According to Sharma and Mishra (2013), *L. plantarum* is suitable for bitter gourd juice fermentation due to its capability of surviving at low pH, high acidic conditions in the fermented juice during cold storage at 4℃. In addition, *L. plantarum* have

ability to produce β-glucosidase enzyme that is an important catalyst in the hydrolysis of glycosides and have function in the liberation of aromatic compounds from glucosides precursors (Singh et al., 2016). Therefore, the fermentation process of bitter gourd using *L. plantarum* would assist to reduce the bitterness caused by some phytochemical compounds such as alkaloids glycosides, saponins and help modify the taste of juice.

There are several studies on fermentation of bitter gourd juice (Gao et al., 2019; Mazlan et al., 2015), however, effects of fermentation factors using *L. plantarum* on bioactive compounds of the juice has not been fully investigated. Therefore, this current work aimed to determine influences of fermentation time, temperature and inoculum volume of *L. plantarum* on amounts of these bioactive compounds in the fermented bitter gourd juice.

Materials and Methods

Materials

Forty-five kg of fresh wild bitter gourd fruits, reached the commercial maturity with green skins, were purchased from a farm in Dong Nai, Vietnam in March, 2019. The size of fruits was around 3 to 5 cm long and 1 to 3 cm in diameter. The fruits free from damage and insects were washed carefully to remove dust and soil and then were stored at -20℃ until experiments could be commenced. All of the following treatments were made in three replicates.

Lactobacillus plantarum was purchased from the Institute of Microbiology and Biotechnology of Vietnam National University, Vietnam. L. *plantarum* was sub-cultured in MRS broth for 2 days to reach 10^7 cells/ ml before fermentation. Cell counting method was used to quantify the number of microorganisms by hemocytometer, using a Thoma counting chamber (Absher, 1973).

Sample preparation

Selected bitter gourds blended using a home blender (Lock & Lock EJM161BLK, Korea) were used for fermentation carried out by *L. plantarum*. Series of bottles that contain bitter gourd pomaces were pasteurized at 85℃ for 5 minutes and were cooled down to room temperature before culturing *L. plantarum* (Huynh and Nguyen, 2017).

Experiment 1 – Bitter gourd juice production with different fermentation time

The bitter gourd bottles were inoculated with 10% of *L. plantarum* and incubated at 30℃ for 12h, 24h, 36h, 48h, 60h, 72h (Thakur and Joshi, 20017). After studied conditions were reached, the collected pomace was further pressed to collect the juice that was then pasteurized at 85℃ for 5 minutes. The fresh bitter gourd juice that was unfermented and pasteurized at 85℃ for 5 minutes was served as the control sample. The juice samples were stored at 4℃ for further analysis.

Experiment 2 – Bitter gourd juice production with different fermentation temperature

The bottles containing the blended bitter gourd were inoculated with 10% of *L. plantarum* and incubated at different temperature conditions (20, 25, 30, 35, 40℃) (Matejčeková et al., 2016) for 24 hours. The fermented juice collected after pressing the pomaces was pasteurized at 85℃ for 5 minutes. The fresh juice heated at 85℃ for 5 minutes was served as control. The juice were then stored at 4℃ for further analysis.

Experiment 3 – Bitter gourd juice production with different inoculum volume (v/w)

The bitter gourd bottles were inoculated with different inoculum volume (1, 5, 10, 15, 20%, v/w) (Mazlan et al., 2015) of *L. plantarum* and incubated at 30℃ for 24 hours. After desirable condition was reached, the pomace was pressed to release the juice that was then pasteurized at 85℃ in 5 minutes. The control sample was the unfermented fresh juice. The juice bottles were stored at 4℃ for further analysis.

All three experiments were independent from each other, in which the constant values kept for the trials were based on the study of Mazlan et al. (2015).

Methods

Determination of pH

The pH values were measured using pH meter (Hanna HI 2216, USA) (Mazlan et al., 2015). Calibration of the pH meter was done prior to pH determination.

Determination of total soluble solids (TSS)

Total soluble solids (TSS) were measured using a refractometer (Atago RX-5000α, USA) at 25℃ and the result was expressed as %.

Extraction of total phenolic content

Total phenolic compounds were extracted basing on a procedure described by Tan et al. (2014) with minor modifications. In details, 5ml of each juice sample was mixed with 10ml of 80% methanol (v/v). The mixture was incubated at 60℃ in the dark condition for 2 hours using shaking water bath (Daihan Scientific MaXturdy 30, Korea) before being centrifuged at 4300 RCF (Hettich Universal 320R, Germany) for 10 minutes at 4℃. The collected supernatants were stored at -20℃ for further measurements.

Determination of total phenolic content

Total phenolic content was determined using Folin – Ciocalteu method (Sutanto et al., 2015) with slight modifications. Briefly, 0.2 ml sample was mixed with 1 mL of Folin – Ciocalteu reagent (Merck, Germany). After 5 minutes, 0.8 ml of aqueous 7.5% (w/v) sodium carbonate (Merck, Germany) was added, vortexed and incubated for 1 hour at room temperature in the dark. The mixture was then transferred to 3.5ml cuvette for absorbance measurement at 765 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (Jasco V-730 UV-vis, Japan). Gallic acid (Sigma Aldrich, Germany) was used to plot the standard curve at the concentrations of 0, 20, 40, 60, 80 and 100 ppm and the result was expressed as mg gallic acid equivalent (GAE) per 100 ml of sample.

Determination of antioxidant capacity

DPPH radical-scavenging activity of the fermented bitter gourd juice was carried out following a method described by Lim et al. (2007) with some modifications. In details, 1 ml of extracted sample was mixed with 3 mL of 0.1mM DPPH stock solution. The mixture was further incubated at 25℃ for 30 minutes in the dark before measuring absorbance at 517 nm.

The percentage of DPPH radical scavenging was determined by the following equation:

$$\%\ DPPH\ scavening = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where: $A_{control}$ is the absorbance of the control; A_{sample} is the absorbance of sample.

Sensory evaluation of bitterness

Ranking test was carried out at the International University in Laboratory La1.601 with 50 untrained panelists chosen randomly to perform the test. The panelists were asked to rate the level of bitterness in several samples. Samples were coded with 3-digit number and arranged randomly.

Data analysis

Statistical analysis of one-way ANOVA was carried out using SPSS software version 20.0 with a level of a confidence of 95% to study the effects of fermentation factors on the bioactive compounds. The results were means ± standard deviation.

Results and Discussion

Effects of fermentation time on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

Over the time, total phenolic contents of fermented bitter gourd juice were found to be significantly ($p < 0.05$) higher than the unfermented sample. This could be due to the lactic acid bacteria activity used as starter culture in the fermentation process. During fermentation, *L. plantarum* would produce several types of enzymes such as decarboxylases and tannases, releasing the corresponding aglycones (Curiel et al., 2015) that may load with phenolic moieties (Bhagavan, 2002), leading to higher amounts of in the total phenolic content (Kwaw et al., 2017).

Table 1. Effects of fermentation time on pH, total soluble solids, total phenolic content and antioxidant capacity of bitter gourd juice

Time (h)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
0	4.98 ± 0.02^{a}	3.14 ± 0.02^{ab}	5.77 ± 0.71^{a}	55.95 ± 4.22^{a}
12	4.76 ± 0.08^{b}	3.20 ± 0.03^{a}	8.84 ± 0.78^{b}	60.46 ± 1.22^{a}
24	4.53 ± 0.03^{d}	3.15 ± 0.05^{ab}	12.12 ± 0.03^{c}	68.97 ± 3.79^{b}
36	4.59 ± 0.06^{cd}	3.09 ± 0.04^{bc}	9.95 ± 0.09^{b}	69.85 ± 1.90^{b}
48	4.67 ± 0.04^{bc}	3.09 ± 0.02^{bc}	9.63 ± 0.84^{b}	70.00 ± 1.59^{b}
60	4.54 ± 0.01^{d}	3.03 ± 0.02^{c}	10.23 ± 0.20^{b}	70.32 ± 2.47^{b}
72	4.50 ± 0.02^{d}	3.02 ± 0.01^{c}	9.68 ± 0.59^{b}	70.88 ± 2.30^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

The antioxidant capacity of fermented bitter gourd juice reached the value which was about 1.2 times higher as compared to the control sample after 24h (Table 1). It could be due to the ability of *L. plantarum* in breaking down the chemical bonds existed in bitter gourd and released more bioactive compounds (Nisa et al., 2019). Moreover, as mentioned in a research of Kubola and Siriamornpun (2008), quercetin and catechin were flavanols that could be found abundantly in bitter melon. They were proven to be powerful scavengers of reactive oxygen species and could help prevent degenerative diseases. According to López de Felipe et al. (2010), *L. plantarum* did not break down catechin and quercetin during fermentation; in contrast these flavanols could promote the sugar consumption and therefore increased the fermentative performance.

The average ranking scores of bitterness among 7 samples were summarized in Table 2. Generally, significant differences ($p<0.05$) in the level of bitterness were observed at different fermentation time. The unfermented sample was ranked as the most bitter (6.58 ± 0.50) and the least bitter belonged to the sample 72h – fermented (1.64 ± 0.83). As mentioned in a research of Okabe et al. (1982), cucurbitacin-like alkaloid momordicine and triterpene glycosides (momordicoside K and L) were the main compounds that were responsible for the bitter flavor in bitter gourd. These compounds were also considered to be the bitterest compounds in the plant kingdom (Johns, 1990). During fermentation, *L. plantarum* would release enzymes such as β – glucosidase that had the ability to hydrolyze those momordicosides to aglycones (Mazlan et al., 2015). Therefore, the bitterness could decline significantly. Although the charantin content increased (Nguyen and Nguyen, 2020), reduction in bitterness was observed. It is understandable that charantin is just one of the glycosides that are responsible for the bitter flavor (Nguyen and Nguyen, 2020). Therefore, the degradation of other glycoside compounds such as saponin and tannin after fermentation of the bitter gourd juice (Olaniyi et al., 2013), could result in bitterness reduction that would not much associated with charantin content in the juice (Nguyen and Nguyen, 2020).

Table 2. The level of bitterness at different fermentation time

Fermentation time (h)	Bitterness
0	6.58 ± 0.50^{a}
12	5.84 ± 1.22^{b}
24	4.76 ± 1.02^{c}
36	3.98 ± 1.25^{d}
48	3.06 ± 1.11^{e}
60	2.14 ± 1.16^{f}
72	1.64 ± 0.83^{g}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

After 72h fermentation, the sample was ranked as the least bitter one (Table 2). As longer fermentation time was taken, microorganisms would have more time for their metabolic activities. The sour taste that resulted from low pH also had effects on the overall taste of the samples. The sample people prefer the most was the one that fermented for 60h.

Effects of fermentation temperature on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

As illustrated in Table 3, the unfermented sample contained least total phenolic content; meanwhile the highest value was obtained after 24h of fermentation at 40℃. It was also recorded that total phenolic content increased by the elevated temperature. During fermentation, phenolic compounds could be hydrolyzed by proteolytic enzymes released from *L. plantarum* to turn into soluble-free phenols and other simpler and biologically more active compounds (Muñoz et al., 2017). In addition, the heat treatment could cause the disruption of plant cell wall by which polyphenol and flavonoid compounds could be released more easily (Shahidi and Yeo, 2016). Also, the release of bound phenolic compounds from the corresponding glycosidic precursors or polymeric forms could take place when there is an increase in phenolic content (Deshaware et al., 2019).

Table 3. Effects of fermentation temperature on pH, total soluble solids, total phenolic content and antioxidant capacity of bitter gourd juice

Fermentation temperature (°C)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
Control	5.68 ± 0.04^{a}	3.29 ± 0.01^{a}	8.52 ± 0.50^{a}	47.37 ± 0.83^{a}
20	4.66 ± 0.02^{b}	3.11 ± 0.03^{c}	10.63 ± 1.18^{ab}	69.45 ± 1.74^{b}
25	4.61 ± 0.04^{b}	3.15 ± 0.02^{c}	10.68 ± 0.96^{ab}	68.17 ± 3.00^{b}
30	4.62 ± 0.03^{b}	3.15 ± 0.04^{c}	12.07 ± 0.75^{bc}	72.06 ± 0.55^{b}
35	4.62 ± 0.01^{b}	3.17 ± 0.02^{bc}	11.77 ± 0.32^{bc}	70.82 ± 5.70^{b}
40	4.63 ± 0.03^{b}	3.24 ± 0.03^{ab}	13.13 ± 0.16^{c}	73.42 ± 0.99^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

As seen from Table 3, the fermented samples had much higher ($p < 0.05$) DPPH scavenging activity compared to the control sample. Muñoz et al. (2017) reported that gallic acid and protocatechuic acid could be hydrolyzed into pyrogallol and catechol due to decarboxylation activity of *L. plantarum* cultures. These simple phenols were considered to be the most potential radical scavengers (Ordoudi and Tsimidou, 2006). Moreover, *L. plantarum* had the ability to breakdown the ester linkages of hydroxycinnamic acids such as caffeic and p-coumaric acid to release more free phenolic acids, consequently, enhancing the antioxidant capacity of the fermented product (Muñoz et al., 2017).

Table 4. The level of bitterness at different fermentation temperature

Fermentation temperature (°C)	Bitterness
Control	5.54 ± 0.65^{a}
20	4.96 ± 0.81^{b}
25	3.76 ± 1.36^{c}
30	2.10 ± 1.13^{e}
35	1.70 ± 0.81^{f}
40	2.94 ± 0.94^{d}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

At different incubating temperature, significant differences ($p<0.05$) in bitterness were observed (Table 4). The most bitter sample recorded was the control one (5.54 ± 0.65) while at 35℃, the least bitter flavor was noted (1.70 ± 0.81). As reported by Olaniyi et al. (2013), fermentation could help decrease the amount of bitter compounds such as saponin and tannin which could be found abundantly in bitter gourd. This action was performed by *L. plantarum* due to the enzyme production; therefore, those compounds could be degraded rapidly. Although the pH value was quite stable, differences in sourness were detected by the panelists. However, during the sensory test, errors might happen due to sour-bitter confusion (Meiselman and Dzendolet, 1967). Various studies have been carried out about this phenomenon in which panelist easily got confusion by sour and bitter flavors (Gregson and Baker, 1973). However, in general, the most preferable sample was the one incubated at 35℃ with the average value of bitterness was 1.70 ± 0.81. This sample was chosen as it could reach the harmonious between bitter and sour flavor

Effects of inoculum volume (v/w) on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

The results obtained from the current work showed that the higher inoculum volume of *L. plantarum* used, the more phenolic compounds could be obtained (Table 5). Specifically, with 20% inoculating ratio, the samples obtained the highest value which was about 1.4 times higher than that of the control sample. As reported by Wardani et al. (2017), the higher amount of initial inoculums would shorten the lag phase of the microorganisms which might make the liberation process of bioactive compounds happened much faster than the degradation. According to Nisa et al. (2019), monomers of phenolic compounds could be liberated by breaking the bond between phenolic compounds with other substances through the fermentation process. When increasing the

inoculum ratio, metabolic activities of microorganism would occur more powerful as they could increase the amount of bioactive compounds via the action of β-glycosidase (Sabokbar and Khodaiyan, 2016).

Table 5. Effects of inoculum volume (v/w) on pH, total soluble solids, total phenolic content, antioxidant capacity and sensory evaluation of bitter gourd juice

Inoculum volume (%, v/w)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
0	5.00 ± 0.01^{a}	3.11 ± 0.05^{a}	9.78 ± 0.93^{a}	61.94 ± 3.83^{a}
1	4.55 ± 0.03^{b}	3.14 ± 0.06^{a}	9.81 ± 0.62^{a}	67.22 ± 2.85^{ab}
5	4.57 ± 0.04^{b}	3.15 ± 0.04^{ab}	10.07 ± 0.83^{ab}	64.62 ± 2.96^{ab}
10	4.58 ± 0.05^{b}	3.30 ± 0.04^{b}	12.11 ± 0.73^{bc}	64.42 ± 1.99^{ab}
15	4.61 ± 0.06^{b}	3.47 ± 0.10^{c}	12.93 ± 0.25^{c}	67.45 ± 0.33^{ab}
20	4.56 ± 0.03^{b}	3.53 ± 0.03^{c}	13.78 ± 0.16^{c}	70.93 ± 3.12^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

Moreover, the breaking down of the cell walls via fermentation could be another factor that led to increasing in TPC. Consequently, the nutraceutical value of bitter gourd juice would be improved due to the improvement of free phenolic acids (Acosta-Estrada et al., 2014).

By increasing the inoculum volume of *L. plantarum*, significant improvements ($p < 0.05$) of antioxidant capacity in the fermented juice as compared to the unfermented one were observed (Table 5). With 20% inoculating ratio, the highest scavenging effect was recorded. This result had a positive correlation with total phenolic content obtained from this experiment. The same phenomenon was also stated in a research of Sabokbar and Khodaiyan (2016) that increasing the level of kefir grain inoculation would lead to an increase in DPPH radical scavenging. Besides, due to the production of tannase, *L. plantarum* was proven to be able to hydrolyze the ester bonds in hydrolyzable tannins and gallic acid esters to release more potent antioxidant compounds (Hur et al., 2014). In addition, according to Gao et al. (2019), the biotransformation of bioactive compounds in bitter gourd such as polyphenols through the fermentation process using *L. plantarum* would also provide stronger antioxidant property for the products.

Table 6. The level of bitterness with different inoculum volume (v/w)

Inoculum volume (%)	Bitterness
0	4.74 ± 1.81^{a}
1	3.60 ± 1.62^{bc}
5	3.92 ± 1.41^{b}
10	3.06 ± 1.50^{cd}
15	2.98 ± 1.35^{cd}
20	2.68 ± 1.73^{d}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

Different inoculum ratio was used to evaluate the level of bitterness (Table 6). As expected, the 20% inoculum volume sample was the least bitter one (2.68 ± 1.73), while the control still remained the most bitter sample (4.74 ± 1.81). With different inoculum volume, significant differences ($p<0.05$) in bitterness were observed. As increasing the initial amount of microorganisms inoculated, the metabolic activity of *L. plantarum* would occur faster and more powerful. As a result, more glycosides responsible for bitterness such as saponin, tannin would be degraded due to enzymes produced by *L. plantarum* (Barthelmebs et al., 2000; Rodriquez et al., 2008; Curiel et al., 2015; Sabokbar and Khodaiyan, 2016). In cocoa beans processing, microbial fermentation is also one of the methods that were applied to reduce the bitterness as it can reduce alkaloids contents (Aliani and Eskin, 2017). Moreover, according to Gao et al. (2019), fermentation with *L. plantarum* could improve the volatile profile of fruits and vegetables. Therefore, it was suggested that to obtain a favorable and harmonious flavor for bitter gourd juice, *L. plantarum* should be used. In general, although the least bitter sample was recorded with 20%, the sample with 15% inoculum volume was most preferable one with the ranking score of 2.98 ± 1.35. This sample seemed to be the most pleasant according to the panelists.

Overall, there was a reduction in bitterness among unfermented and fermented samples in three experiments. This result indicated that during fermentation, the level of bitterness decreased significantly due to the hydrolysis of glycosides which were responsible for the bitter flavor in bitter gourd (Nguyen and Nguyen, 2020).

Conclusions

In this study, the fermentation process using *Lactobacillus plantarum* significantly affected ($p < 0.05$) pH, total soluble solids, and the amounts of total polyphenols, the bioactive compounds, antioxidant capacity and bitterness level of the bitter gourd juice. Fermentation process not only enhanced the bioactive compounds but also increased the antioxidant capacity of the juice. Therefore, it is suggested that fermentation of bitter gourd juice using *L. plantarum* should be considered in production due to its debittering effect as well as the potential health benefits, especially for diabetes patients.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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