Indonesian Journal of Pharmaceutical Science and Technology
Journal Homepage : http://jurnal.unpad.ac.id/ijpst/
Research Article

Tea Fermentation of *A. malacencis* with *Lactobacillus plantarum* as a starter: In vitro study as a functional drink

Pratiwi Apridamayanti[1*], Rafika Sari[1]
[1]Department of Pharmacy, Medical Faculty, University of Tanjungpura, Pontianak, Indonesia
Submitted 17 February 2022; Revised 07 February 2023; Accepted 18 March 2023; Published 13 February 2024
*Corresponding author: apridamayanti@pharm.untan.ac.id

Abstract
Some herbal teas contain antioxidant compounds that can neutralize the free radicals. *Aquilaria malaccencis* is used to make teas in Indonesia and other countries and has been identified having antioxidant properties. This study aimed to determine the characteristics of tea made from *A. malaccencis* leaves and fermented with *Lactobacillus plantarum* for 48, 96, and 192 hours. The features investigated were antioxidant activity measured with DPPH assay, total phenol, and flavonoid content measured spectrophotometer. The fermentation results at 48, 96, and 192 hours were a pale yellow, sour aroma, and sour taste. Total LAB content of 8.49 x 106 CFU/mL, 7.42 x 106 CFU/mL, and 2.6 x 1010 CFU/mL, respectively. Antioxidant activity (IC_{50}) at 48, 96, and 192 hours was 439.444 µg/ml; 235.309 µg/ml and 190.33 µg/ml. Total phenolic content (mg/g GAE) at 48, 96 and 192 hours was 22.561; 18.173, and 21.14. Total flavonoid content (mg/g QE) at 48, 96, and 192 hours was 1.901; 1.938, and 3.76. This research concluded that *L. plantarum*, a starter for fermented tea made from *A. malaccensis* Lam. leaves, could produce appropriate characteristics for its functional food consumption. The best fermentation time was 192 hours with an IC_{50} value of 190.33±1.64 µg/ml, total phenol content of 36.346 mg/g GAE, and total flavonoid content of 3.876±0.317 mg/g QE.
Keywords: Antioxidant activity, Tea fermentation, Total flavonoid content, Total phenolic content

Teh fermentasi daun *A. malacencis* dengan starter *Lactobacillus plantarum* : Studi in vitro sebagai minuman fungsional

Abstrak
Teh herbal mengandung senyawa antioksidan yang dapat menetralisir radikal bebas. *Aquilaria malaccencis* digunakan untuk membuat teh di Indonesia dan negara lain dan telah diidentifikasi menunjukkan sifat antioksidan. Penelitian ini bertujuan untuk mengetahui karakteristik teh yang dibuat dari daun *A. malaccencis* dan difermentasi dengan *Lactobacillus plantarum* selama 48, 96, dan 192 jam. Tujuan dari penelitian ini adalah untuk mengetahui aktivitas antioksidan yang diukur melalui uji DPPH, kandungan fenol, dan flavonoid total diukur secara spektrofotometri. Hasil fermentasi pada jam ke 48, 96, dan 192 adalah kuning pucat, aroma asam, dan rasa asam. Kandungan total bakteri asam laktat berturut-turut sebesar 8,49 x 106 CFU/mL, 7,42 x 106 CFU/mL, dan 2,6 x 1010 CFU/mL. Aktivitas antioksidan (IC_{50}) pada 48, 96, dan 192 jam berturut-turut adalah 439,444 g/ml; 235.309 g/ml; dan 190,33 g/ml. Kandungan total fenol (mg/g GAE) pada 48, 96, dan 192 jam berturut-turut adalah 22,561; 18,173; dan 21,14. Kandungan flavonoid total (mg/g QE) pada 48, 96 ,dan 192 jam bertutrut-turut adalah 1,901; 1,938; 3,76. Penelitian ini menyimpulkan bahwa *L. plantarum* sebagai starter untuk teh fermentasi berbahan *A. malaccensis* Lam. dapat menghasilkan karakteristik yang sesuai untuk konsumsi sebagai minuman fungsional. Waktu fermentasi terbaik adalah 192 jam dengan nilai IC_{50} 190,33 ± 1,64 g/ml, kandungan fenol total 36,346 mg/g GAE, dan kandungan flavonoid total 3,876 ± 0,317 mg/g QE.
Kata Kunci: Aktivitas antioksidan, Teh fermentasi, Total Fenol, Total Flavonoid

1. Introduction

During the Covid-19 pandemic announced by WHO in March 2020, the level of immunity level needs to be increased, and one way to increased immune system is to consume healty food and drink. Immunity is closely related to the body's immune systems; these reflect the nutritional value of food consumed and its functional aspects. Functional foods contain physiologically active compounds that provide benefits beyond essential nutrients.[1] In Indonesia, one of the functional foods consumed is a drink made from fermented milk that can suppress the growth of harmful bacteria in the intestines via the active action of lactic acid bacteria.[2] Fruit and vagatables fermented with lactic acid bacteria can produce bioactive compounds with antioxidant, antibacterial, anti-inflammatory, anticancer, and anti-viral properties.[3]

Some herbal teas are functional drinks containing antioxidant compounds that neutralize free radicals.[4,5] Fermentation in teas has been claimed to increase antioxidant activity via the metabolic results of microorganisms produced during the fermentation process.[6] *Lactobacillus plantarum* is a bacteria used in food fermentation that can increase antioxidant activity by forming lactic acid, phenolics, vitamin C, and vitamin E.[7,8] Research into the Lactobacillus plantarum fermentation process has reported excellent organoleptic qualities, including sour taste, brownish color, distinctive fermented aroma, and suitable homogeneity.[9] Research into the antioxidant activity of juice probiotic drinks with the addition of *Lactobacillus plantarum* showed that as fermentation time lengthens, antioxidant activity, total phenols, and total flavonoids had increase.[10] Research into red dragon fruit extract (*Hylocereus polyrhizus*) with addition of Lactobacillus plantarum culture found that it produced the best probiotic drink quality in chemical, physical and microbiological analysis and panelist preference measurements, including color, aroma, and taste parameters.[8]

Aquilaria malaccensis Lam (*A. malaccencis*) is found across India, Myanmar, Malaysia, Penincula, Sumatra, Borneo and the Philippines.[11] The plant's leaves contain steroids, saponins, tannins, phenols, terpenoids, quinones and flavonoids.[12,13] *A. malaccencis* leaf tea is used as a body fitness drink and has been shown to have antioxidant activity and analgesic, antipyretic, anti-inflammatory, antimicrobial, and antihyperglycemic properties.[13] The results of previous studies have indicated that tea made from the leaves of A. malaccensis is popular and acceptable to local communities. Antioxidant activity of IC_{50} value 73.02 µg/ml has been identified in A. malaccensis leaf tea.[14] Previous studies have reported that aquimavitalin obtained from the isolation of *A. malaccencis* seed has an inhibitory effect on mast cell degranulation (IC_{50} value of 10 µg/ml) and that phorbol ester isolated from *A. malacencis* seed can inactivate HepG2 (hepatoma), MDA-MB-231 (breast) and A549 (lung) cancer cell lines. Plants of the same genus, *A. malacencis* is A. cressna, are known to have antioxidant activity of IC_{50} value 47.18 g/ml, identified using the DPPH method. The antioxidant activity of A. malaccensis water extract is 128.63 ± 6.7 µg/ml.[15,16,17] It is known that the biological activity possessed from a literature search is expected that this research sample can provide good benefits for health.

The benefits of A. malaccensis tea as a health drink can be increased by fermentation using lactic acid bacteria, namely *Lactobacillus plantarum*. Fermented *A. malaccensis* tea can be used as a functional food because of the fermentation outputs produced and having nutritional value. Its nutritional value is related to the chemical content possessed by *A. malacencis,* based on research by Apridamayanti et al., 2018, which is known *A. malacencis* contain flavonoids, phenols, alkaloids, anthraquinones, and terpenoids. According to Tania et al., 2022, fermentation can affect nutritional components and produce primary and secondary metabolites. It also has physiological and antioxidant properties that can improve the immune system.[58,59]

Lactic acid bacteria can stimulate the immune response through endotoxic

lipopolysaccharide, peptidoglycan, and lipoteichoic acid. In the intestine, peptidoglycan can stimulate the surface of the intestinal mucosa, in which glucan is found in the cell walls. Lactic acid bacteria can stimulate macrophages to produce interleukins with the effect of increasing lymphocyte cell proliferation.[18]

T lymphocytes release interferons to activate macrophages, and B lymphocytes produce antibodies that play a role in humoral-specific immunity.[18] Some studies have been carried out on the effects of lactic acid bacteria in test animals. Lactic acid bacteria can increase amounts of IL-8, IL-6, IL-10, IL-1B, and TNF-alpha, all of which are involved in the immune system.[19,20,21,22,23,24,25] The absorption mechanism of probiotics is thought to be through M cells found in the intestinal tract into the lymph follicles, where signal stimulation occurs, releasing pre- and pro-inflammatory cytokines.[26]

There is no study related to *Lactobacillus plantarum* as a fermentation starter for tea made from *A. malaccensis* leaves. Therefore, it is necessary to research and determine the antioxidant activity and total phenol and flavonoid content of *Lactobacillus plantarum* in fermented teas after fermentation for different periods. The results of this study established the best length of fermentation in providing antioxidant activity so that it can be used effectively as a functional drink.

2. Method

2.1. Tools

Autoclave (HL® 36Ae-China), hot plate (SI Analytics® GmbH D-55122), incubator (Memmert® E24899), laminar air flow (Cabinet®), micro pipettes with volume 10–100µL and 100–1000 µL (Rainin® E1019705K), oven (Memmert® UP400), pH meter (Hanna®), UV-visible spectrophotometer (Shimadzu), analytical balance (Ohauss® PA2102).

2.2. Materials

Aluminum chloride (Merck®), Aquadestilata (Wida-Wi®), gallic acid (Merck®), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich®), sodium acetate (Merck®), quercetin (Merck®), media de Mann Rogosa Sharpe Agar (MRSA) (Merck®), media de Mann Rogosa Sharpe Broth (MRSB) (Merck®), methanol p.a. (Merck®), NaCl 0.9% (Otsu-RL®), Na_2CO_3 (Sigma-Aldrich®), Folin-Ciocalteu reagent (Merck®), skimmed milk (Lactona Mirota®).

2.3. Methods

The samples used comprised tea made from *A. malaccensis* leaves and *Lactobacillus plantarum* as a starter in the fermentation process. Observations of tea characteristics, IC_{50} values, total phenolics and total flavonoids were made at various fermentation times.

2.3.1. Preparation of plants and bacteria

A. malaccensis leaves were collected from Kubu Raya, West Borneo, Indonesia. The plants' authenticity was verified in the biology laboratory of the Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak, Indonesia. The Lactobacillus plantarum bacterium was isolated from *ce hun tiau* (a local drink in west Borneo, Indonesia) in the biology laboratory of the Faculty of Medicine, Tanjungpura University Pontianak.

2.3.2. Preparation of bacterial starter culture

Lactobacillus plantarum bacteria were rejuvenated in MRSA medium, then transferred to MRSB medium, and incubated at 37°C for 48 hours. A 5 mL sample was collected into a test tube containing a 7 mL MRSB medium, set at 37°C for 48 hours, and then used as stock culture. A solution of skimmed milk 10%, sugar 3%, and Aquadestilata was pasteurized and cooled. Stock culture 5% was put into the skimmed milk solution and incubated at 37°C for 48 hours.[27]

2.3.3. Preparation of fermented tea

Prepared samples of 1.2 grams of *A. malaccensis* leaves were added to 100 mL of water, heated until boiling, then brewed. 50 ml tea was placed into glass bottles, and 10 grams of sugar was added. *Lactobacillus plantarum* bacteria 10% were added total

volume of mix solution is 60 ml, and then the sample was fermented for 48 hours, 96 hours, and 192 hours at room temperature.[27,28]

2.3.4. Observation of tea organoleptic

After fermentation with five senses, observations were made of the colour, aroma and taste of *A. malaccensis* tea.

2.3.5. pH measurement

After fermentation, the pH of the *A. malaccensis* tea was measured at room temperature using a pH meter in triplicate.

2.3.6. Measurement of total lactic acid bacteria (LAB)

After fermentation, tests were carried out on starter cultures of *Lactobacillus plantarum* and *A. malaccensis* tea. The calculation of colonies was carried out according to the Harrigan method with modifications.[29] The sample dilution series was made up of 10-10 using a 0.9% NaCl dilution solution. Each dilution series was pipetted as 0.1 mL samples added to 15 ml of MRSA medium in quadrant strokes. Incubation was carried out at 37°C for 48 hours. The total number of bacteria was expressed in CFU/mL.

2.3.7. Antioxidant activity test

Tests were carried out on *A. malaccensis* leaf tea after fermentation. The tea solution was made to a specific concentration, then pipetted as 1 mL samples, reacted with 3 mL of 38 µg/ml DPPH solution, and incubated at room temperature for 30 minutes. Uptake was measured at a maximum wavelength of 514.5 nm. Absorbance was used to measure the per cent inhibition of DPPH uptake. In this study, experiments were carried out in triplicate. The calculation of the inhibition percentage is added into the regression equation (y = bx + a) with the concentration of tea as the X axis and the percentage inhibition value as the Y axis. The following equation is used to determine the IC_{50} of each sample:

$$\%\ Inhibition = \frac{DPPH\ Abs - Sample\ Abs}{DPPH\ Abs}\ x\ 100\%$$

where DPPH Abs = DPPH radical uptake; Sample Abs = absorption of tea samples in radical DPPH.[30]

2.3.8. Determination of total phenolic content (TPC)

Total phenolic content was expressed as gallic acid equivalent (GAE) percentage from the standard curve of gallic acid. This process is based on the Chang method with minor modifications. Gallic acid stock solution concentration of 100 µg/ml and a series of solution concentrations of 15 µg/ml, 25 µg/ml, 35 µg/ml, 45 µg/ml, and 55 µg/ml were used for the calibration curve. Samples of 1 mL of solution were added to 500 µL of Folin-Ciocalteu reagent, 2 mL Na_2CO_3 10%, and 6.5 mL Aquadestilata heated at 50°C for 5 minutes and incubated for 10 minutes. The solution was measured at a maximum wavelength of 748.5 nm. The standard curve equation was obtained from linear Regression between gallic acid levels (X) and absorbance (Y). *A. malaccensis* tea samples before and after fermentation were made in 1000 µg/ml concentration and treated similarly with gallic acid. In this study, experiments were carried out in triplicate. The total phenolics content is calculated from the standard gallic acid curve using the following formula:[31]

$$TPC = \frac{c\ x\ v\ x\ fp\ x\ 10^{-6}}{w(gram)}\ x\ 100\%$$

where TPC = total phenolics content (% w/w); w = sample weight (g); v = volume of sample extract (mL); fp = dilution factor; c = gallic acid equivalent (µg/mL) from the equation for the standard gallic acid curve.

2.3.9. Determination of total flavonoid content (TFC)

TFC content was determined according to the Chang method and expressed as quercetin equivalent (%) from the quercetin standard curve equation. Quercetin solution with a concentration of 1000 µg/ml was used to make a series of concentrations of 9 µg/ml, 12 µg/ml, 15 µg/ml, 18 µg/ml, 21 µg/ml and 24 µg/ml. To 2 mL samples of the solutions, 0.1 mL of 10% $AlCl_3$ reagent, 0.1 mL sodium acetate 1 M, and 2.8 mL aquadestilata were

added. The solution samples were incubated for 30 minutes and measured at a maximum wavelength of 433.5 nm. The standard curve equation was obtained from linear Regression between quercetin (X) and absorbance (Y). Before and after fermentation, tea made from A. malaccensis leaves was then used in 1000 µg/ml concentrations and treated similarly to the quercetin samples. In this study, experiments were carried out in triplicate. The total flavonoid level was calculated from the standard curve of quercetin using the following formula:[31]

$$TFC = \frac{c \, x \, v \, x \, fp \, x \, 10^{-6}}{w(gram)} x \, 100\%$$

2.3.10. Results analysis

The data obtained from our observations of characteristics and measurements of IC_{50}, TPC and TFC values in *A. malaccensis* tea after fermentation with *Lactobacillus plantarum* are presented in the form of figures and tables. Data were recorded as mean ± standard deviations of three determinations of absorbance. The linear correlation coefficient (R^2) value was calculated using MS Office Excel 2017. The linear regression equation for a straight line is y = bx + a where y = absorbance extract, b = slope of calibration curve, x = concentration of extract, and a = intercept. Statistical analytics of antioxidant activity, TPC and TFC with ANOVA testing followed post hoc test analysis with a significance level of 95%.

3. Result

Observations showed that longer fermentation could reduce the intensity of tea and lighten the colour, as seen in Figure 1. Organoleptic observation results for the fermented A. malaccensis leaf tea are presented in Table 1.

3.1. Measurement of tea pH

The results of pH in this study are shown in Table 2. Table 2 in this study shows that fermentation duration does not change the pH.

3.2. Measurement of total lactic acid bacteria (LAB)

The test results showed that as the length of the fermentation process increased, LAB increased from 8.49 x 106 (48 hours); 7.42 x 106 (96 hours) and 2.6 x 1010 CFU/ml at 192 h (eight days) of fermentation. The result of this study can be seen in Table 3.

3.3. The antioxidant activity identified using DPPH methods

The DPPH (2,2-diphenyl-1-

A *B* *C* *D*

Figure 1. The colour of *A. malaccensis* leaf tea during fermentation: (a) *A. malaccensis* leaf tea before fermentation; (b) *A. malaccensis* tea after fermentation for 48 h; (c) *A. malaccensis* tea after fermentation for 96 h, and (d) *A. malaccensis* tea after fermentation for 192 h.

Table 1. Organoleptic observation results of A. malaccensis leaf tea

Starter		*A. malaccensis* leaf tea after fermentation		
		48 h	96 h	192 h
L. plantarum	Colour	Pale brown	Very pale brown	Pale yellow with a hint of brown
	Taste	Sweet and sour	Sweet and sour	Spoiled with a hint of sweet
	Smell	Sour	Sour	Sour, but not sharp

Table 2. pH of *A. malaccensis* leaf tea solution after fermentation

Replication	pH after fermentation of *A. malaccensis* leaf tea		
	48 h	96 h	192 h
1	3.7	3.7	3.6
2	3.7	3.7	3.6
3	3.7	3.6	3.6
Average	3.7 ± 0	3.66 ± 0.05	3.6 ± 0

picrylhydrazyl) radical method was used to test antioxidant activity. IC_{50} values after fermentation in this study can be seen in Table 4.

3.4. Determination of total phenolic and flavonoid content

The regression curve of gallic acid content is y = 0.01282x + 0.0261 and regression value is R = 0.999. The quercetin regression curve is $y = 0.0304x + 8.7904x10^{-3}$ and regression value is R = 0.999. This study's total phenol and flavonoid content results are presented in Tables 5 and 6.

4. Discussion

4.1. Observation of tea organoleptic

Observations showed that longer fermentation could reduce the intensity of tea colour and lighten the colour, as shown in Figure 1. The colour of the tea is influenced by the breakdown of the components in solution by microbes, resulting in reduced colour intensity.[32] In accordance with Haryanto's research, 2018, kombuca *Hibiscus rosa-sinensis* L tea faded during fermentation which was carried out until day 14. The ability of the consortium of microorganisms in kombucha to degrade the color of the substrate used as the basic ingredient for kombucha so that the longer the fermentation time, the color of the hibiscus kombucha will fade.[32] The change of colour of the tea is also influenced by the solution's pH, which affects one component in the tea.[27] *A. malaccensis* leaves contain tannin/catechin group compounds, including epicatechin gallate, epigallocatechin gallate, and epicatechin.[33,34] These tannin compounds can be influenced by the presence of an acid, impacting the colour brightness of fermented tea.[35] Other studies have revealed that tannins/catechins are converted into thearubigin and theaflavin compounds during fermentation. The arubigin is a brownish pigment that will reduce the colour intensity of tea in an acidic environment; meanwhile, theaflavin is a yellowish pigment that does not lose colour intensity, so the resulting colour of the fermented tea is yellowish.[34,36]

The aroma produced is related to the taste of the fermented tea. During fermentation, *Lactobacillus plantarum* bacteria produce lactic acid, acetic acid, gluconate acid, glucuronic acid, propionic acid and butyric acid.[37,38,39] The higher the organic acid content, the sourer the taste and aroma. Organoleptic observation results for the fermented *A. malaccensis* leaf tea are presented in Table 1.

4.2. Measurement of tea pH

Change in pH is related to the tea's

Table 3. Antioxidant activity of A. malaccensis leaf tea after fermentation

Starter	Replication	IC_{50}		
		48 h	96 h	192 h
L. plantarum	1	233.208	83.095	191.81
	2	607.769	271.129	188.57
	3	477.355	351.704	190.60
	Average	439.444 [a]	235.309 [a]	190.33 [b]
	SD	190.136	137.840	1.64

Note: Values are mean ± standard deviation (n = 3). Values within treatments in a column with different superscript lowercase letters (a–b) differ significantly ($p < 0.05$).

Table 4. Total phenol level of *A. malaccensis* leaf tea after fermentation

Starter	Replication	Phenol after fermentation		
		48 h (mg GAE/ gram sample)	96 h (mg GAE/ gram sample)	192 h (mg GAE/ gram sample)
L. plantarum	1	28.273	33.273	33.820
	2	30.148	37.648	44.367
	3	30.227	64.445	30.852
	Average	29.549 [a]	45.112 [b]	36.346 [c]
	SD	0.932	16.876	7.103

Note: Values are mean ± standard deviation (n = 3). Values within treatments in a column with different superscript lowercase letters (a–c) differ significantly ($p < 0.05$).

taste. According to Fessard et al., 2017 in the fermentation process, there is a change in the level of acidity, with *L. plantarum* bacteria reducing the edge of the medium to pH 3.7 after fermentation.[40] In line with this research, the pH after 24, 96, and 192 hours of fermentation in the present study was 3.7, 3.6, and 3.6, respectively (see Table 2). A study conducted by Li et al. of the fermentation of apple juice using L. plantarum bacteria obtained a pH of 3.68 after 72 hours.[41] *L. plantarum* presumably produced organic acids such as lactic acid, acetic acid, gluconate acid, glucuronic acid, propionic acid, and butyric acid during fermentation.[39,42] The lactic acid produced will dissociate and create H^+ and $CH_3CHOHCOO^-$, so that as more lactic acid is made, the H+ ions released in the medium will also be higher and cause a decrease in pH.[10]

4.3. Measurement of total lactic acid bacteria (LAB)

Lactobacillus plantarum formed a convex opaque white colony of about 2-3 mm depth.[43] The test results showed that as the length of the fermentation process increased; LAB increased to 2.6 x 1010 CFU/ml at 192 h (eight days) of fermentation. The result of this study can be seen in Table 3. In line with Wistiana and Zubaidah 2015 study results, the maximum number of lactic acid bacteria was found on the eighth day of fermentation, with a total of 9.90×105 CFU/ml, as compared to 4.30×105 CFU/ml on the fourteenth day of fermentation.

4.4. Antioxidant activity test

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method was used to test antioxidant activity. DPPH compounds will react with antioxidant compounds by taking hydrogen atoms from antioxidant compounds to form electron pairs.[45] As an antioxidant, the active compound will reduce DPPH free radicals (2,2-diphenyl-1-picrylhydrazyl) to diphenyl picrylhydrazyl and fade the colour of the solution. The more antioxidant compounds interact with DPPH, it's can reduce the ability of radicals from DPPH,

Table 5. Total flavonoid level of *A. Malaccensis* leaf tea after fermentation

Starter	Replication	Flavonoid after fermentation		
		48 h (mg GAE/ gram sample)	96 h (mg GAE/ gram sample)	192 h (mg GAE/ gram sample)
L. plantarum	1	5.401	3.705	4.172
	2	5.730	3.146	3.541
	3	5.483	4.554	3.916
	Average	5.555[a]	3.802[b]	3.876[b]
	SD	0.165	0.708	0.317

Note: Values are mean ± standard deviation (n = 3). Values within treatments in a column with different superscript lowercase letters (a–b) differ significantly ($p < 0.05$).

the impact of the interaction is the higher antioxidant activity of these compounds.[46]

The fermentation process used in this study combined the water kefir and kombucha methods. This study used bacteria preparation from a water kefir strain, while the form and duration of fermentation used the kombucha technique. Fermentation with water kefir strains takes 24–48 hours, while kombucha takes 7–12 days.[28] According to previous studies, different fermentation times and temperatures using Lactobacillus plantarum is different. The results of research conducted by Ariyanto, 2021, the difference between fermentation time and temperature is related to the rate of destruction by microbes. The length of the fermentation period and storage temperature can affect the number of available microbes, in this study it was found that fermented pineapple juice at 4°C can be stored for 28.32 weeks and at 10°C can be stored for 8.67 weeks to get the best product quality9. IC_{50} values after fermentation in this study can be seen in Table 4 and indicate that the optimum fermentation time was eight days, with resulting antioxidant activity of 190.33 ± 1.64µg/ml. Research conducted by Pratama et al., 2015 found a lower IC_{50} value in mangosteen rind fermentation on day eight of fermentation (54.51 µg/ml) compared to day 14 (92.43 µg/ml).[4]

In the study of Azizah et al., 2012 it was known that fermentation on pineapple skin using *Saccharomyces cerevisiae* bacteria during fermentation showed no change in pH, namely 3.5-3.7 with a fermentation time of 24-72 hours, this happened because the fermentation environmental conditions were good for live bacteria *Saccharomyces cerevisiae*. In line with this, in the research by Wilujeng and Wikandari, 2013 fermented Arabica coffee (Coffea arabica) using *L. plantarum* bacteria for 24-96 hours did not change with a value of 4.25-4.79; and research by Pratiwi et al., 2012. Fermentation of kombucha drink from seaweed for 4-16 hours showed that the pH during fermentation was 3.41-3.09 with starter Acetobacter xylinum.[60,61,62]

The increase in radical scavenging activity in DPPH compounds in fermented drinks can be seen in the research by Hassym et al., 2017 using *Acetobacter xylinum* bacteria in the fermentation process for 1-10 days giving a percent inhibition value of 90.835% -91.853% with a tendency for the product's pH to be at a value of 3.11-2.93. Hapsari et al., 2021 research on red galangal fermented beverage products with *Acetobacter xylinum* bacteria has free radical scavenging activity with a fermentation time of 8 days with an inhibitory activity of 89.75% and a decrease in antioxidant activity on the 10th day of 79.46%. so that the optimum time for making fermented products is obtained on the 8th day with pH level 3.56.[63,64]

The increased antioxidant activity changes the pH of the fermentation medium, causing it to become more acidic and making phenolic compounds more stable.[47] Based on research by Hur et al., 2014 and Diaconeasa et al., 2015, anthocyanin is a durable compound in low pH.[48,49] The pH value is one of the most important environmental parameters affecting food fermentation and is closely related to microbial growth and changes in phytochemical structure during fermentation.[50] The stability of phenolic compounds also prevents bacteria from bio-transforming their system to produce free phenolics, increasing antioxidant activity.[44] Microbial enzymes including glucosidase, amylase, cellulase, chitinase, inulinase, phytase, xylanase, tannase, esterase, invertase and lipase are produced by fermentation. They can hydrolyze glucosides and break down plant cell walls or starch. These enzymes play a role in disintegrating the plant cell wall matrix and facilitating flavonoid extraction from plant cells48. Another mechanism for increasing the antioxidant activity of plant-based foods using fermentation may be structural changes in phytochemicals48. According to Hur et al., 2014 increasing antioxidant activity is influenced by various factors such as species of lactic acid bacteria, pH, fermentation time, kind of food, and aerobic conditions.[48]

Lactic acid bacteria can control fermentation through simple phenolic structure and the depolymerization of high molecular

phenolic compounds.[50] Nkhata et al., 2018 reported that fermented soy foods were attributed to the antioxidative activity. The authors found changes of glycosylated isoflavones into aglycones during fermentation.[51] Fermented soybean foods contain aglycones as the predominant isoflavone structures. β-glucosidase is present in LABs such as *Lactobacillus acidophilus, Lactobacillus casei, L. plantarum, Lactobacillus fermentum, Bifidobacterium animalis subsp. lactis,* and *Bifidobacterium longum* can increase the content of the isoflavone aglycone in soymilk during fermentation. This ability may be due to their high α-galactosidase activity, and the released isoflavone aglycone can act as an antioxidant. In contrast, research by Watson et al. reported that the amount of flavonol glycosides decreased by up to 18% of the total phenolic compounds in green tea, caused by oxidative degradation.[41,52] During fermentation with lactic acid bacteria, phenolic compounds are synthesized, with the liberated phenolic compounds having antioxidant abilities. The principal structure of simple phenol and flavonoid compounds increases antioxidative activity in plant-based foods.[48]

4.5. Determination of total phenolic and flavonoid content

Phenolics, including phenolic acids, tannins, lignin, and flavonoids, are plants' most extensive secondary metabolites.[53] The principle of measuring total phenolic content with Folin-Ciocalteu reagents is based on the strength of the reducing hydroxy phenol group in an alkaline atmosphere and is characterized by the formation of complex blue compounds that indicate a solution containing phenolics.[54] The principle of determining the total flavonoid content with aluminium chloride ($AlCl_3$) is based on the formation of yellow complexes between aluminium chloride and quercetin compounds.[55]

In line with Hapsari et al., 2021 study, the results showed that there was an effect of the duration of fermentation and the total content of phenolic compounds where on days 1-8 the phenol content increased. This was due to the increase in total phenol in red galangal kombucha because during fermentation, the enzymes released by bacteria and yeast will degrade complex compounds in red galangal such as alpinia, galangin, camphorol into simple compounds. According to Coelho et al., (2020) During the fermentation process, the amount of phenolic compounds increased due to hydrolysis of sugar by yeast and lactic acid bacteria enzymes. Total phenolic content is higher than total flavonoids during fermentation in this study, it's can cause macromolecules from plant cells can transform into other structural metabolites.[64,65] Research by Li et al., 2019 concludes that *L. plantarum* ATCC14917 fermentation modified the phenolic composition of apple juice, and as such, fermentation may be employed as a valuable and straightforward method of enhancing the bioavailability of polyphenols in apples. Lin et al., 2109 study known that in the apple juice fermentation process there was an increase in gallic acid compounds by 102.9%; quercetin by 22.4%; 3-O-caffeoylquinic acid 121.4%; 5-O-caffeoylquinic acid aeamount 611.4%.[41] This study's total phenol and flavonoid content results are presented in Tables 5 and 6. Table 4 shows that the total of phenolic compounds increased 15.56% during fermentation from 48 h to 96 h and then decreased 8.77% to 192 h. Table 5 shows that there was a decrease in total flavonoids during the additional fermentation time at 48, 96 and 192 hours of 5.555 mg/g QE, 3.802 mg/g QE and 3.876 mg/g QE, respectively, equivalent to reduced yields of 17.53% and 16.79%, respectively. The best product, with an IC_{50} value of 190.33 ± 1.64µg/ml, has a total phenolic content of 36.346 mg/g GAE and a total flavonoid content of 3.876 ± 0.317 mg/g QE, results from 192 hours of fermentation.

The decrease in total flavonoid content after fermentation is suspected to be because *L. plantarum* cannot produce enzymes (or produces enzymes in smaller amounts), so sugars and complex flavonoid compounds cannot be broken down to increase total flavonoids.[10] In research by Li et al.,2019 flavonoid content in apple juice experienced

a surprising drop, with a decrease in value of 34.8%.[41] A study found that fermented barberry juice experienced decreased total phenol, anthocyanin, and ascorbic acid.[53,56]

During fermentation, the enzymatic oxidation process also affects total phenolic content, causing polyphenols to oxidize and decreasing bioactive components such as tannins. The total phenolics content produced was lower than before.[56] The presence of lactic acid as the end product of a fermented product is thought to cause the bacteria to be unable to biodegrade phenolic structures. This study identifies that antioxidant activity increases during fermentation even though the total flavonoid content decreases by 17.53% and 16.9% to 96 hours and 192 hours, respectively.

There was a decrease in the study's total phenol and flavonoid content, but this did not cause a reduction in antioxidant activity. As reported by Khan et al., 2013 during the fermentation process of lactic bacteria, they can use the compounds contained in the medium to produce large amounts of free aglycones with low steric resistance.[57] According to Li et al.,2019 antioxidant activity remains good because, during the fermentation process, lactic bacteria produce metabolites with good antioxidant properties, and thus, antioxidant activity is maintained during fermentation.[41]

5. Conclusion

Using *L. plantarum* as a starter for fermented tea of *A. malaccensis* leaves can produce appropriate characteristics for consumption as a functional food. The best product, with an IC_{50} value of 190.33 ± 1.64µg/ml, has a total phenolic content of 36.346 mg/g GAE and a total flavonoid content of 3.876 ± 0.317 mg/g QE, was obtained from the treatment of 192 hours of fermentation.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Medicinal Faculty of Tanjungpura University.

CONFLICT OF INTEREST

We declared that there is no conflict of interest during research activity and publication.

FUNDING

This research was fully funded by the Medicinal Faculty of Tanjungpura University Indonesia under Contract No. SP.DIPA-023.17.2.677517/2020.

Referensi

1. Gur J, Mawutu M, Martirosyan. FFC'S Advancement of functional food definition: Review article. Functional food in Health and Disease; 2018:8(7):385-397.
2. Muchtadi DM. Functional foods and bioactive compounds. 1st ed. Bandung: CV Alfabeta; 2012. p. 3–9.
3. Ryu JY, Kang HR. Changes over the fermentation period in phenolic compounds and antioxidant and anticancer activities of blueberries fermented by Lactobacillus plantarum. J Food Sci. 2019. July 17:0(0):1–10.
4. Pratama N, Pato U, Yusmarini Y. Study of making kombucha tea from mangosteen peel (Garcinia mangostana L.). Student online journal. 2015;2(2).
5. Pratama AN dan Busman H. Potensi antioksidan kedelai (Glycine max L) terhadap penangkapan radikal bebas:Literature review. Jurnal Ilmiah Kesehatan Studi Husada; 2020. Juni 30:11(1):497-504.
6. Chakravorty S, Bhattacharya S, Chatzinotas A, Chakraborty W, Battacharya D, Gachhui R. Kombucha tea fermentation: Microbial and biochemical dynamics. Int.J.of Food Microbiology. 2016. March 2:220(2): 63-72.
7. Diasari, NR, Nurrahman N, Yusuf M. Antioxidant Activity And Physical Properties Soyghurt Edamame With Red Beet. Edible: Jurnal Penelitian Ilmu-Ilmu Teknologi Pangan, 2021, Juni:10(1): 6-16
8. Eka PO, Ekawati P, Pranata FS. Quality and activity of antioxidants from probiotic drinks with variations of red dragon fruit (Hyloreceus polyrhizus). J.

Technobiology. 2014;1–15.
9. Ariyanto YS, Meryandini A, Sunarti TC .ApplicationLactobacillusplantarumNHC6 as a Probiotic in Pineapple Juice. Sumberdaya HAYATI. 2021. Juni:7(1): 1-8.
10. Primurdia E, Kusnadi J. Antioxidant activity of probiotic drink from date extract (Phoenix dactilyfera L.) with isolates of L. plantarum and L. casei. J Food Agroindustry. 2014;2(3):98–109.
11. Susilo A, Kalima T, Santoso E. Taxonomic status and population of Aquilaria and Gyrinops species. Bogor: IPB Press; 2014.
12. Silaban S. Phytochemical screening and antioxidant activity test of A. malaccensis leaf ethanol extract (Aquilaria malaccensis Lamk). Thesis. Sumatera Utara University, Medan; 2013.
13. Begum Y. Study on agarwood (Aquilaria malaccensis) to evaluate antibacterial and antioxidant activities of n-hexane, chloroform, and ethyl acetate extracts. PharmaTutor. 2016. Februari:4(2):47–50.
14. Simatupang J, Batubara R, Julianti E. Consumers' preference level for A. malaccensis (Aquilaria malaccensis Lamk.) tea based on shape and size and antioxidant content. Thesis. Sumatra Utara University, Medan; 2015.
15. Korinek M, Wagh VD, Lo IW, Hsu, YM, Hsu HY, Hwang TL. Antiallergic phorbol ester from the seeds of Aquilaria malaccensis. Int. J. Mol. Sci. 2016. March 21:17(3):390¬–398.
16. Wagh VD, Korinek M, Lo IW, Hsu YM, Chen SL, Hsu HY. Inflammation modulatory phorbol esters from the seeds of Aquilaria malaccensis. J Nat Prod. 2017. April 26:80(5):1421–1427.
17. Wang S, Yu Z, Wang C, Wu C, Guo P, Wei J. (2018). Chemical constituents and pharmacological activity of agarwood and Aquilaria plants. Molecules. 2018. February 7:23(2), 342.
18. Maldonado GC, Novotny NI, Carmuega E, de Moreno de LeBlanc A, Perdigon G. Role of probiotics and functional foods in health: gut immune stimulation by two probiotic strains and a potential probiotic yoghurt. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders). 2015. March 1st:15(1), 37-45.
19. Dvary RK, Batish VK, Grover S. Immunomodulatory activity of two potential probiotic strains in LPS-stimulated HT-2gcell. Genes Nutr; 2014. March 30:9(389):1-16.
20. Shirazinia RDVM, Golabchifar AA, Fazeli MR. Efficacy of probiotics for managing infantile color due to their anti-inflammatory properties: A meta-analysis and systematic review. Clin Exp Pediatr. 2021. April 12:64(12):642-651.
21. Ren J, Zhao Y, Huang S, Dan LV, Yang F, Lou L, Zheng Y, Zhang J, Liu S, Zhang N, Bachert C. Immunomodulatory effect of Bifidobacterium breve on experimental allergic rhinitis in BALB/c mice. Experimental and therapeutic medicine. 2018. September 6:16:3996-4004.
22. Gomez NC, Ramiro JMP, Quecan BXV, Franco BDGM. Use of potential probiotic Lactic Acid Bacteria (LAB) biofilms for the control of Listeria monocytogenes, Salmonella typhimurium and Escericia coli O157.H7 biofilm formation. Frotiers in Microbiology; 2016. Juni 10:7(863):1-15.
23. Ozuslam MA and Gunyakti A. Lactobacillus fermentum starins from human breast milk with probiotic properties and cholesterol-lowering effect. Food sci Biothecnol. 2019. Oktober 16:28(2): 501-509.
24. Papadimitri OUK, Zoumpopoulou G, Foligne B, Alexandraki V, Kazou M, Pot B, Tsakaludou E. Discovering probiotic microorganisms: Invitro, invivo genetics and omics approaches: review article. Frontiers in microbiology. 2015. February 17:6:58:1-28.
25. Peng K, Koubaa M, Bals O, Vorobiev E. Recent insights in the impact of emerging technologies on lactic acid bacteria: A review. Food research international. 2020. November:137:109544.

26. Lopez LS, Mendoza AH, Garcia HS, Haro VM, Belinda VC, Cardove AFG. The effect of consuming probiotic-fermented milk on the immune system: A review of scientific evidence. Nutrients. 2015. February 3:67:1-13.
27. Mahadi I, Sayuti I, Habibah I. Pengaruh variasi jenis pengolahan the (Camellia sinensis L. Kuntze) dan konsentrasi gula terhadap fermentasi kombucha sebagai rancangan kerja peserta didik (LKDP) biologi SMA. Biogenesis; 2017:13(2):p.93-102.
28. Maufiroh. Pengaruh variasi konsentrasi gula dan lama fermentasi kombucha the daun mengkudu (Morinda citrofilia L) terhadap kandungan vitamin C dan total asam organic sebagai sumber belajar biologi. Skripsi. Malang:Universitas Muhammadiyah Malang; 2019.
29. Adeniran HA and Ajifolokun OM. Microbiological studies and sensory evaluation of breadfruit and cassava co-fermented into gari analog. Nigerian Food Journal. 2015. June 33:(1):39-47.
30. Sanera F. Antioxidant and anti-inflammatory activity test of ethanol extract of karas leaves (Aquilaria Malaccensis Lamk), one plant that produces agarwood. Skripsi. Pontianak: Tanjungpura University; 2017.
31. Hanani E. Phytochemical analysis. Jakarta: EKG; 2017. p. 10–13.
32. Haryanto LT. Pengaruh Lama Fermentasi Terhadap Kandungan Vitamin C Dan Perbandingan Aktivitas Antioksidan Kombucha Teh Bunga Sepatu Merah (Hibiscus Rosa-Sinensis L.) Dengan Teh Bunga Sepatu Pink (Hibiscus Rosa-Sinensis Cv Natal). Skripsi. Yogyakarta:Sanata Dharma University.
33. Adam AZ, Lee SY, Mohamed R. Pharmacological properties of agarwood tea derived from aquilaria (Thymelaeaceae) leaves an emerging contemporary herbal drink. J Herb Med. 2017. December:10:37–44.
34. Guerrouj K, Sanchez-Rubio M, Taboada-Rodriguez A, Cava-Roda, Marin RM, Iniesta F. Sonication at mild temperature enhaces bioactive compound and microbiological quality of orange juice. Food and bioproducts processing. 2016. July:99:20-28.
35. Lestari OA, Palupi NS, Setiyono A, Kusnandar F, Yuliana ND. In vitro antioxidant potential and phytochemical profiling of Melastoma malabathricum leaf water extract. Food Science and Technology. 2022. February 1: 42:1-9.
36. Campos-vega R, Vazquez-sanches K, Lopez-Barrera D, Loarca-Pina G, Mendoza-Diaz S, Dave Omah B. Stimulated gastrointestinal digestion and in vitro colonic fermentation of spent coffe (Coffea arabical). Bioaccessibility and intestinal permeability food research international. 2015. November:77(2):156-161.
37. Suhardini PN, and Zubaidah E. Studi aktivitas antioksidan dan kombucha dari berbagai jenis daun selama fermentasi. Jurnal pangan dan agroindustry. 2016. January:4(1):221-229.
38. Arasu MV, Al-dhabi NA, Ilavenil S, Choi KC, Srigopalram S. Invitro importance of probiotic Lactobacillus plantarum related to medical field. Saudi journal of biological science. 2016. January:23(1):6-10.
39. Maryati Y, Nuraida L, Dewanti HR. A study in vitro of lactic acid bacteria (LAB) isolates on cholesterol-lowering ability in the presence of oligosaccharides. Agritech. 2016. May:36(2):196–205.
40. Fessard A, Kapoor A, Patche J, Assemat S, Hoarau M, Bourdon M, et al. Lactic fermentation as an efficient tool to enhance the antioxidant activity of tropical fruit juices and tea. Microorganisms. 2017. May 10:5(23):1–20.
41. Lie Z, Teng J, Lyu Y, Hu X, Zhao Y, Wang M. Enhanced antioxidant activity for apple juice fermented with Lactobacillus plantarum ATCC14917. Molecules. 2019. December 24:24(51):1–12.
42. Suhardini PN, and Zubaidah E. Studi aktivitas antioksidan dan kombucha dari berbagai jenis daun selama fermentasi. Jurnal pangan dan agroindustry. 2016:January:4(1):221-229.

43. Dewi IB. Produksi etanol dari hasil hidrolisis pati tepung kulit singkong (Manihot sp) menggunakan Bacillus stearothermophillus, Aspergillus niger dan Rhizopus oryzae. Thesis; 2013: Universitas Kristen Duta Wacana. Yogyakarta.
44. Wistiana D, and Zubaidah E. Chemical and microbiological characteristics of various leaves high in phenol during fermentation. J Food Agroindustry. 2015;3(4):1446–1457.
45. Fang J, Wang Z, Wang P, Wang M. Extraction, structure and bioactivities of the polysaccharides from Ginkgo biloba: A review. International Journal of Biological Macromolecules. 2020. November 1:162:1897-1905.
46. Theodora D. Review on indonesian soybean as protein source and ingredient for tempe production. Thesis; 2020:Swiss German University. Banten
47. Ayu S, Yan R, Eka L. Determination of optimal antioxidant activity in local kombucha black tea in Bali with variations in fermentation time. J. Pharm Udayana. 2013;2(1).
48. Hur SJ, Lee SY, Kim YC, Choi I, Kim GB. Effect of fermentation on the antioxidant activity in plant-based food. J.Food Chem. 2014. October 1:160:346-356.
49. Diaconeasa Z, Leopald L, Rugina D, Ayvaz H, Socaciv C. Antiproliferative and antioxidant properties of Athocyanin rich extract from blueberry and blackcurrant juice. Int.J.Mol.Sci. 2015. January 25:16:2352-2365.
50. Hashemi SMB, Khaneghah AM, Barba FJ, Nemati Z, Shokofti SS, Alizadeh F. Fermented sweet lemon juice (Citrus limetta) using Lactobacillus plantarum LS5:chemical compotition, antioxidant and antibacterial activities. Journal of functional food. 2017. November:38(A):409-414.
51. Nkata SG, Ayua E, Kamau E, Shingiro JB. Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzym. Food Sci Nutr. 2018. October 16:6:2446-2458.
52. Chahrour M, Assi S, Bejjani M, Salhab AANH, Fares M, Khachfe HH. A bibliometric analysis of COVID-19 research activity: a call for increased output. Cereus. 2020. March 21:12(3).
53. Watson RR. Polyphenols in plants: isolation, purification and extract preparation. USA: Academic Press; 2014.
54. Kumar S and Pandey AK. Chemistry and biological activities of flavonoids an overview:Review article. The scientific world journal. 2013. December 29:2013:1-16.
55. Hashemi MB, Seyed, Mahmoodi M. Fermentation of barberry juice to produce probiotic beverage. Curr Nutr Food Sci. 2017. August 1:13(3):204–11.
56. Palupi NS, Hunaedi D, Susanto N. Optimization of green tea extraction based on the polyphenol content, antioxidant activity, and sensory profile. Journal of industrial and beverage crops; 2021:8(2):87-98.
57. Khan MK, Huma ZE, Dangles O. A comprehensive review on flavanones, the major Citrus polyphenols. Journal of food composition and analysis. 2014:33(1):85-104.
58. Tania M and Parhusip AJN. Studi Literatur Perbandingan Mutu Mikrobiologis Dan Fisikokimia Minuman Fermentasi Kefir Dari Beberapa Jenis Susu. Jurnal Teknologi Pangan dan Kesehatan. 2022:4(1):25-36.
59. Apridamayanti P, Sanera F, Robiyanto R. Antiinflammatory Activity of Ethanolic Extract from Karas Leaves (Aquilaria malaccensis Lamk.). Pharmaceutical Sciences and Research, 2018. December:5(3):152 – 158.
60. Azizah N, Al-Baarry AN, Mulyani S. Pengaruh Lama Fermentasi Terhadap Kadar, Alkohol, Ph, Dan Produksi Gas Pada Proses Fermentasi Bioetanol Dari Whey Dengan Substitusi Kulit Nanas. Jurnal Aplikasi Teknologi Pangan. 2012. Agust 29:1(2):72-77.
61. Wilujeng AAT dan Wikandari PR. The Effect Of Fermentation Time Of Arabica

Coffee (Coffea Arabica) With Lactobacillus Plantarum B1765 Lactic Acid Bacteria To The Product Qualities. UNESA Journal of Chemistry. 2013. September:2(3):1-10.
62. Pratiwi A, Elfita E, Aryawati R. Pengaruh Waktu Fermentasi Terhadap Sifat Fisik dan Kimia pada Pembuatan Minuman Kombucha dari Rumput Laut Sargasssum sp. Maspari Journal. 2012. Apr :4(1):131-136.
63. Hassmy NP, Abidjulu J, Yudistira A. Analisisaktivitas Antioksidan Pada Teh Hijau Kombucha Berdasarkan Waktu Fermentasi Yang Optimal. 2017. November:6(4):67-74.
64. Hapsari MW, Rizkiprilisa W, Sari AP. Pengaruh Lama Fermentasi terhadap aktivitas antioksidan minuman fermentasi kombucha lengkuas merah (Alpinia purpurata). AGROMIX Journal. 2021. September 28:12(2):146-149.
65. Coelho RMD, Almeida A, do Amaral RQG, da Mota RN, de Sousa PHM. Kombucha: review. International Journal of Gastronomy and Food Science. 2020. December:176(2020):138-141.