Analysis of Snakefruit Vinegar with Variation Time and Concentration of *Lactobacillus plantarum* Inoculation During Alcohol Fermentation

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Abstract. The purpose of this study was to analyze the effect of *L.plantarum* inoculation at the alcohol fermentation stage on the quality of wine snakefruit vinegar. The results showed that the total phenolic content (TPC) increased in snakefruit wine with 2% *L.plantarum* concentration with the difference in inoulation time, namely 106.72 to 100.58 mg GAE/g. Antioxidant activity was evaluated with DPPH in vitro and expressed in IC50. The antioxidant activity increased from 50.99 to 47.19 ppm at concentrations of 2% and 4%, respectively, and decreased from 51.81 to 35.60. The TPC decreased in 4% and 6% cultures with the longer the fermentation time. There was an increase in TPC at a concentration of 4% with the decrease in pH values from 5.66% to 5.97% with the increase in 2% concentration. The total antioxidant activity of snakefruit wines was significantly increased with 4% concentration and 5% concentration of S. cerevisiae. In addition, there was a significant increase in the antioxidant activity at 6% concentration. In conclusion, the use of mixed cultures during alcoholic fermentation of snakefruit vinegar could be a new method for producing high quality vinegar.

Keywords: *L.Plantarum, Malolactic* Fermentation, Mixed Culture, Snakefruit, Vinegar.

1. Introduction

Vinegar is a traditional condiment produced from raw materials such as rice, malt, apples, alcoholic liquids and various other vegetable ingredients. Vinegar made from juice is known as cider vinegar [1] and is made through two fermentations, namely alcoholic and acetic [2]. Vinegar is defined as an acidic liquid produced through a two-stage fermentation process [3,4]. For anaerobic fermentation, yeast converts sugar into ethanol, while for aerobic processes, acetic acid bacteria (AAB) oxidise ethanol into acetic acid [5]. Yeasts, particularly Saccharomyces cerevisiae, are responsible for alcoholic fermentation and

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contribute to the creation of important aroma compounds like aldehydes, esters, fatty acids, and high alcohols [6].

One stage at the end of alcoholic fermentation, carried out by Lactic Acid Bacteria (LAB) species is malolactic fermentation, where this stage has an important role in the formation of product characteristics [7]. Malolactic fermentation generally occurs in the wine making process. The primary microorganisms implicated in malolactic fermentation during winemaking have been determined to be four types: Leuconostoc, Pediococcus, Lactobacillus, and Oenococcus [8].

It has been demonstrated that Lactobacillus spp. is resistant to the conditions involved in wine fermentation and possesses a number of advantageous traits that would make it an appropriate malolactic starter [9]. Citrate metabolism, amino acid metabolism, polysaccharide metabolism, polyol metabolism, aldehyde catabolism, glycoside hydrolysis, ester synthesis and hydrolysis, and phenolic degradation are examples of secondary metabolic reactions that are crucial for the development of aroma, taste, and functionality. peptidolysis, lipolysis, proteolysis, and acid [10].

Lactic acid bacteria (LAB) have recently gained popularity for their ability to bioconvert phenolics. Previous research has revealed that different LAB have the ability to de-carboxylate, de-esterify, de-methylate, and de-glycosylate dietary polyphenols [11][12]13]. LAB has been found to be capable of producing biotransformation of polyphenols by the action of various glycosylhydrolases by releasing aglycones from glycol-conjugated phenolics [14][15].

Cider vinegar's final quality is governed by the chemical complexity produced by the fermenting process. Most vinegar fermentations comprise two stages of liquid culture fermentation, including alcoholic fermentation, which is the anaerobic conversion of fermentable carbohydrates to ethanol by yeast, typically S.cerevisiae. Fermentation with various microbial strains produces a wider range of metabolites and sensory qualities. Chinese cereal vinegar and sherry vinegar are both generated through multi-strain collaborative fermentation, and the fermentation strains involved are lactic acid bacteria, which have benefits and high activity [16].

L.plantarum is employed in fermented food biotechnology and is becoming more relevant in the winemaking process. Like other Lactobacillus, *L.plantarum* can survive the harsh environment of grapes [8][17][18][19]. Many natural fermentation systems contain *L.plantarum* and S.cerevisiae, including wine and kefir. Lactic acid bacteria and S.cerevisiae can form a mutually beneficial connection during wine production [20]. Lactobacilli and yeast can support each other's survival during kefir production [1]. Although, in recent years, research on vinegar fermentation has been carried out [21][24]23], there has not been enough scientific research regarding the study of *L.plantarum* inoculation at the fermentation stage in vinegar production. This situation can

be considered an important shortcoming for the food industry. This study intends to examine the influence of *L.plantarum* inoculation at the alcohol fermentation stage on the quality of snakefruit vinegar made with different quantities and *L.plantarum* inoculation times.

2. Methodology

2.1. Materials

Snakefruit was obtained from Suwaru Village, Malang, East Java. Sacharomycess cerevisiae 3004 and *Lactobacillus plantarum* 0026-CCRC10069 were employed in alcohol fermentation for the production of snakefruit vinegar. Starter was obtained from the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. The reagents used are PP indicator (Phenolphthalein), NaOH 0.1 N, DPPH (Diphenylpicrylhydrazyl), ascorbic acid, methanol, gallic acid, Folin Ciocalteau, and Na_2CO_3.

2.2. Methods

Preparation of snakefruit juices and Inoculum. First, snakefruit is mixed with drinking water and ground in a ratio of 1:2 (w/v). The mixture is filtered through a multi-layered filter cloth. After that, Diammonium hydrogen phosphate 0.2%, sucrose 12.5% until the total dissolved solids were 15°Brix, and Na-bisulfite 200 mg/L were added to the snakefruit juice and pasteurized at 70°C for 15 minutes. Snakefruit juice is filtered again before adding the starter. YPD media (10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone) was used to grow Sacharomycess cerevisiae. DeMan Rogosa Sharp Broth (MRSB) media to grow *L.plantarum* [24].

Table 1. Chemical analysis snakefruit wine after fermentation

Concentration *L.plantarum*	Time Inoculation	pH	Total Acidity (%)	Total Soluble Solid (°Brix)	Total Sugar (%)	Alcohol (%)
2%	0	3.66 ± 0.01^{c}	0.19 ± 0.02^{a}	4.52 ± 0.01^{b}	0.55 ± 0.03^{b}	5.66 ± 0.03^{b}
	12	4.13 ± 0.01^{g}	1.16 ± 0.02^{c}	4.67 ± 0.11^{cd}	0.45 ± 0.03^{a}	5.97 ± 0.01^{f}
	24	4.21 ± 0.06^{h}	1.31 ± 0.74^{de}	4.74 ± 0.14^{de}	0.43 ± 0.04^{a}	5.71 ± 0.01^{c}
4%	0	3.35 ± 0.01^{a}	0.97 ± 0.05^{ab}	4.27 ± 0.02^{a}	0.52 ± 0.01^{b}	5.75 ± 0.02^{d}
	12	3.98 ± 0.01^{f}	1.29 ± 0.04^{d}	4.37 ± 0.07^{a}	0.44 ± 0.04^{a}	5.89 ± 0.06^{e}
	24	3.87 ± 0.01^{e}	1.39 ± 0.08^{ef}	4.34 ± 0.04^{a}	0.40 ± 0.01^{a}	5.97 ± 0.01^{f}
6%	0	3.33 ± 0.02^{a}	1.00 ± 0.03^{b}	4.84 ± 0.03^{e}	0.55 ± 0.03^{b}	5.67 ± 0.01^{b}
	12	3.74 ± 0.02^{d}	1.34 ± 0.25^{def}	4.74 ± 0.04^{de}	0.45 ± 0.03^{a}	5.43 ± 0.01^{a}
	24	3.49 ± 0.01^{b}	1.39 ± 0.01^{f}	4.57 ± 0.03^{bc}	0.41 ± 0.01^{a}	5.69 ± 0.01^{f}

Alcohol Fermentation of Vinegar. Vinegar production begins with the alcohol fermentation stage with Sacharomycess cerevisiae 2% and *L.plantarum* (2%; 4%; and 6%) used during alcohol fermentation with different inoculation times (0 hour, 12 hours, and 24 hours). Alcohol fermentation for 7 days and at room temperature (33 - 35°C). Sampling was carried out every day during fermentation.

pH, Total Soluble Solid, and Total Sugar Analysis. pH was measured using a pH-meter (HP 9000) and total soluble solids were measured using hand refractometer [25]. Total sugar analysis using the anthrone method [26], Sample solution 1 mL was added to 5 mL of anthrone reagent, then closed and agitated. Solution was boiled in a water bath at 100°C for 12 minutes. Solution was cooled and read at wavelength 628 nm using a spectrophotometer.

Alcohol analysis. 100 ml of sample is put in distillation flask, add 100 ml distilled water into the distillation flask, pour as much sample 50 ml into Erlenmeyer and distilled at 80oC, then move it to the pycnometer that has been dried and weighed. Weigh pycnometer containing distillate and record weight do the same procedure on distilled water as a comparison [25].

Analyze the total phenolic content and antioxidant activity. Total phenolic content was determined using Follin-ciocalteau and gallic acid standards. 0.5 mL of material was combined with 5 mL of Follin-Ciocalteau reagent and incubated for 3 minutes at room temperature. 2 mL of 150 g/L Na2CO3 solution was added, diluted with distilled water to 10 mL, and incubated at room temperature for 60 min.

Table 2. Total phenolic content and antioxidant activity snakefruit wine

Concentration *L.plantarum*	Time Inoculation	Total phenolic content (mg GAE/g)	Antioxidant activity IC50 (ppm)
2%	0	106.72 ± 0.18^{e}	50.99 ± 8.60^{bcd}
	12	105.03 ± 0.24^{d}	41.30 ± 6.14^{ab}
	24	100.58 ± 0.73^{c}	47.19 ± 7.83^{bcd}
4%	0	65.50 ± 0.26^{b}	51.81 ± 1.92^{cd}
	12	63.37 ± 0.06^{a}	48.34 ± 1.21^{bcd}
	24	66.40 ± 0.30^{b}	35.60 ± 1.85^{a}
6%	0	104.34 ± 0.10^{d}	46.29 ± 5.90^{bc}
	12	108.73 ± 1.67^{f}	69.16 ± 4.90^{e}
	24	112.22 ± 0.45^{g}	56.86 ± 2.15^{d}

A spectrophotometer was used to measure the sample at a wavelength of 760 nm. Total phenolics were determined using the gallic acid equation (GAE) from the calibration curve and expressed in mg GAE/g [24].

Statistical Analysis. Data is presented as mean±standard deviation, with triple repetitions. Data were evaluated using two-way analysis of variance (ANOVA) and Duncan's multiple range test with a significance threshold of $p<0.05$. SPSS 27.0 (IBM SPSS, New York, USA) was used to conduct statistical analyses.

3. Result and Discussion

3.1. pH, Total Soluble Solid, Total Acidity, and Total Sugar

The pH of wine snakefruit was analyzed on the last day of alcoholic fermentation using a pH meter. Based on Figure 1A, the average pH value during the alcohol fermentation process varies based on the *L.plantarum* concentration and inoculation time. Wine's pH at the end of alcoholic fermentation ranges from 3.33 to 4.21. The pH was significantly different (Table 1) with an interaction between concentration and inoculation time of *L.plantarum* in alcohol fermentation ($p<0.05$). pH variations are connected to total acidity changes. The findings revealed that increasing *L.plantarum* concentrations increased overall acidity (Table 1) while decreased pH values during snakefruit vinegar fermentation. Lactic acid bacteria's capacity to generate organic acids and release H+ ions increases overall acidity while decreasing pH [27]. A decrease in pH values can also be caused by sugar consumption and subsequent acid production by *L.plantarum* [28].

The total average dissolved solids can be seen in Figure 1D. The lowest mean total dissolved solids was found in the 4% *L.plantarum* inoculation treatment, namely around 4.27 to 4.37°Brix. Inoculation of 2% *L.plantarum* varied from 4.52 to 4.74°Brix, while inoculation of 6% *L.plantarum* ranged from 4.87 to 4.84□Brix. Table 2 displays statistical test results indicating a significant difference ($p<0.05$) in TSS due to the interaction between concentration and inoculation time of *L.plantarum* during alcohol fermentation. The decrease in total soluble solids during fermentation is considered to be caused by yeast metabolizing sugar, the primary dissolved solids component in the medium except colors, vitamins, and minerals, into alcohol and CO2.

The total sugar content changes due to fermentation by yeast. The average total sugars are in Figure 1C. Total sugar in 2% *L.plantarum* inoculation ranged from 0.43% to 0.55% from the three different inoculation times. At a concentration of 4% it ranges from 0.40% to 0.52%, and at a concentration of 6% it ranges from 0.41% to 0.55%. Table 1 shows a significant difference ($p<0.05$) in total sugar due to the interaction between *L.plantarum* concentration and time of inoculation. Research on citrus vinegar using mixed cultures resulted in a decrease in TSS and total sugar due to yeast activity during alcoholic fermentation [24].

3.2. Alcohol

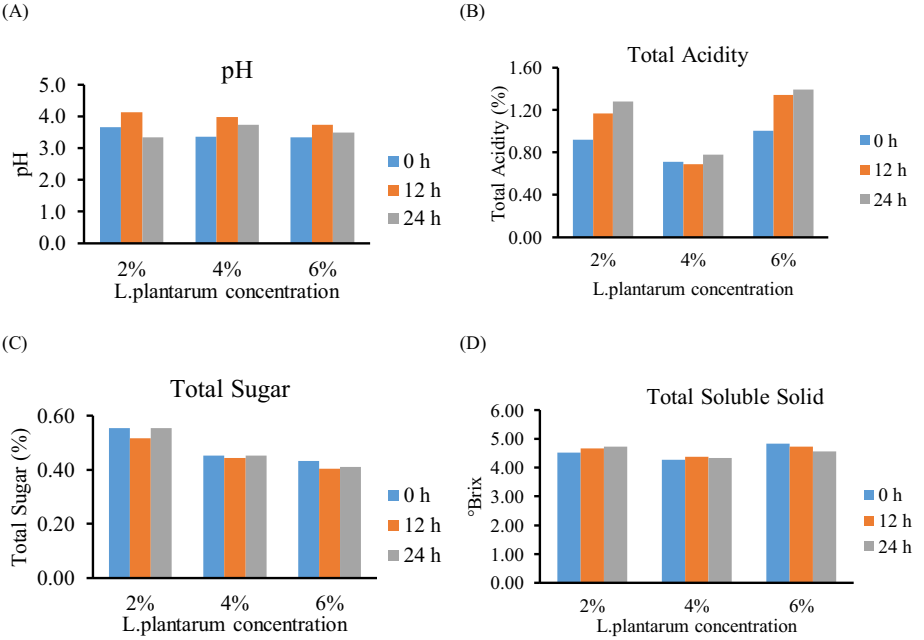
Alcoholic fermentation was complete after 7 days for all MLF inoculation treatments. As can be seen in Figure 1F, the alcohol yield did not have much difference in all samples. Alcohol in 2% *L.plantarum* inoculation ranged from 5.66% to 5.97% from different third inoculation times. At a concentration of 4% it ranges from 5.75% to 5.97%, and at a concentration of 6% it ranges from 5.43% to 5.67%. A 6% concentration of *L.plantarum* resulted in the lowest alcohol percentage. The difference in inoculation time for *L.plantarum* is around 5.43%-5.97%. Thus, the culture mixture had negligible influence on S.cerevisiae metabolism. *L.plantarum* is frequently present in grapes and contributes to spontaneous MLF [29]. Alcohol content is related to total dissolved solids and total sugar. Alcoholic fermentation, the initial step, is when yeast Saccharomyces typically turns sugar into ethanol [30].

3.3. Antioxidant Activity and Total Phenolic Content

Many plant-based substances' polyphenol concentration can be impacted by fermentation. TPC results varied in snakefruit wine with differences in concentration and inoculation time of *L.plantarum* (Figure 1E). TPC decreased in snakefruit wine with 2% *L.plantarum* inoculation with increasing inoculation time, namely 106.72 to 100.58 mg GAE/g. Other research indicated that olives' TPC decreased after fermentation with *L.plantarum* PTCC 1058 [9,10]. The presence of lactic acid bacteria helps to the conversion of simple phenolics and depolymerization of large molecular weight phenolic compounds in apple juice, which explains the lower TPC [32]. There was an increase in TPC in 4% *L.plantarum* inoculation, namely 65.50 to 66.40 mg GAE/g, and 6%, namely 104.34 to 112.22 mg GAE/g with increasing inoculation time. Table 2 displays statistical test results indicating a significant difference ($p<0.05$) in TPC due to the interaction between concentration and inoculation time of *L.plantarum* during alcohol fermentation. The higher *L.plantarum's* ability to synthesize more volatile phenols from phenolic acids may account for its greater TPC [33]. Lactic acid bacteria can activate enzymes that convert phenolic compounds into simple complexes [34]. Furthermore, the decrease in TPC and TFC of fermented apple juice had no effect on antioxidant activity in this study. It is possible that lactic acid bacteria use glucose molecules in phenolic compounds to produce free aglycones with a higher number of hydroxyl groups [35], allowing for increased antioxidant activity during apple juice fermentation [10][°11].

Antioxidant activity was tested with DPPH in vitro and expressed in IC50. Differences in concentration and inoculation time of *L.plantarum* have an impact on the antioxidant activity of wine snakefruit. Figure 1G shows that antioxidant activity increased from 50.99 to 47.19 ppm at 2% *L.plantarum* concentration with different inoculation times. Antioxidant activity increased at a concentration of *L.plantarum* of 4% with the difference in inoculation time, namely from 51.81 to 35.60 ppm. Different results showed that there was a concentration of *L.plantarum* of 6% with the longer the inoculation time, the antioxidant activity decreased from 46.29 to 56.86 ppm. In general, the snakefruit wine

produced has very strong to strong antioxidant activity because the value is between 35.60 ppm to 69.16 ppm (weak antioxidant activity if >100 ppm). Antioxidant activity was significantly different due to the interaction between concentration and inoculation time of *L.plantarum* during alcohol fermentation ($p<0.05$). Fermentation with *L.plantarum* improved the juice's free radical scavenging effect [32]. As a result, the addition of *L.plantarum* to alcoholic fermentation can greatly boost the antioxidant activity and phenolic content of wine snakefruit. Antioxidant activity is related to TPC, where an increase in antioxidant activity accompanied by an increase in TPC has been proven by several studies [10,15,16]. Fermentation promotes the biotransformation of snakefruit's bioactive components, such as polyphenols, into stronger antioxidants, resulting in improved antioxidant capabilities in snakefruit wine. In addition, the fermented samples showed strong antioxidant capacity.



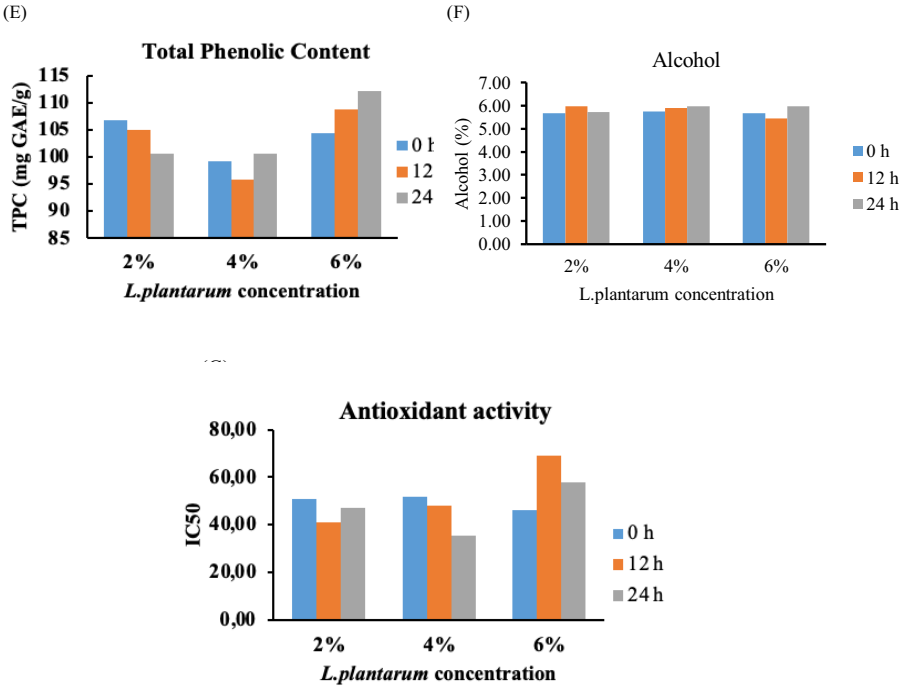


Figure 1. Changes of pH (A), total soluble solid (B), total acidity (C), total sugar (D), total phenolic content (E), alcohol content (F), and antioxidant activity (F) in snakefruit vinegar with different concentrations of *L.plantarum* at various fermentation times

4. Conclusion

This study demonstrates that using a mixed culture of S.cerevisiae and *L.plantarum* during the alcoholic fermentation of snakefruit vinegar has a substantial impact on the quality of the resulting snakefruit wine. Snakefruit's total phenolic content and antioxidant activity increased considerably after mixed culture in alcoholic fermentation. The use of mixed cultures could be a new method for producing high quality vinegar. Further research should focus on the use of other lactic acid bacteria as mixed cultures.

Authors' Contributions

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