Enhancing *Lacticaseibacillus rhamnosus* ATCC 9595 growth in *Aloe vera* juice fermentation with stingless bee honey

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Abstract. Consumers are increasingly seeking probiotic beverages for better health. Fermented *Aloe vera* juice is one such beverage. This study investigated *Lacticaseibacillus rhamnosus* ATCC 9595 bacteria's activity in fermented *Aloe vera* juice with added stingless bee honey (SBH). Six formulations used as additives in *Aloe vera* juice: Formula A: 10% honey + 5% sugar; B: 5% honey + 5% sugar; C: 10% honey; D: 10% honey + 5% sugar + salt + vanilla; E: 2.5% honey + 10% sugar; and F (control): 10% sugar + ascorbic acid + salt + vanilla. *Aloe vera* juice was fermented with 5% (v/v) of *L. rhamnosus* ATCC 9595 (10^7 CFU/mL) for 48 hours at 37 ºC. The observed variables were optical density (OD), pH value, total *L. rhamnosus*, and total lactic acid. The results demonstrated that the addition of SBH increased *L. rhamnosus* growth. Formula D (10% honey + 5% sugar) resulted in a higher *L. rhamnosus* growth rate, with 7.52 Log CFU/mL total bacteria, 0.23 OD_{600} nm, 0.64% of total acid, and a pH value of 3.70 than Formula F as a control without SBH. This study indicated that SBH is an inducer in *L. rhamnosus* growth in *Aloe vera* juice fermentation.

1 Introduction

Consumers tend to look for food products that can provide health benefits for their lives. These benefits are generally obtained through preventing nutrition-related diseases or overcoming stress due to modern lifestyles. Functional foods encompass a variety of nutrients that confer supplementary health benefits beyond basic nutritional value [1]. Probiotic-rich foods contain a minimum concentration of 6-7 log cfu/mL or g of probiotic microorganisms. Consuming foods with a high microbial count can positively impact the

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microbiota in the gut. The foundation of the functional diet is the inclusion of probiotics, which enhance immune function, increase cognitive response, guard against infection, and enhance overall health [2].

Probiotics are frequently present in dairy products. Moreover, fruit juices may also serve as a viable medium for the development of probiotic products, owing to their nutritional attributes and the presence of bioactive compounds, including vitamins, phenolic acids, flavonoids, and antioxidants. Fruit drinks containing probiotics can benefit persons with lactose intolerance, milk allergies, and vegetarian diets [3].

Aloe vera gel can be processed into fermented food by adding probiotics to *Aloe vera* juice. The carbohydrates in *Aloe vera* gel, particularly in the form of mannose polymers (acemannans), can serve as a substrate for probiotics. Additionally, combining *Aloe vera* and probiotics can form a potential synbiotic, a combination of probiotics and prebiotics with beneficial effects. *Lacticaseibacillus rhamnosus,* a probiotic that belongs to the Lactic Acid Bacteria (LAB) group, can be used in this approach [4].

Sweeteners are required in *Aloe vera* juice to enhance its flavor. Honey can be a sugar substitute sweetener and an energy source for LAB growth. Honey is mostly composed of fructose (38%) and glucose (31%), with just 8% sucrose [5]. These contents can be utilized as a simple sugar source and substrate for bacteria in fermentation. Honey also contains vitamins such as thiamin, riboflavin, ascorbic acid, pyridoxine, and vitamin K. Furthermore, honey comprises approximately 180 distinct compounds, including water, sugars, free amino acids, proteins, enzymes, essential minerals, vitamins, and various phytochemicals [6]. In light of these properties, a research study was undertaken entitled "The Growth Characterization of *Lacticaseibacillus rhamnosus* ATCC 9595 in *Aloe vera* Juice Fermentation with Stingless Bee Honey Supplementation". The objective of this study was to evaluate the activity of *L. rhamnosus* bacteria in fermented *Aloe vera* juice supplemented with stingless bee honey.

2 Materials and methods

2.1 Materials

The primary materials utilized in the investigation were *Aloe vera*, stingless bee honey and an isolate of *Lacticaseibacillus rhamnosus* ATCC 9595. The *Aloe vera* was the chinensis (L.) Baker variety obtained from CV Mount Vera Sejati in Gunungkidul, Yogyakarta, Indonesia. The stingless bee honey (*Heterotrigona itama*) was collected from the Griya Kenari beekeeper in Bantul, Yogyakarta, Indonesia. The isolate of *Lacticaseibacillus rhamnosus* ATCC 9595 was from the Research Center for Food Technology and Processing, National Research and Innovation Agency.

Several analyses used the analytical-grade materials listed below, such as De Man Rogosa and Sharpe (MRS) Broth (Merck, Germany) and De Man Rogosa and Sharpe (MRS) Agar (Merck, Germany).

2.2 Methods

2.2.1. Aloe vera var. chinensis juice preparation and formulation

The preparation of *Aloe vera* juice began with selecting the fresh, dark green *Aloe vera* leaves without any wounds, each weighing approximately 1 kg. After that, the *Aloe vera* gel was separated from the leaf skin for further processing. *Aloe vera* gel was then washed to remove any dirt. It was subsequently immersed in a citric acid solution

The citric acid solution was made from diluted citric acid powder using water in a ratio of 1 g of citric acid for 1 kg of *Aloe vera* gel. Soaking was carried out for ± 12 h and washed three times. Immersion in citric acid solutions has been shown to inhibit browning reactions and preserve the antioxidant properties of *Aloe vera* gel. Subsequently, the *Aloe vera* gel was immersed in distilled water for two hours to remove residual citric acid. Following this, the gel was subjected to boiling and continuous stirring until the water reaches a boil. Thereafter, the *Aloe vera* gel was weighed and processed using a blender to achieve a uniform consistency.

The *Aloe vera* juice formula used differs in concentration in honey. *Aloe vera* juice formulation was made using six variations: Formula A: 10% honey + 5% sugar; Formula B: 5% honey + 5% sugar; Formula C: 10% honey; Formula D: 10% honey + 5% sugar + salt + vanilla; Formula E: 2.5% honey + 10% sugar; Formula F: 10% sugar + ascorbic acid + salt + vanilla. The formulated juice was then boiled for 10 min, cooled, and finally packed in a sterile plastic cup using a packing machine.

2.2.2. Preparation of inoculum

The following steps were taken in the preparation of inoculum: One mL of *Lacticaseibacillus rhamnosus* ATCC 9595 overnight culture was inoculated into 10 mL of de Man Rogosa Sharpe (MRS) Broth medium and incubated for 24 h at 37 °C. The optical density (OD) of *L. rhamnosus* culture was determined using a microplate reader at a wavelength of 600 nm. The absorbance value obtained was then compared to the absorbance value of the McFarland standard to determine the number of cell colonies. The bacterial suspension used for fermentation was 7 Log CFU/mL.

2.2.3. Fermentation of Lacticaseibacillus rhamnosus ATCC 9595 and Aloe vera var. chinensis juice

First, 50 mL of *Aloe vera* juice was pasteurized at 90 ºC for 3 min using an autoclave and then placed in a sterile 100 mL flask. After pasteurization, it was allowed to cool to 40 °C. Next, 1 mL of overnight *L. rhamnosus* culture (10^7 CFU/mL) was inoculated and incubated for 48 h at 37 °C. Each *Aloe vera* juice formula was then inoculated with 2% (v/v) of *L. rhamnosus* and fermented for 48 h at 37 °C. Sampling for fermentation parameter analysis was conducted at 0, 24, and 48 h.

2.2.4. Analysis of Optical Density (OD) and pH measurement

The OD value of the *L. rhamnosus* culture was determined using a microplate reader at a wavelength of 600 nm. Meanwhile, each experimental group's pH value was determined by using a digital pH meter.

2.2.5. Analysis of Lacticaseibacillus rhamnosus ATCC 9595 growth by Total Plate Count (TPC)

The total plate count technique was used to count total lactic acid bacteria on MRS Agar media. To generate a 10^{-4} serial dilution, one mL of fermenting sample was transferred to a test tube containing 9 mL of 0.85% NaCl. The last two dilutions of each fermented sample (0, 24, and 48 h) were collected at 1 mL and inoculated on MRS Agar medium using the pour plate technique. The plates were then incubated at 37 °C for 24 h. The number of *L. rhamnosus* colonies was determined using a colony counter.

2.2.6. Analysis of total titratable acidity

One mL of the material was collected and put in a 10 mL volumetric flask. The distilled water met the standards. Following that, 2-3 drops of 1% PP (phenolphthalein) indicator were added, and the solution was titrated with NaOH 0.1 N (standardized first with oxalic acid 0.1 N) until it became pink.

2.2.7. Data analysis

The data was analyzed statistically using one-way analysis of variance (ANOVA) in CoStat Software, followed by the Duncan post-hoc test at a significance threshold of 5% if the findings differed substantially across samples and if the results significantly differed between samples.

3 Result and discussion

3.1 Effect of fermented *Aloe vera* juice on optical density value

Optical density analysis was performed on *Aloe vera* juice fermentation at 0, 24, and 48 h (Table 1). At 0 h of fermentation, the formula showed a low absorbance value due to the *L. rhamnosus* bacteria not experiencing growth and metabolism yet. Optical density values equal to or greater than 0.1 in Formulas A, B, C, D, E, and F were relatively steady throughout fermentation. The average turbidity of *Aloe vera* juice was found to be 0.13 up to 0.23. A separate investigation found that LAB strains with an OD value of ≥ 0.1, are tolerated at low pH [7].

Table 1. Value of Optical Density$_{600\ nm}$ ($OD_{600\ nm}$) based on variations in the *Aloe vera* juice formulation and fermentation time

Fermentation Time (h)	*Aloe vera* juice formulation					
	Formula A	Formula B	Formula C	Formula D	Formula E	Formula F
	$OD_{600\ nm}$ value					
0	0.18 ± 0.01^{bc}	0.18 ± 0.01^{b}	0.19 ± 0.03^{c}	0.18 ± 0.03^{b}	0.16 ± 0.01^{b}	0.13 ± 0.02^{a}
24	0.18 ± 0.01^{ab}	0.17 ± 0.00^{ab}	0.19 ± 0.02^{c}	0.18 ± 0.00^{bc}	0.18 ± 0.01^{bc}	0.15 ± 0.01^{a}
48	0.15 ± 0.02^{a}	0.15 ± 0.02^{a}	0.14 ± 0.04^{a}	0.23 ± 0.05^{a}	0.20 ± 0.04^{a}	0.19 ± 0.12^{a}

Notes :

- **Formula A** : 10% honey + 5% sugar; **Formula B**: 5% honey + 5% sugar; **Formula C**: 10% honey; **Formula D**: 10% honey + 5% sugar + salt + vanilla; **Formula E**: 2.5% honey + 10% sugar; **Formula F**: 10% sugar + ascorbic acid + salt + vanilla.
- Different notations on the same row indicate significantly different effects with a p-value <0.05 at the α=0.05

The bacterial density in *Aloe vera* juice was determined through optical density analysis to measure its turbidity. Turbidity can detect microbes in a liquid. The growth of bacterial cells in a liquid medium increases the turbidity and affects the amount of light transmitted through the medium [8]. Insoluble solids and other microorganisms in the *Aloe vera* juice formula contribute to its turbidity. During fermentation, LAB experiences growth by utilizing available substrate, leading to an increase in turbidity in *Aloe vera* juice.

In Formulas D, E, and F, the OD value increases in line with the length of fermentation time. This result was conducted with the theory. However, Formulas A, B, and C experienced a slight decrease in OD value during fermentation. The decrease may have been caused by light dissipating from the detector by one bacterial cell and then being

dissipated back to the detector by other bacterial cells, resulting in the OD value not increasing. Furthermore, fibers from the *Aloe vera* gel were detected during the OD measurement which may have led to inaccuracies in the cell density results at 0, 24, and 48 h of fermentation. Another study reported that relying solely on the OD test for accurately measuring bacterial population density may not be advisable [9].

3.2 Effect of fermented *Aloe vera* juice on pH value

The total acid content of *Aloe vera* juice Formulas A, B, C, D, E, and F, as measured at 0, 24, and 48 hours post-fermentation, indicate the metabolic activity of *Lacticaseibacillus rhamnosus* bacteria in the transformation of the substrate present in *Aloe vera* juice (Table 2). As the fermentation time increases, the amount of acid increases, leading to a decreased pH value. The average pH of *Aloe vera* juice after 48 h of fermentation ranges from 3.32 to 3.97.

Table 2. The pH value of different *Aloe vera* juice formulations and fermentation times.

Fermentation time (h)	*Aloe vera* juice formulation					
	Formula A	**Formula B**	**Formula C**	**Formula D**	**Formula E**	**Formula F**
	pH value					
0	3.82 ± 0.01^{b}	3.97 ± 0.02^{c}	3.81 ± 0.06^{b}	3.67 ± 0.02^{a}	3.86 ± 0.07^{bc}	3.63 ± 0.05^{a}
24	3.77 ± 0.04^{c}	3.97 ± 0.01^{d}	3.74 ± 0.01^{bc}	3.70 ± 0.04^{c}	3.78 ± 0.01^{c}	3.60 ± 0.00^{a}
48	3.53 ± 0.01^{d}	3.49 ± 0.03^{c}	3.43 ± 0.01^{ab}	3.32 ± 0.01^{a}	3.42 ± 0.01^{b}	3.66 ± 0.00^{e}

Notes :

- **Formula A** : 10% honey + 5% sugar; **Formula B**: 5% honey + 5% sugar; **Formula C**: 10% honey; **Formula D**: 10% honey + 5% sugar + salt + vanilla; **Formula E**: 2.5% honey + 10% sugar; **Formula F**: 10% sugar + ascorbic acid + salt + vanilla.
- Different notations on the same row indicate significantly different effects with a p-value <0.05 at the α=0.05

During the 0 to 48 h fermentation, the pH of Formulas A, B, C, D, and E of *Aloe vera* juice decreased, indicating an increase in acidity due to the breakdown of the juice's substrate by *L. rhamnosus* into lactic acid. This is a common outcome of the fermentation process. The pH value of *Aloe vera* juice is inversely related to its total acid content. The higher acid values correspond to lower pH values. The reduction in pH observed during the fermentation of *Aloe vera* juice can be ascribed to the activity of lactic acid bacteria, specifically through glycolysis. This process converts the glucose from the juice into lactic acid, lowering the pH. The resulting low pH value can inhibit the proliferation of pathogenic bacteria [10].

In Formula F, pH was observed to slightly increase from 24 to 48 h of fermentation, which amounted to 0.06. It may be attributed to the depletion of substrates that could be converted into lactic acid [10]. This decrease in acidity leads to a subsequent increase in pH. Additionally, fluctuating *L. rhamnosus* during fermentation in Formula F may have impacted the pH value, indicating suboptimal growth at 24 h of fermentation.

3.3 Effect of *Aloe vera* juice on growth of *Lacticaseibacillus rhamnosus* ATCC 9595

The growth pattern of *L. rhamnosus* ATCC 9595 during the fermentation of *Aloe vera* juice exhibits variability, attributed to its capacity to utilize the substrate sources within the juice (Table 3). This study found that *L. rhamnosus* growth in Formulas A, B, C, D, and E

increases with longer fermentation. The ratio of sugar and honey was the same in formulas A and D, but formula D also included salt and vanilla, while formula E did not.

The growth of *L. rhamnosus* in Formula D during 48 h fermentation was 7.52 Log CFU/mL, slightly higher than in Formula E (7.38 Log CFU/mL). Similarly, the growth of *L. rhamnosus* in Formula C during the 48 h fermentation was 7.4 Log CFU/mL, slightly higher than in Formula B (7.3 Log CFU/mL). In formula F, the growth of *L. rhamnosus* decreased after 24 h of fermentation and then increased after 48 h. This fluctuating growth pattern may be due to LAB adapting to the environment. It was proposed that the acidic nature of the *Aloe vera* juice resulted from the incorporation of ascorbic acid.

Table 3. Effect of *Aloe vera* juice formulation and fermentation time on the number of *Lacticaseibacillus rhamnosus* ATCC 9595 colonies

Fermentation Time (h)	***Aloe vera* juice formulation**					
	Formula A	**Formula B**	**Formula C**	**Formula D**	**Formula E**	**Formula F**
	Total of LAB (CFU/mL)					
0	4.57 ± 0.08^{a}	4.68 ± 0.03^{ab}	4.63 ± 0.01^{ab}	4.96 ± 0.19^{b}	4.94 ± 0.19^{b}	4.70 ± 0.01^{b}
24	6.21 ± 0.06^{b}	6.38 ± 0.13^{b}	6.44 ± 0.22^{b}	6.36 ± 0.05^{b}	6.63 ± 0.15^{b}	3.40 ± 0.12^{a}
48	7.28 ± 0.03^{b}	7.30 ± 0.02^{b}	7.40 ± 0.01^{c}	7.52 ± 0.01^{d}	7.38 ± 0.03^{c}	3.54 ± 0.09^{a}

Notes:

- **Formula A**: 10% honey + 5% sugar; **Formula B**: 5% honey + 5% sugar; **Formula C**: 10% honey; **Formula D**: 10% honey + 5% sugar + salt + vanilla; **Formula E**: 2.5% honey + 10% sugar; **Formula F**: 10% sugar + ascorbic acid + salt + vanilla.
- Different notations on the same row indicate significantly different effects with a p-value <0.05 at the α=0.05

As *Aloe vera* juice contains minimal carbohydrates, additional substrates such as sugar and honey are necessary to promote the growth of *L. rhamnosus* [11]. These added substrates significantly affect the *L. rhamnosus* growth during the fermentation. *L. rhamnosus* obtains its energy from sugar and honey. Sugar contains sucrose, while honey contains sucrose, fructose, glucose, oligosaccharides, minerals, phenolic compounds, enzymes, and water. High sugar concentrations in *Aloe vera* juice fermentation can change the environmental conditions, leading to cell dehydration and shrinkage due to plasmolysis. Sugar concentrations that are too high cause LAB cells to undergo lyse (rupture) due to differences in osmotic pressure (solute concentration on both sides of the cell membrane) [12]. The 48 h growth of LAB in Formula F fermentation was not optimal.

The presence of salt, vanilla, and ascorbic acid in *Aloe vera* juice may also influence the growth of *L. rhamnosus*. In addition to the flavor of *Aloe vera* juice, salt can make out water and nutrients from *Aloe vera*. Salt can remove water from fruit that contains dissolved solids such as proteins, carbohydrates, and minerals, which are important for the growth of LAB [13]. The addition of vanilla to *Aloe vera* juice aims to add flavor. In *Aloe vera* juice, a formula that uses the addition of salt and vanilla is in formulas D and F. Ascorbic acid is a compound with the formula $C_6H_8O_6$ with a 6-carbon lactone ring structure. The synergistic effects of ascorbic acid and lactic acid in *Aloe vera* juice demonstrate antimicrobial activity against *Listeria monocytogenes* and *Escherichia coli* [14].

3.4 Effect of *Aloe vera* juice fermentation on total lactic acid

Table 4 shows the *Aloe vera* var. *chinensis* juice formulation and fermentation time affect the total lactic acid produced. Fermentation is a biochemical process during which bacteria metabolize carbohydrates, resulting in the production of acids. In this study, total lactic acid

was measured using the titration method. This method can determine the percentage of total lactic acid concentration produced.

Table 4. Effect of concentration of *Aloe vera* var. *chinensis* juice formulation and fermentation time on the total lactic acid produced

Fermentation Time (h)	*Aloe vera* juice formulation					
	Formula A	**Formula B**	**Formula C**	**Formula D**	**Formula E**	**Formula F**
	Total Lactic Acid (%)					
0	0.25 ± 0.05^{a}	0.27 ± 0.08^{ab}	0.26 ± 0.02^{ab}	0.26 ± 0.02^{b}	0.26 ± 0.07^{b}	0.23 ± 0.01^{ab}
24	0.45 ± 0.08^{b}	0.37 ± 0.01^{b}	0.39 ± 0.02^{b}	0.46 ± 0.08^{b}	0.50 ± 0.05^{b}	0.46 ± 0.13^{a}
48	0.66 ± 0.13^{b}	0.61 ± 0.10^{b}	0.69 ± 0.05^{c}	0.64 ± 0.02^{d}	0.63 ± 0.00^{c}	0.49 ± 0.04^{a}

Notes:

- **Formula A**: 10% honey + 5% sugar; **Formula B**: 5% honey + 5% sugar; **Formula C**: 10% honey; **Formula D**: 10% honey + 5% sugar + salt + vanilla; **Formula E**: 2.5% honey + 10% sugar; **Formula F**: 10% sugar + ascorbic acid + salt + vanilla.
- Different notations on the same row indicate significantly different effects with a p-value <0.05 at the α=0.05

According to the data, the total acid content of fermented *Aloe vera* juice increased with fermentation time. This observation aligns with prior research that indicates a correlation between longer fermentation periods and elevated total acid concentrations in fermented beverages. The testing of total lactic acid revealed that the content in *Aloe vera* juice fermented for 48 h ranged between 0.49% and 0.69%. The rise in acidity of *Aloe vera* juice can be ascribed to the heightened activity of lactic acid bacteria (LAB), which metabolize glucose into lactic acid [4]. Lactic acid bacteria as a natural antibacterial component produced by LAB. Foodborne pathogens such as *Klebsiella pneumonia*, *Shigella sonnei*, *Staphylococcus epidermidis*, *Bacillus cereus*, and *Candida albicans* can all be inhibited in their growth by LAB [15].

4 Conclusion

The growth parameters of *Lacticaseibacillus rhamnosus* ATCC 9595 exhibited variability when cultured in various formulations of *Aloe vera* juice supplemented with stingless bee honey. As the fermentation time increased, the Optical Density, total acid, and number of *L. rhamnosus* ATCC 9595 also increased, while the pH value declined, *L. rhamnosus* growth was inhibited. The highest *L. rhamnosus* ATCC 9595 growth rate was observed in Formula D (10% honey + 5% sugar + salt + vanilla) with 7.52 log10 CFU/mL total lactic acid bacteria, 0.23 OD_{600} nm, 0.64% total acid, and a pH of 3.70

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Conflict of interest

All authors hereby declare that no conflict of interest exists.

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