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Effectiveness of Tea Leaf (*Camellia sinensis*) Liquid Smoke as an Antiseptic

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Abstract

Microbes are often the cause of infectious diseases; one method of prevention is to use antiseptics. Excessive use of antiseptics can cause mild to severe side effects, so an alternative antiseptic is needed. Liquid smoke is an alternative raw material to produce antiseptics because its main component can be used as an inhibitor of microbial growth. This study aims to determine the effectiveness of tea-leaf liquid smoke as an antiseptic against microbial growth in vivo and in vitro. The method for producing liquid smoke via pyrolysis involves distilling grade 3 liquid smoke to generate grade 1 and grade 2 liquid smoke. The in vitro test uses a microbial growth inhibition test with grade 1 and 2 liquid smoke concentrations of 35%, 50%, and 75%. The microbes used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, and *Aspergillus flavus* ATCC 9643. The in vivo test uses the swab test method on the palm of the hand. The results of the in vitro test showed that the largest inhibition zone of tea leaf liquid smoke was obtained using grade 2 liquid smoke with a concentration of 75%. The results of the in vivo test showed that the effectiveness of tea leaf liquid smoke was 80% against bacteria and 85% against fungi. Based on the results of the study, it can be concluded that tea leaf liquid smoke (*Camellia sinensis*) has potential as an antiseptic.

Keywords: liquid smoke, tea leaves, antiseptic, microbes.

1. Introduction

The most appropriate process to prevent the spread of bacteria, fungi, and viruses is to wash your hands using soap and running water. Get used to using masks or washing hands with soap or antiseptic hand sanitizer gel preparations (hand sanitizer) after every activity [1, 2]. Antiseptic is a hand sanitizer product with the main content of alcohol, which can kill or inhibit the growth of microorganisms [3]. Excessive use of alcohol can result in increased skin permeability by eliminating lipids in the stratum corneum layer, which can trigger Systematic Contact Dermatitis (SCD) [4].

The use of antiseptic raw materials in general is still dominated by using alcohol, so alternative raw materials are needed that can be used as antiseptics, especially hand sanitizers other than alcohol. One of the materials under consideration is liquid smoke. In general, liquid smoke is the result of condensation or condensation of vapour from biomass combustion through a pyrolysis process. This combustion is carried out either indirectly or directly from materials that contain a lot of carbon and other compounds [5]. The selection of liquid smoke as an antiseptic raw material comes from previously conducted research. It is known that liquid smoke from bamboo stems [6], coffee skin [7], bamboo leaves [8], pine fruit [9], and palm oil [10] can be an antiseptic.

The effectiveness of liquid smoke as an antiseptic is because liquid smoke contains antibacterial compounds, namely phenols and acids. These phenol and acid fractions can inhibit the growth of microorganisms [11]. Considering some research results that show that the chemical content, especially phenol, in liquid smoke functions as a disinfectant, it is possible to conduct further research on the utilization of tea-leaf liquid smoke as an antiseptic. Tea leaf (*Camellia sinensis*) is one of the natural ingredients that can be used as an antiseptic because it has phenol compounds that can dam- age mycobacterial cell membranes [12]. Therefore, this study aims to determine the effectiveness of tea-leaf liquid smoke as an antiseptic raw material.

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2. Methodology

2.1. Preparation of Tea Leaf Liquid Smoke

The tea leaves utilized as the primary ingredient for liquid smoke are the trimmings discarded from five-year-old tea plants sourced from the Indonesia Research Institute for Tea and Cinchona. The raw material in the form of dried tea leaves is weighed as much as 3 kg, then put into a pyrolysis furnace made of stainless steel equipped with a cylindrical pyrolysis tube (retort) with a height of about 60 cm and a diameter of 40 cm equipped with 2 thermocouples, an electric heating device, a condenser, and a distillate collection flask. Pyrolysis was carried out at 250–450 °C for 7-8 hours. The pyrolysis process is carried out in a special furnace that does not allow the involvement of oxygen in the pyrolysis process [13]. The initial pyrolysis liquid smoke (grade 3) still contains tar, acidity, and odor. Because the smoke is highly unpleasant, it must be filtered using the distillation procedure to generate grade 1 and grade 2 liquid smoke. Grade 2 liquid smoke is obtained by distilling grade 3 liquid smoke once, while grade 1 liquid smoke is obtained by distilling grade 3 liquid smoke twice [10].

2.2. In Vitro Testing

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, and *Aspergillus flavus* ATCC 9643 were investigated in vitro for their antibacterial effectiveness. The test microbes used were identified by gram staining for bacteria and methyl blue staining for fungi. Microbial identification employs colony morphology, cell morphology, and gram staining techniques to verify the absence of contamination in the utilized microbes. To determine the ability of tea leaf liquid smoke to inhibit bacteria using the disc paper method while inhibiting fungi using the well method with a 6 mm diameter disc paper [14]. The work was done under sterile conditions to avoid contamination from other microbes.

The inhibition test used grade 1 and 2 tea leaf liquid smoke with concentrations of 35%, 50%, and 75%. Observations on the media were made after 24 hours of incubation. The diameter of the inhibition zone, or clear zone, that appears around the disc paper is an indication of the microbial sensitivity to the antimicrobial material used as test material and is expressed by the diameter of the inhibition zone. The zone of inhibition formed around the disc paper was measured by vertical diameter and horizontal diameter in mm units using a caliper [15].

Table 1. Microbial inhibition response based on clear zone diameter [16]

Diameter of clear zone (mm)	Microbial inhibition response
< 5	Weaker
5 – 10	Medium
10 – 20	Strong
> 20	Very strong

2.3. In Vivo Testing

In vivo tests were carried out by administering liquid smoke to the palms of respondents according to the grade and optimal concentration of the in vitro test results [8]. The number of research samples was 24 respondents, who were divided into two groups of 12 people each for the administration of liquid smoke as a test material and 70% alcohol as a control. Swabbing of respondents' palms was carried out before and after antiseptic application, and then the results of the swab suspension were planted on nutrient agar (NA) medium and potato dextrose agar (PDA) medium in a pour plate to calculate the effectiveness of microbial reduction. The questionnaire parameters were liking for color, aroma, dryness effect, itching effect, and burning [10].

2.4. Data Analysis

Microsoft Excel organizes and evaluates data in a table format, including measurements of the inhibition zone, calculations of colony numbers, and questionnaire evaluations of tea-leaf liquid smoke as an antiseptic.

3. Result and Discussion

3.1. Physical Properties of Liquid Smoke

The yield of liquid smoke produced from the pyrolysis process of tea leaves using a reactor with a capacity of 3 kg is 46.28%. The pyrolysis apparatus, the length of the combustion, the kind of raw material, and the initial moisture content of the raw tea leaf materials all have an impact on the quality and yield of liquid smoke. The liquid smoke used in this study is distilled liquid smoke at a temperature of 100–190°C. This distillation aims to separate the carcinogenic tar con- tent in the liquid smoke. The physical properties of tea-leaf liquid smoke can be seen in Table 2, which include color, aroma, and pH.

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Table 2. Physical properties of liquid smoke

Physical properties	Liquid smoke type		
	Grade 3	*Grade 2*	*Grade 1*
Color	Brownish-yellow	Clear yellow	Translucent yellow
Aroma	Strong smoke odor	Smells lighter of smoke	Smells lighter of smoke
pH	5,4	2,8	2,7

The distilled liquid smoke (grade 2 and grade 1) has a lighter color than the liquid smoke before distillation (grade 3). This happens because grade 3 liquid smoke still contains impurities such as tar, while grade 2 and 1 liquid smoke have reduced tar and benzopyrene content. The aroma produced in liquid smoke before and after distillation remains the same, which has a distinctive odor [17]. The purification of liquid smoke to produce grade 2 liquid smoke is done by distillation at a temperature of 130–145 °C for 6 hours. At this stage, distillation is carried out to increase the acquisition of acetic acid and ensure that there are no more impurities, such as tar and benzopyrene. The liquid smoke produced in this grade is clear yellow, but the odor of liquid smoke in grade 2 is lighter than in grade 3. The pH value in grade 2 also increased to 2.8 due to the increase in acid content in grade 2 liquid smoke.

Grade 1 liquid smoke is obtained by distilling the pyrolyzed liquid smoke at 170-190°C for 4 hours. The purpose of this stage of distillation is to increase the recovery of acid and phenol content in liquid smoke and ensure that there are no impurity compounds in this grade so that it is safe to use (18). The grade 1 liquid smoke produced has a clearer color than the other grades and has a lighter smoke aroma as well when compared to the smoke from the other grades' liquids. The pH value of this grade is 2.7, indicating that the acid content and phenol content of the liquid smoke are increasing.

3.2. Identification of Test Microbes

Microbial identification was carried out on *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, and *Aspergillus flavus* ATCC 9643 macroscopically and microscopically. The results of macroscopic and microscopic observations can be seen in Table 3 below.

Table 3. Results of macroscopic and microscopic observations of test microbes

No	Microbe type	Morphology of colonies	Cell morphology	Staining result
1.	*Escherichia coli* ATCC 25922	Round, smooth, convex, milky white, flat	Stem, monobacilli	Colored red
2.	*Staphylococcus aureus* ATCC 25923	Round, smooth, convex, milky white, flat	Rod, monobacillus	Purple-colored
3.	*Candida albicans* ATCC 10231	Round, white in color, convex, soft, flat	ound, purple, has pseudohyphae and blastophores	Purple-colored
4.	*Aspergillus flavus* ATCC 9643	Round filamentous, white (young) and greenish (old), convex	Spherical with short conidia stalks	Blue colored

Based on the results in Table 3, it appears that *E. coli* ATCC 25922 is a gram-negative bacterium because it has a thin cell wall that is between two layers of the cell membrane. *S. aureus* is a gram-positive bacterium because it has a thick cell wall, a cell membrane layer, and no outer membrane [19]. Gram staining results show that *S. aureus* ATCC 25923 is purple because the crystal violet dye is retained in alcohol, as opposed to *E. coli* ATCC 25922, which is red because the crystal violet dye fades in alcohol and allows the safranin dye to be absorbed [20].

The results of macroscopic observations of *C. albicans* ATCC 10231 have round colonies, white in color with smooth textured colonies, and convex elevations, this is because *C. albicans* is a yeast [21]. The results of gram staining of *C. albicans* ATCC 10231 with oval-shaped, purple-colored colonies indicate a gram-positive bacterial group. These gram-positive

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bacteria are able to retain crystal violet dye. The macroscopic observation of *A. flavus* ATCC 9643 is a round-shaped colony, with a colony size of 0.5 - 2 cm, with a white color when incubated for 24 hours and a greenish color when incubated for 48 hours, convex elevation and has a velvety texture. Meanwhile, microscopic results with methyl blue staining, the results obtained round spores with short conidia stalks. Methyl blue staining in fungi will distinguish which yeast cells are still alive and which are dead because methyl blue will give col- or when oxidation occurs. This reduction is what causes the color to disappear and oxidation will cause a blue color [22].

The data obtained from microbial identification, which includes the examination of colony morphology, cell morphology, and gram staining, confirms that all microbial cultures are free from impurities and contaminants. This ensures that the selected treatment will produce optimal outcomes for the specific microorganism being targeted.

3.3. In Vitro Test

The presence of a clean zone around the disk or well indicates the outcome of the antimicrobial activity test. The results of in vitro testing of tea-leaf liquid smoke can be seen in Table 4. Based on Table 4, it can be seen that the diameter of the clear zone formed is getting bigger as the concentration increases. This means that the greater the concentration, the greater the diameter of the clear zone produced. Lala [23] stated that the higher the concentration of liquid smoke added to the product, the lower the acid value, or pH. The higher the concentration of ingredients, the higher the content of antibacterial active substances. The addition of antibacterial compound concentrations is thought to increase the penetration of antibacterial compounds into microbial cells, which will damage the cell's metabolic system and can result in cell death.

Table 4. Results of measuring the diameter of the liquid smoke inhibition zone against test microbes

Liquid smoke type	Concentration (%)	Test microbial species and inhibition zone diameter (mm)			
		Escherichia coli ATCC 25922 (mm)	*Staphylococcus aureus* ATCC 25923 (mm)	*Candida albicans* ATCC 10231 (mm)	*Aspergillus flavus* ATCC 9643 (mm)
Grade 1	35	1,3 (L)	2,3 (L)	0 (L)	9,8 (S)
	50	1,8 (L)	3,0 (L)	1,0 (L)	16,1 (K)
	75	3,0 (L)	5,8 (S)	1,5 (L)	17 (K)
Grade 2	35	1,3 (L)	4,8 (L)	0,8 (L)	16,1 (K)
	50	2,0 (L)	5,8 (S)	1,5 (L)	23,3 (SK)
	75	3,5 (L)	9,0 (S)	3,8 (L)	25,6 (SK)

Based on the inhibition response criteria according to Andriani et al. [16], the grade 1 and 2 tea leaf liquid smoke against *Escherichia coli* ATCC 25922 has an inhibition response that is classified as weak (<5 mm), against S*taphylococcus aureus* ATCC 25923 classified as weak to moderate (5-10 mm), against *Candida albicans* ATCC 10231 classified as weak, and against *Aspergillus flavus* ATCC 9643 classified as moderate to very strong (>20 mm).

Liquid smoke has inhibition against microbial growth, which can be caused by the content of phenol and acetic acid, which can function as antibacterial. The mechanism of phenol compounds and their derivatives as disinfectants is to damage bacterial cell membranes causing leakage of organic ions, nucleotides, coenzymes, and amino acids out of bacterial cells and prevent essential substances from entering the cell. This causes disruption of the bacterial growth system and can cause cell death. Acetic acid is one of the organic acids commonly used as an antibacterial because it is able to reduce pH, causing instability in the cell membrane [17].

Phenol compounds and their derivatives easily form protein complexes through hydrogen bonds. At low levels, protein-phenol complexes will form weak bonds and immediately decompose into cells, so that protein denaturation occurs in the bacterial cell wall. At high levels, the presence of phenolic compounds can cause bacterial cells to lyse [24]. Acetic acid in liquid smoke also has an important role as an antibacterial because it has an effect on destabilizing the function and structure of components in bacterial cells [25].

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3.4. In Vivo Test

The test of the effectiveness of tea-leaf liquid smoke as an antiseptic in vivo was carried out by applying tea-leaf liquid smoke to the palms of the respondents. This in vivo test was conducted to support the results of the liquid smoke effectiveness test on microbial growth in vitro. The research respondents were 24 people consisting of 2 groups, namely respondent group A (swab the respondent's palm before and after using 70% alcohol) as many as 12 respondents and respondent group B (swab the respondent's palm before and after using 75% tea leaf liquid smoke) as many as 12 respondents, in each group evenly distributed male and female respondents.

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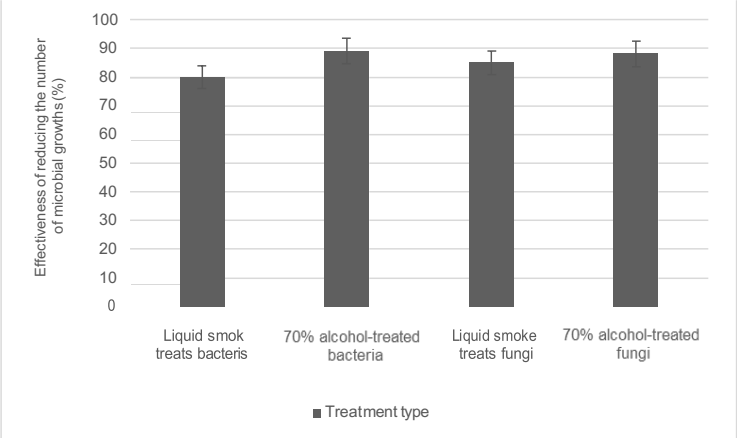

Figure 1. Effectiveness of Tea Leaf Liquid Smoke on Microbial Growth

In order to calculate the percentage of the efficacy of tea leaf liquid smoke in preventing bacterial and fungal growth, the number of bacterial and fungal colonies decreased during the in vivo effectiveness test of tea leaf liquid smoke as an antiseptic. The results in Figure 1 show that the 75% tea leaf liquid smoke treatment decreased the number of bacterial colonies by 80% and decreased the number of fungal colonies by 85%, while the 70% alcohol treatment showed a decrease in the number of bacterial colonies by 89% and fungi by 87.2%.

The decrease in the amount of microbial growth with tea-leaf liquid smoke indicates that tea-leaf liquid smoke has the ability to inhibit microbial growth. The results of this study are in accordance with research conducted by Oktarina et al. [26] that showed that liquid smoke containing phenol compounds and acetic acid effectively inhibits the growth of *Escherichia coli*. Compound phenol in liquid smoke can form phenol-protein complex bonds that cause protein coagulation so that the cell membrane is lysed. According to Erlytasari et al. [27], phenol compounds contained in liquid smoke can bind to bacterial proteins through hydrogen bonds, causing the protein structure to be damaged.

The decrease in the number of fungal colonies using 75% tea-leaf liquid smoke was lower compared to 70% alcohol. This shows that 70% alcohol is still superior in inhibiting the growth of fungal colonies compared to 75% tea-leaf liquid smoke. According to research conducted by Putri et al. [28], antiseptics containing 70% alcohol content will be more sensitive and work faster to precipitate proteins and lipid membranes in microbes. In addition, 70% alcohol is considered superior to inhibiting microbial growth because 70% alcohol is obtained from the results of purification through several stages, including evaporation, distillation, dehydration, and recryption stages known as the refinery process (29).

3.5. Questionnaire Results

In this study, questionnaire data was also collected from respondents regarding their level of preference for the physical characteristics of tea-leaf liquid smoke. This evaluation was performed to see if the direct application of tea-leaf liquid smoke to the respondent's hand had any effects. In Figure 2, it can be seen that overall respondents liked the color of tea leaf liquid smoke (92%), liked the aroma of tea leaf liquid smoke (75%), liked the tea leaf liquid smoke product because it did not give the effect of dryness on the hands (92%), and liked the bamboo stem liquid smoke product because it did not give the effect of a burning sensation (100%).

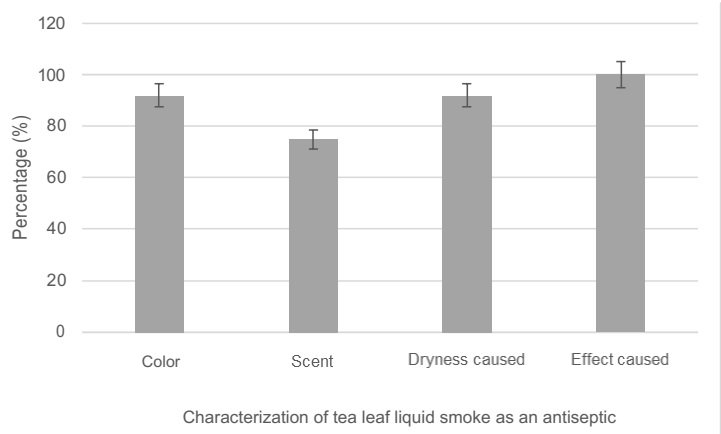

Figure 2. Questionnaire for Respondents' Assessment of Tea Leaf Liquid Smoke

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Based on Figure 2, 92% of respondents liked the color of the 75% concentration of tea leaf liquid smoke with yellowish brown color criteria. Aznuri et al. [30] stated that the level of preference for the color of the antiseptic gel is green and bright because it more appealing compared to dark colors that are less attractive to the public. In this study, tea-leaf liquid smoke added 1% orange perfume and increased the respondents' level of preference for the antiseptic aroma of tea-leaf liquid smoke by 75%. This is in accordance with the research of Rindarwati and Noviyanto [31]. Regarding consumer interest in the use of antiseptics, people like antiseptics that are attractive and refreshing. This is also similar to research conducted by Handayani et al. [32] regarding the characteristics of antiseptic ingredients that people like, namely that people prefer antiseptics that can provide an attractive aroma, such as the scent of lemon or others. Rindarwati and Noviyanto [31] also stated that the comfort factor of using antiseptic products must prioritize color, texture, and aroma and not cause side effects on the skin.

4. Conclusion

The tea leaf liquid smoke exhibited the most significant inhibition zone when grade 2 liquid smoke with a concentration of 75% was used. Liquid smoke from the pyrolysis of tea leaves (*Camellia sinensis*) as an antiseptic raw material is effectively used to inhibit the growth of bacteria and fungi.

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The Effect of Photoperiod on the Growth of *Stevia rebaudiana* In Vitro

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Abstract

Stevia rebaudiana, the source of non-caloric natural sweeteners in the form of steviol glycosides, is a plant with a poor germination rate. Therefore, micropropagation is a potential alternative method to propagate the plants in a large number. Light is an important factor for photosynthesis, so changing the intensity, quality, and duration of lighting can affect plant growth. Photoperiod, the duration of light within 24 hour period, has been known to influence the growth of S. *rebaudiana* grown in ex vitro conditions. The purpose of this study is to investigate the effect of various photoperiod (8, 12, and 16 hours) on the growth of S. *rebaudiana* in vitro. The node segments from ex vitro grown S. *rebaudiana* plants were cultured on solid MS media supplemented with 1.13 mg/L BA and 0.35 mg/L IAA. The shoots were rooted on solid half-strength MS media containing 0.1 mg/L IAA. For acclimatization, the rooted shoots were grown on a mixture of fertile soil, burnt rice husk, cocopeat, and manure. The photoperiod treatment was applied from the beginning to the end of the experiment. Our results showed that the highest shoot length was found under 16 hour photoperiod. On the other hand, root number and root length were not affected by photoperiod. Additionally, a 16 hour photoperiod increased shoot length (5.9 cm) compared to a 12-hour (3.48 cm) and 8-hour photoperiod (3.08 cm) after 5 weeks of acclimatization. A 16 hour photoperiod also produced highest total leaf fresh weight (0.2 g). However, different photoperiods did not significantly affect leaf number and leaf area. In conclusion, 16 hour photoperiod is the best condition for *S. rebaudiana* micropropagation.

Keywords: *Stevia rebaudiana*, photoperiod, growth

1. Introduction

The increasing interest in non-sugar sweeteners in recent years has led to the growing popularity of stevia as a sweetener. Stevia is derived from the leaves of *Stevia rebaudiana*, a flowering plant native to Paraguay and belonging to the Asteraceae family [1]. The sweetness of stevia is attributed to a group of diterpene compounds called steviol glycosides, including stevioside, rebaudioside A, rebaudioside M, and others [2]. Steviol glycosides are calorie-free, up to 400 times sweeter than sucrose, and have positive health effects such as decreasing insulin levels and reducing inflammation [3-5].

S. rebaudiana is not extensively cultivated from seeds due to their low germination rate [6]. Instead, *S. rebaudiana* are commonly propagated using stem cuttings. However, it produces limited number of new individual plants. Another method employed for the propagation of *S. rebaudiana* is micropropagation. This method involves growing small parts from a plant into new individuals in a sterile nutrient medium, often supplemented with plant growth regulators, which enables the generation of a larger quantity of plants in a relatively short time [7].

Photoperiod is an important light factor influencing the growth and development of *S. rebaudiana*, in addition to light quality and intensity [8-10]. It is well established that extending the photoperiod can lengthen the photosynthesis period and enhance dry matter accumulation in numerous plant species [11]. Studies on the effect of photoperiod on the growth of *S. rebaudiana* in ex vitro conditions have been conducted previously [8, 12-13]. However, the impact of photoperiod on the in vitro growth of *S. rebaudiana* has not been extensively explored. Therefore, the aim of this study is to evaluate the

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influence of photoperiod on the growth *S. rebaudiana* shoot and root in vitro, as well as the growth of in vitro grown *S. rebaudiana* after acclimatization.

2. Methodology

2.1. Explants Source

The source of explants used in this study were 3-week-old *S. rebaudiana* BL clone obtained from the Biotechnology and Bioindustry Research Center, Bogor, Indonesia. Stem node segments were used as explants for in vitro shoot initiation.

2.2. Treatment and Growth Conditions

This study employed three treatments: 8-hour, 12-hour, and 16-hour photoperiods. The light source used was fluorescent lamps, with an intensity of 48 W/m^2. The room temperature was maintained at 23 ± 2°C. These treatment and growth conditions were applied during the shoot initiation, rooting, and acclimatization phases.

2.3. Explant Sterilization

The cut node segments were washed with soap in running water for 30 minutes, then immersed in a 0.1% fungicide solution for 5 minutes. Under aseptic conditions in a laminar airflow cabinet, the explants were immersed in 70% alcohol for 1 minute, then washed in a 0.79% NaClO solution with two drops of Tween 20 for 10 minutes, and finally rinsed three times with sterile distilled water. The sterilized node segments were cut into 1-2 cm sizes.

2.4. Effect of Photoperiod on In Vitro Shoot Initiation

The node segments were cultured on MS medium (Murashige and Skoog, 1962) containing 1.13 mg/L benzyladenine (BA), 0.35 mg/L indole-3-acetic acid (IAA), 30 g/L sucrose, and 8.5 g/L agar. The medium's pH was adjusted to 5.8 ± 0.2. The medium was autoclaved at 121 °C and 124 kPa for 15 minutes before use. After four weeks, the shoot length, number of nodes per shoot, and number of branches per shoot were measured.

2.5. Effect of Photoperiod on In Vitro Rooting

The 1-3 cm shoots obtained from the initiation phase were cut and cultured on half-strength MS medium containing 0.1 mg/L IAA, 30 g/L sucrose, and 8.5 g/L agar. The medium's pH was adjusted to 5.8 ± 0.2. The medium was autoclaved at 121 °C and 124 kPa for 15 minutes before use. After four weeks, the number of roots per shoot and root length were measured.

2.6. Effect of photoperiod on acclimatization

The in vitro shoots with developed roots were rinsed with sterile distilled water and planted in a growth medium composed of a mixture of fertile soil, burnt rice husk, cocopeat, and manure. Liquid NPK fertilizer and 0.1% fungicide were sprayed at the beginning of acclimatization. Watering was done initially and every two weeks. After five weeks, the change in shoot length, change in nodes number, total area as well as total fresh weight of the leaves that emerged during acclimatization per shoot were measured.

2.7. Statistical analysis

The experiment was conducted using a completely randomized design (CRD). The biological replicates used for in vitro shoot initiation, in vitro rooting, and acclimatization were 14, 11, and 8 replicates, respectively. Significant differences were analyzed using one-way ANOVA, followed by Tukey's HSD test ($p < 0.05$) using IBM Statistics SPSS 25 software.

3. Result and Discussion

3.1. Effect of Photoperiod on In Vitro Shoot Initiation

Ideal in vitro shoot growth is important for *S. rebaudiana* micropropagation because a higher number of nodes or branches produced leads to a higher rate of plantlet multiplication. Regulating growth conditions such as lighting duration is one way to obtain optimal in vitro shoot growth. *S. rebaudiana* explants treated with a 16 hours photoperiod for four weeks resulted in taller shoots (5.93 cm) compared to the 8-hour (3.08 cm) and 12-hour (3.48 cm) photoperiods (Figure 1a). However, photoperiod did not have a significant effect on the number of nodes per shoot and the number of branches per shoot (Figure 1b and c).

These results support the findings of the positive effect of long photoperiod on plant's growth [14-17]. It has been demonstrated that longer photoperiod increased the daily availability of light for photosynthesis which improved the growth performance of plants [15,18]. This might explain the increase of in vitro *S. rebaudiana* shoot's length in our result.

3.2. Effect of Photoperiod on In Vitro Rooting

In vitro rooting is a crucial stage prior to acclimatization. Plantlets with roots usually have a greater chance of survival during acclimatization. In this study, photoperiod does not affect the number of roots per shoot and root length. The average number of roots per shoot for the 8-hour, 12-hour, and 16-hour photoperiods were 2.36, 2.55, and 2.64, respectively (Figure 2a). Meanwhile, the average root length for the 8-hour, 12-hour, and 16-hour lighting treatments were 1.13 cm, 0.84 cm, and 0.92 cm, respectively (Figure 2b).

There have been several reports suggesting that longer photoperiod can also increase root growth by increasing photosynthesis duration [19-21]. However, our research did not yield the same results as there was no impact of photoperiod on rooting. It probably was not due to the sample size, as our sample size was not too small (30 plants per treatment). The photosynthetic products from the shoots reaching the roots might be not substantial enough to make a significant difference.

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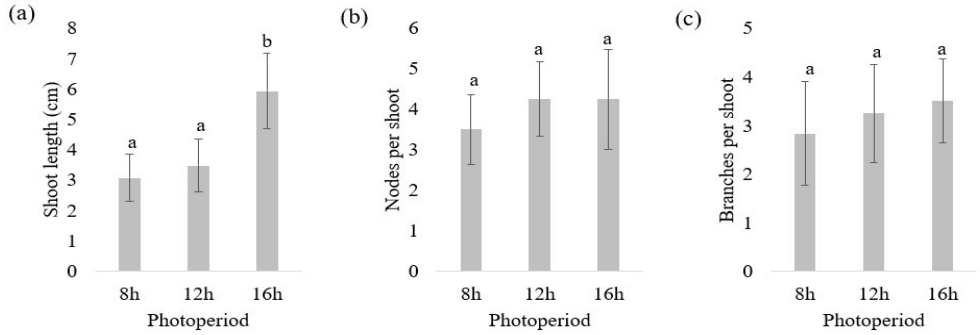

Figure 1. The effect of different photoperiod treatment on (a) in vitro shoot length, (b) the number of nodes per in vitro shoot, and (c) the number of branches per in vitro shoot after four weeks. Data represents mean ± standard deviation (n = 14). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

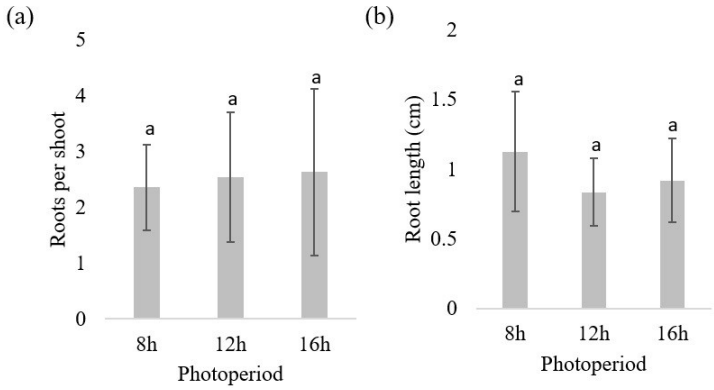

Figure 2. The effect of different photoperiod treatment on (a) the number of in vitro roots per shoot and (b) in vitro root length after four weeks. Data represents mean ± standard deviation (n = 11). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

3.3. Effect of Photoperiod on Acclimatization

Acclimatization is a stage carried out before the micropropagated plantlets are exposed to ex vitro conditions. After 5 weeks of acclimatization, the plantlets become more vigorous in all treatments (Figure 3). Photoperiod did not significantly affect the number of nodes (Figure 4a). However, there was a trend where longer photoperiods resulted in higher shoot length (Figure 4b), which might be due to increase in photosynthesis duration [15,18]. The number of nodes and total leaf area per shoot were also not influenced by photoperiod (Figure 4c-d). The highest total leaf fresh weight was obtained with a 16-hour photoperiod treatment (0.2 g) (Figure 4e), consistent with previous findings. This also might be due to increase in photosynthesis duration, which in turn causes the accumulation of biomass in the leaves [22-23]. Thus, the most effective photoperiod for the growth enhancement of *S. rebaudiana* plantlets during the acclimatization process is 16 hours.

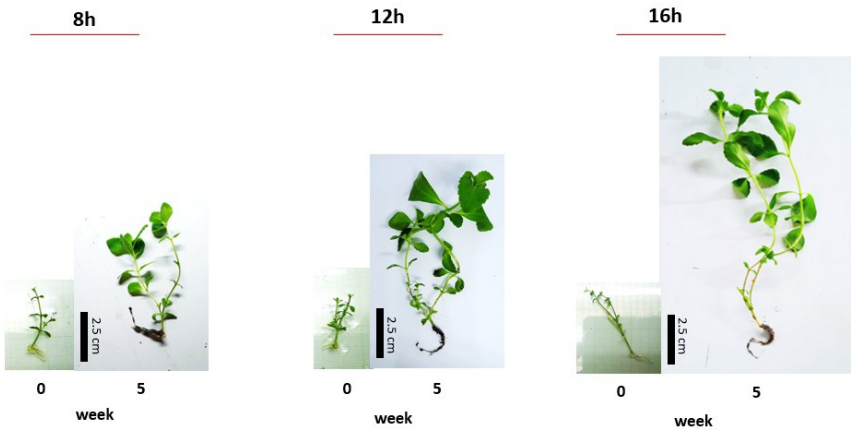

Figure 3. *S. rebaudiana* planlets morphology before and after five weeks acclimatization under all photoperiod treatments.

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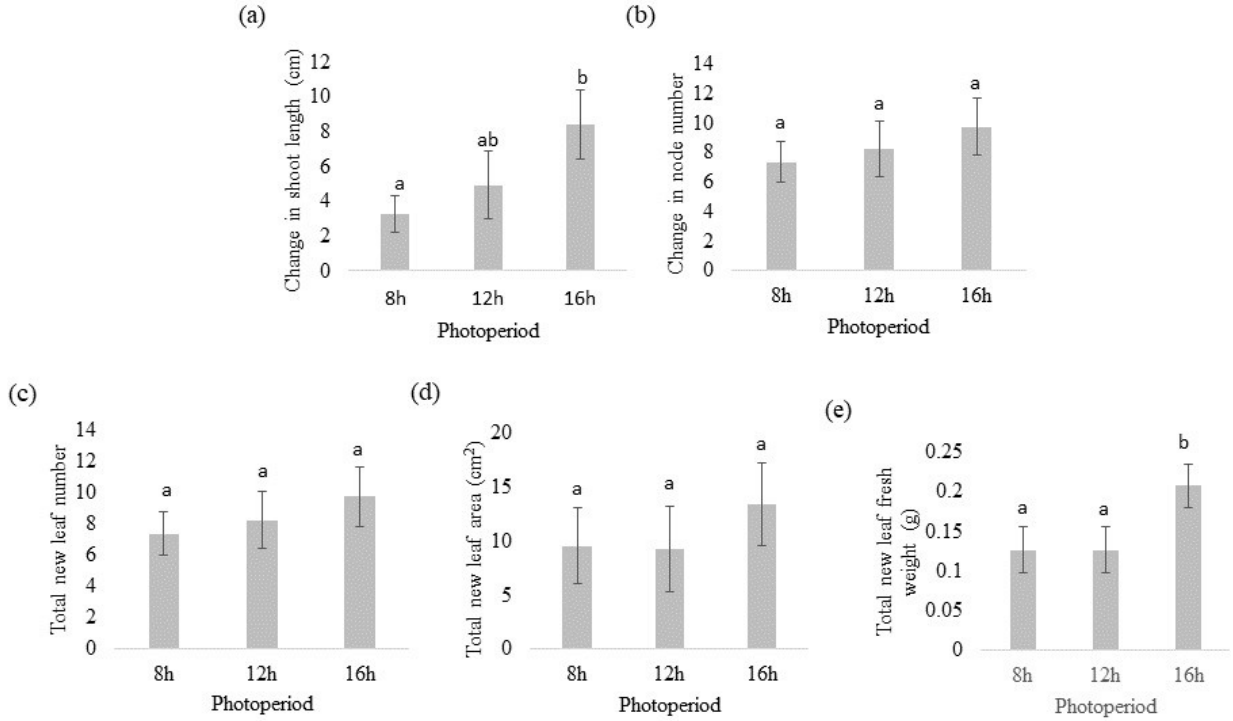

Figure 4. The effect of different photoperiod treatment on (a) the change in shoot length, (b) the change in node number, (c) total number of the new leaves, (d) total area of the new leaves, and (e) total fresh weight of the new leaves of *S. rebaudiana* plantlets after five weeks acclimatization. Data represents mean ± standard deviation (n = 8). Different letters indicate significant differences using Tukey HSD test ($p < 0.05$).

4. Conclusion

A 16-hour photoperiod increases shoot length during the in vitro shoot initiation phase, making this photoperiod most suitable for shoot multiplication. On the other hand, a 16-hour photoperiod also increases shoot length and leaf fresh weight during acclimatization. The increase in leaf biomass will enhance the total steviol glycoside production in *S. rebaudiana*. The results of this study indicate that photoperiod has an impact on the in vitro growth of *S. rebaudiana*.

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Artificial Neural Networks (ANN) to Model Microplastic Contents in Commercial Fish Species at Jakarta Bay

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Abstract

Jakarta Bay is known as one of the marine ecosystems that have been contaminated by microplastics. Despite massive loads of microplastic contamination, Jakarta Bay is also habitat to potential commercial fish species, including anchovy *Stolephorus commersonnii* and mackerel *Rastrelliger kanagurta*. While information on the microplastic contents and their determining factors is still limited, the goal of this study was to use artificial neural networks (ANN) as a novel and useful tool to model the determinants of microplastic content in fish in Jakarta Bay, using fish weight and length as proxies. Inside the stomachs of *S. commersonnii* and *R. kanagurta*, the order of microplastics from the highest to the lowest was fiber > film > fragment > pellet. Based on the RMSE values of 3.199 for *S. commersonnii* and 2.738 for *R. kanagurta*, the ANN model of fish's weight + length ~ pellet was found to be the best fitted model to explain the correlation of fish weight and length with microplastic content in the stomach. The results indicate that ANN is suitable for solving large, complex problems in determining and projecting microplastic contents and provides better estimates that can be used to manage R. *kanagurta* and S. *commersonnii* along with microplastic contamination threats.

Keywords: anchovy, fiber, mackerel, pellet, RMSE

1. Introduction

During the year 2015, the Indonesian marine ecosystems had a high quantity of commercial fish, including anchovy and mackerel species (scientifically known respectively as *Stolephorus* sp. and *Rastrelliger* sp.). *Stolephorus* sp. are little commercial fish with a strong market demand. Additionally, anchovies serve as a food source for other fish species that consume them as prey. *Stolephorus* sp. is a tiny pelagic fish that contributes significantly to fisheries productivity in Indonesian waters. It is typically caught with a lift net and has an economic worth of USD 5.77-6.92 per kilogram [1, 2]. In 2015, production totaled 6,474.5 tons, accounting for 28% of total small pelagic fish production [3]. Actual real production may be larger than the estimate because fishermen rarely land their anchovies at fish landing sites or sell the fish directly to anchovy collectors at sea, therefore production is not fully documented at the fishing port. In addition to being marketed fresh, *Stolephorus* sp. is salted [4] to extend storage time. The fish body is the most commonly consumed portion, while the fish head is an underutilized by-product. It is estimated that 15% [5] of anchovy resources are by products of anchovy meats and discarded parts [6]. Besides *Stolephorus* sp., *Rastrelliger* sp. [7] species is also known as the most common commercial fish in Indonesia. The potential mackerel resources in the Java Sea reaches 450.400 tons/year and make this species as the mostly caught with purse seine in the waters of western Indonesia.

Microplastics sized 1 mm-5 mm are some of the most persistent and ubiquitous contaminants in oceanic waters and on beaches. According to recent reports on marine biota, microplastics have contaminated various forms of marine life. Microplastics are derived from textiles, personal care products, industrials, and household garbage, which are carried by rivers, winds, floods, and storms, and eventually end up in marine environments. Another source of microplastics in the water is natural phenomena such as floods [8], heavy rainfall, tsunamis, and cyclones [9]. Degradation of microplastics in the oceanic environment began with water temperature, ultraviolet light, currents, and the activities of decompositions by marine microorganisms. Microplastics floating on the surface

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of oceanic water may enter the food chain if consumed by zooplankton or small pelagic fishes like anchovies which exhibit subsurface foraging behavior [10]. Recently, Indonesia has become one of the risked countries by produce 0.48-1.29 million metric tons per year (MMT/year) plastic marine debris on the last 2010 [11].

Fish-microplastic data is important for studies on fish ecology. Meanwhile, processing data, information, and knowledge entails estimating parameters in mathematical space. Since data from fisheries [12], chemical, and atmospheric studies are indirect observations using secondary data, extrapolating their findings to a biological measurement scale distinguishes adequate from overly ambitious and deficient models. Uncertainty in data, ambiguity in information, missing data points, and unclear goals all contribute to the complexity. As a result, a paradigm change from traditional model-driven methodologies to artificial intelligence (AI) tools is required. Artificial Neural Networks (ANN) approaches are used in one AI approach. Data processing with ANNs (Neural Networks) is done either directly on a chip or through software. ANN implementations in software are widely used and have been successful in forecasting the stock market, forex, sunspots, diabetes onset, separating renal cell cancer from cyst, identifying acute myocardial infarction, and classifying iris data. As a result, ANN has been frequently used in fishery research. Kang et al. [13] employed ANN to examine numerous hydrological and environmental variables, and their correlation, to improve fishery estimation for the ecological health evaluation of streams and water resource management in South Korea.

Jakarta Bay is considered to be the largest contributor of plastic to the marine environments in Indonesia and thus it has raised the level of concern. Cordova and Nurhati [14] confirmed plastics as the most common debris entering Jakarta Bay representing 59% (abundance) or 37% (weight) of the total collected debris. Dwiyitno et al. [15] reported that plastic debris in Jakarta Bay ranged from 7,400 to 10,300 particles/km^2. Those plastic debris can potentially contribute to the microplastic contents in Jakarta Bay. Several studies have reported the occurrence of microplastics in surface water or sediment in Jakarta Bay. Manalu et al. [16] have reported the abundance of microplastic in sediment of Jakarta Bay ranging from 18,405 to 38,790 particles/kg dry sediment. Microplastic loads in Jakarta Bay were followed by microplastic contents in marine creatures, including commercial fish species. According to Susanti et al. [17], *Lutjanus vitta* populating Jakarta Bay has contained film microplastics of 2-21 particles/fish and fiber of 1-10 particles/fish. Meanwhile Efadeswarni et al. [18] confirmed that more than half of the fish species studied in Jakarta Bay contained microplastic in the following order: fiber > film > fragment, with the greatest concentrations equaling 137 particles/fish and the lowest being 8 particles/fish.

Despite growing reports on microplastic contents in fish, limited research has informed the model that can depict the determinant factors and proxies of microplastic contents in fish. Taking into consideration, this study used an ANN as a novelty and tool to model the determinants of microplastic contents in fish utilizing fish weight and length as determinant variables affecting microplastic contents. Microplastic content in fish can be discovered and calculated immediately by using the correct modeling tools. The modeled fish in this case is mackerel *Rastrelliger* sp. and anchovy *Stolephorus* sp., which are commercial species commonly available and consumed in Indonesia.

2. Methodology

2.1. Study Area

The fish sampling site location was in the water of Jakarta Bay, Indonesia. It is a shallow bay with an average depth of 15 m, its shoreline is about 149 km long and covers an area of an approximately 595 km^2. The bay is located north of Jakarta, the capital of Indonesia. Thirteen rivers around Jakarta Bay are known to discharge a large amount of anthropo- genic material from land-based sources into the bay, including industrial effluent, sewage, and agricultural discharges.

2.2. Fish Survey and Collection

Sampling was performed in November 2022 in Jakarta Bay. Fish were collected from water using fishing nets. The fish species was identified using identification book to determine the fish species and confirmed as *Rastrelliger* sp. and *Stolephorus* sp. [19, 20]. The collected fish samples then stored in cooler box and transported into the laboratory for further microplastic analysis.

2.3. Fish Preparation

Sampled fish in laboratory was measured first for its weight and length with units of gr and mm. Then to avoid contamination, sampled fish was soaked in filtered Milli-Q water and rinsed in flow water [21]. The analyzed parts of the sampled fish were the stomach since the microplastic was accumulated inside fish body in this part. The stomach was isolated as a whole up to 0.5 g in total and placed in an Erlenmeyer jar with 5 ml 0.5% dissolved sodium sulfate and 10 ml NaOH at fume hood, then kept in it on the room temperature for 24 hours. After 24 hours, the jar was gently shaken and let to in- cubate for another 24 hours until all contents dissolved. After the incubation fully dissolved, 1 ml of sample was placed into Sedgwick rafter (triplet) and observed with magnification 10x on the microscope. Types of microplastic shapes including fiber, film, fragment, and pellets were calculated and denoted as particles/fish [22].

2.4. Microplastic Quality Assurance

To prevent the contamination of samples from other possible microplastic sources that were not related to the study,

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including microplastic from air and clothing, a microplastic quality assurance procedure was implemented. During the sample preparation, the sampled fish were tightly packed in a Petri dish. High-density polyethylene (HDPE) materials were avoided during this process, and only glassware was used during sample preparation. Procedural blanks were implemented as controls for laboratory contamination. The blank procedure was carried out exactly as the field samples were, but without the fish samples [23].

2.5. ANN Analysis

ANNs are mathematical models inspired by the human brain. They are able to recognize behavioral patterns and learn from their interactions with the environment. Back Propagation (BP) and multiple layer perceptrons (MLPs) neural network were constructed and computed using R version 3. The ANN model consists of three feed-forward layers: input, hidden and output [24]. The input layer was composed of weight and length of fish variables. The number of nodes in the hidden layer was determined by testing the performance of the models using arrange of node numbers. The dependent variable that is the microplastic types including fiber, fragment, film, and pellet represented the output layer. A neuron is the basic processing unit of an ANN and performs two functions: collecting the inputs and producing the output. Each input is multiplied by connection weights, and its products and biases are added and then passed through an activation function to produce an output as microplastic contents, as shown in Figure 1 [25].

2.6. Data Analysis

One way ANOVA combined with Tukey's HSD (honestly significant difference) pairwise comparison were used to test the differences in microplastic content among types, with a significance level of $P < 0.05$.

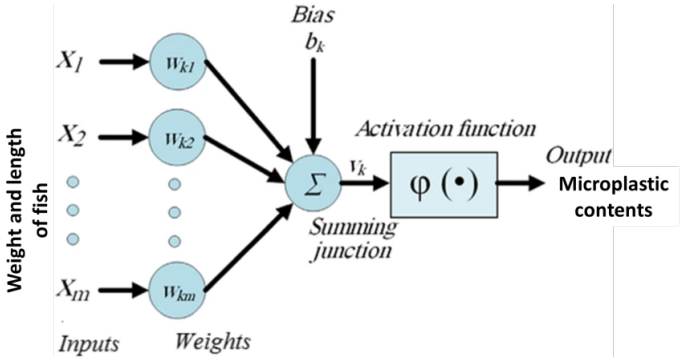

Figure 1. Structure of ANN model [25].

3. Result and Discussion

3.1. Fish's Weight and Length and Microplastic Quantity

The identification confirms that the sampled fishes were *Commersonnii's* Anchovy *Stolephorus commersonnii* (Lacepède, 1803) and Indian Mackerel *Rastrelliger kanagurta* (Cuvier, 1816). There were 15 individuals collected for both species. The average and 95% confidence interval (CI) for weight and length of *S. commersonnii* were respectively 1.07 gr with 95% CI (0.705, 1.45) and 65.0 mm with 95% CI (58.7, 71.4). The weight and length of the sampled fish were comparable to data reported from previous research either at global, regional or national water within Southeast Asia (Table 1). The *S. commersonnii* weight and length recorded in this study seems to be smaller in comparison to individuals recorded in Demak water. The smaller sizes can be due to the several reasons. First, Jakarta Bay is a marine ecosystem that has received pollution [26] and reductions of natural habitats that may reduce the presences of larger individuals [27]. Second reason is due to the overfishing of larger individuals that cause only smaller fishes available [28]. As reported globally, overfishing will result in smaller fish and causing fish to get smaller [29, 30]. While in contrast, *R. kanagurta* (Table 1)recorded in Jakarta Bay was in fact larger than in other location in Indonesia, in this case in Demak waters. The recorded individuals of *R. kanagurta* have an average length of 163.72 mm with 95% CI (148, 180) and weight of 97.25 gr with 95% CI (77.8, 117). This size classes dominated by adult individuals indicates that this species has not yet experienced overfishing in Jakarta Bay.

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Table 1. Weight and length of *Stolephorus* sp. and *Rastrelliger* sp.

Species	Locations	Weight (gr)	Length (mm)	References
Stolephorus sp.	Terengganu, Malaysia	1.57 gr	51.0 – 76.0 mm	[31]
	Demak, Indonesia	1.97 - 8.8 gr	34.17 - 76.69 mm	[32]
	Jakarta Bay, Indonesia	0.35 – 3.56 gr	47.43 - 103.08 mm	This study
Rastrelliger sp.	Demak, Indonesia	40 - 70 gr	140 – 180 mm	[7]
	Suez Bay, Egypt	29 - 339 gr	150 – 320 mm	[43]
	Jakarta Bay, Indonesia	85 - 127 gr	153.6 – 187.8 mm	This study

Figure 2 and Figure 3 depict microplastic quantity in the stomach of *R. kanagurta* and *S. commersonnii*. It is clear that the order of microplastic from the highest to the lowest was in order of fiber > film > fragment > pellet. This confirms that fiber is the common microplastic accumulated inside the stomach of *S. commersonnii* with an average of 42.20 particles/fish and the least microplastic was pellet with an amount of 1.81 particles/fish in average. The amount of microplastics were significantly different (Table 2) among types except between fiber and film. This indicates that the stomach of *S. commersonnii* contains approximately the same amount of fiber and film since those microplastic types had no significant differences ($P > 0.05$) in quantity. Similar to *S. commersonnii*, microplastic contents of fragment and pellet in stomach of *R. kanagurta* are quite similar and show no differences ($P > 0.05$).

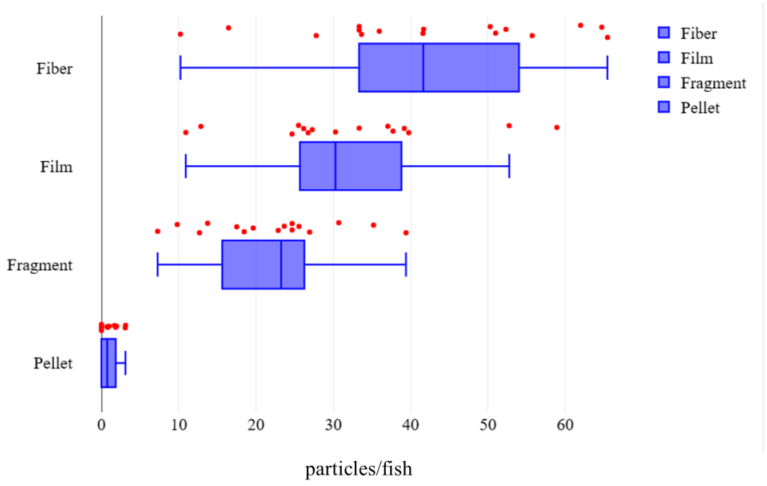

Figure 2. Microplastic quantity (particles/fish) in stomach of *S. commersonnii.*

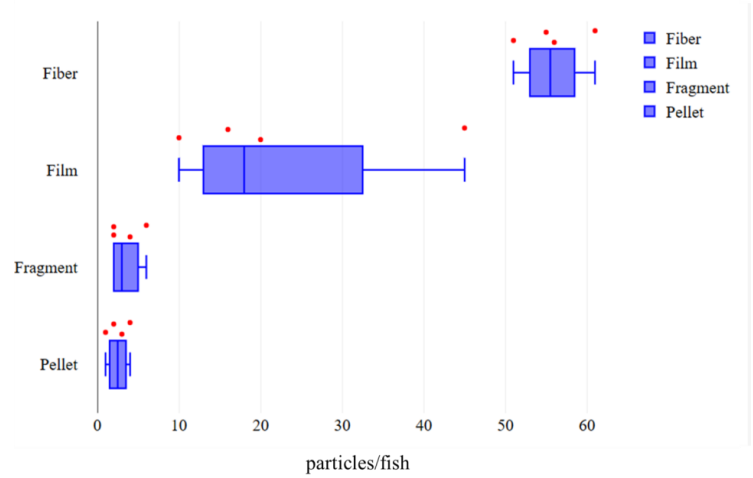

Figure 3. Microplastic quantity (particles/fish) in stomach of *R. kanagurta*

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Table 2. Tukey's HSD comparison of microplastic quantity among microplastic types

Species	Pairwise comparisons	P values	Significance
S. commersonnii	Fiber : film	0.279	Not significant
	Fiber : fragment	0.000	Significant
	Fiber : pellet	0.000	Significant
	Film : fragment	0.026	Significant
	Film : pellet	0.000	Significant
	Fragment : pellet	0.000	Significant
R. kanagurta	Fiber : film	0.000	Significant
	Fiber : fragment	0.000	Significant
	Fiber : pellet	0.000	Significant
	Film : fragment	0.002	Significant
	Film : pellet	0.001	Significant
	Fragment : pellet	0.995	Not significant

In Jakarta Bay, fiber is observed as the most common microplastic found in the stomach of studied commercial fishes which is consistent with findings from other locations [33]. The microplastic content will reflect the surrounding habitats, anthropogenic activities, and plastic material used. Jakarta Bay is a fishing ground that uses a variety of fishing nets mostly made of plastic fibers. According to Wu et al. [34], several marine activities including fisheries are responsible to the production of microfibers. Ranging from fishing nets and ropes to laundry and municipal waste from nearby fishermen villages may be possible sources of fiber in the marine environment.

3.2. ANN Models of Microplastic in R. kanagurta *and* S. commersonnii *Stomach*

ANN models for each microplastic content inside the fish stomach include film, fragment, film, and pellet, as shown in Figure 4 for *S. commersonnii* and Figure 5 for *R. kanagurta*. The ANN models consist of one hidden layer and three nodes. The best and fitted models were indicated by the lowest prediction error and root mean square error (RMSE) according to previous study and analysis [33, 34]. The RMSE for *S. commersonnii* was following order from the highest to the lowest as follows (Table 3): fiber > film > fragment > pellet. The RMSE values then in the order of 50.671, 37.473, 16.080, and 3.199. Similar patterns were observed for *R. kanagurta* with The RMSE values in the order of 55.863, 26.367, 3.872, and 2.738. Since the lowest RMSE value was observed for fish weight-length and pellet model, then ANN model can be used to estimate pellet contents using fish's weight and length as determinant factors. The second fitted model was observed for fragment. Meanwhile, fish's weight and length cannot be used to forecast the fiber and film in both *R. kanagurta* and *S. commersonnii* stomach due to its high RMSE values. This is because the lower RMSE values representing the goodness of fit [35]. RMSE benchmark refers to forecast and can therefore negate the disadvantages that may arise when correlating models developed for examines with different values [36].

Table 3. Prediction error and root mean square error (RMSE) of developed ANN models

Species	Error types	ANN Models			
		Weight + length ~ fiber	Weight + length ~ film	Weight + length ~ fragment	Weight + length ~ pellet
S. commersonnii	Prediction error	4943.459	2667.939	462.52	14.364
	RMSE	50.671	37.473	16.080	3.199
R. kanagurta	Prediction error	6020.508	1301.508	18.008	7.000
	RMSE	55.863	26.367	3.872	2.738

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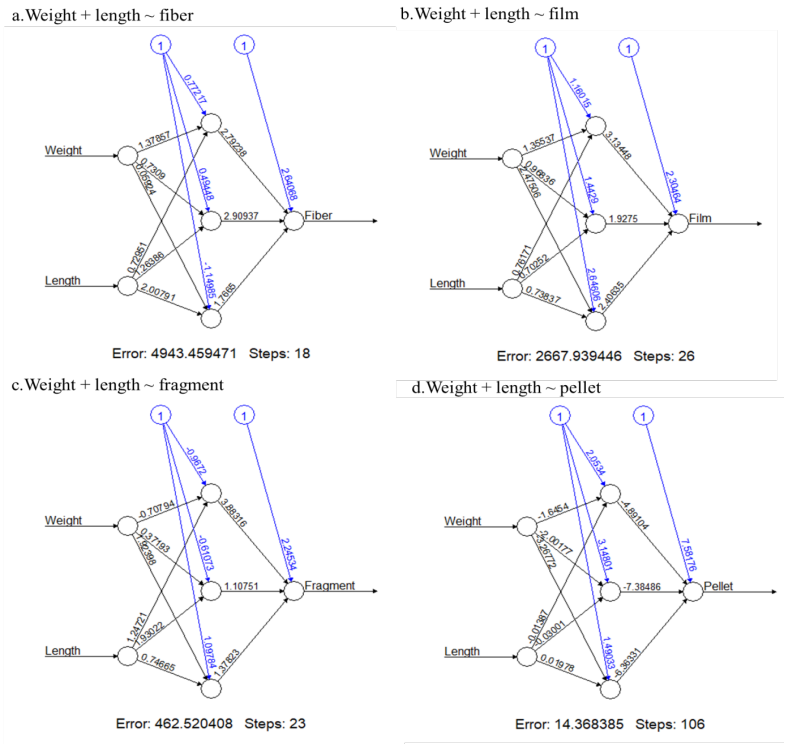

Figure 4. Structures of ANN models for (a) fish's weight + length ~ fiber, (b) fish's weight + length ~ film, (c) fish's weight + length ~ fragment, and (d) fish's weight + length ~ pellet for *S. commersonnii*.

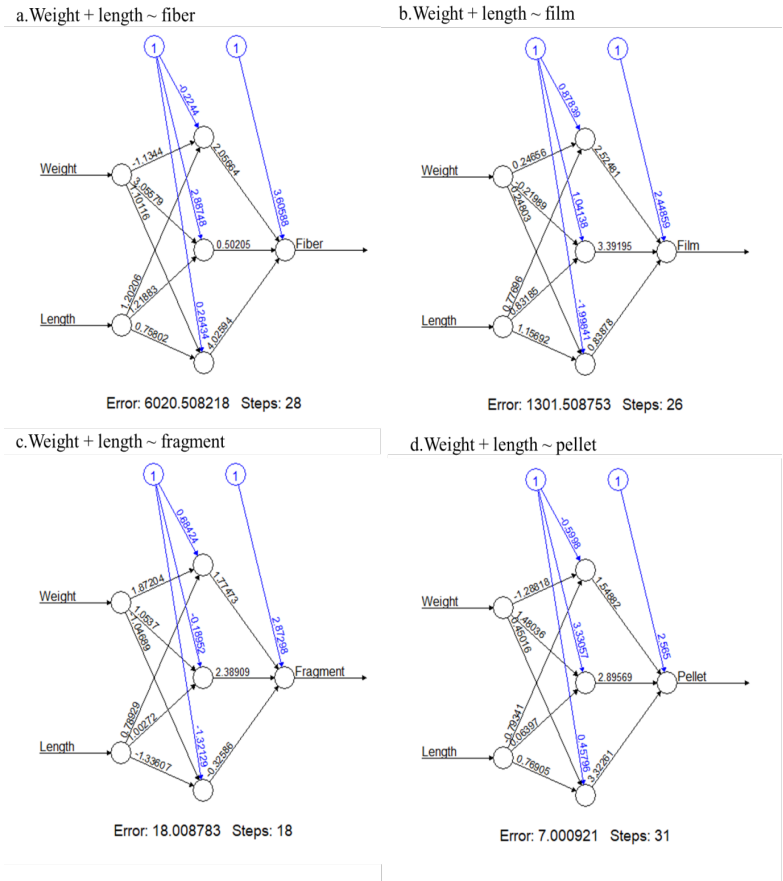

Figure 5. Structures of ANN models for (a) fish's weight + length ~ fiber, (b) fish's weight + length ~ film, (c) fish's weight + length ~ fragment, and (d) fish's weight + length ~ pellet for *R. kanagurta*.

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Despite the increasing number of studies on the use of ANN to model various environmental phenomena [37], including fishery studies [38], the uptake of microplastic by fish generally exhibit nonlinear behaviors due to the significant fish physiological and environmental variability. The nonlinear models, in this case ANN, provide more accurate estimates than linear models in the estimation analysis. ANN is considered well suited to modelling ecological data [39]. While the accuracy of ANN model was determined by the numbers of hidden layer [40]. In this study, only singular hidden layer was used. Further studies should be carried out to develop ANN models with multiple hidden layers. Despite its limitations, ANN was considered a well-suited method to be applied in the fishery model study [41, 42].

4. Conclusion

Among the commercial fish species studied, *S. commersonnii* sizes in Jakarta Bay were dominated by small individuals, possibly due to a lack of adult individuals, while *R. kanagurta* in Jakarta Bay were predominantly adult individuals. Fibers were known as the most common microplastic observed in the stomachs of both commercial species. For *S. commersonnii*, fiber and film were recorded at the same quantity. In the case of *R. kanagurta*, the amount of fragment and pellet were found in equal amounts. The length and weight of the fish were associated with the microplastic contents. Based on the ANN model and RMSE values, significant associations were recorded for pellets. In conclusion, the weight and length of fish can be used to predict the pellet content accurately, in both *R. kanagurta* and *S. commersonnii* in Jakarta Bay. Since *R. kanagurta* has the lowest RMSE values, the weight and length within ANN model are more suitable to be applied to *R. kanagurta*.

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Relationship of Land Cover Heterogeneity and Insecticide Use with Arthropod Community Structure in Rice Agroecosystems

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Abstract

Rice agroecosystems generally experience two kinds of environmental stresses, i.e., a decrease in the heterogeneity of land vegetation types due to the development of settlements in the border zone and the excessive use of insecticides. Both of these factors are known to affect the structure of the arthropod community in rice agroecosystems. However, studies related to the effects of these two stresses in shaping the structure of arthropod communities have not been widely carried out, especially during the planting period in the rainy season. This study aims to analyze the relationship between land cover heterogeneity and insecticide use with the taxonomic and functional diversity of arthropods in the alpha, beta, and gamma dimensions. The study was conducted during the rainy season (October–March) on four rice agroecosystem sites in Bandung, West Java. Data was collected 30 and 50 days after planting (DAP). Arthropod samples were identified, and their taxonomic and functional diversity was calculated using the Shannon entropy formula (D). In general, land cover heterogeneity was positively correlated with the value of arthropod taxonomic and functional diversity, especially in the early vegetative phase of rice plants (30 DAP). In contrast, insecticides (g/m^2) were negatively correlated with the value of arthropod diversity. The composition of arthropod species locally was relatively the same except in the rice agroecosystem with the lowest land cover heterogeneity and the highest use of insecticides. Additionally, the coefficient value of the insecticide variable has more influence on the value of arthropod diversity than the variable coefficient of land cover heterogeneity.

Keywords: arthropods, biodiversity, insecticide, land cover heterogeneity, rice agroecosystem

1. Introduction

Each year, Indonesia faces the threat of a decrease in national rice production. According to the Central Statistics Bureau [1], rice production declined nationally during 2018-2021, presenting a threat to national food security. The decline in rice production may be caused by several factors, including climate change, water and soil pollution, arthropod pests, plant diseases, and land conversion [2-5].

Attack by arthropod pests is one variable that significantly affects the rice agricultural sector. Arthropod pests cause annual losses of 18–26% of the world's rice production, or USD 470 billion [6]. An imbalance in the structure of the arthropod community in the rice agroecosystem is the cause of the rise in arthropod pest attacks on plants. According to Mori [7], the transformation of paddy fields into settlements as a result of urbanization is one of the factors that alter the structure of the arthropod community.

The heterogeneity of vegetation types forming land cover in the paddy field border zones has tended to decrease due to the conversion of paddy fields into settlements [7]. The phenomena of biotic homogeneity, or the tendency of organisms in ecosystems to homogenize as a result of perturbations by human activity, has been caused by the conversion of paddy fields into settlements, which is common in urban areas. The city and district of Bandung in West Java are among the many locations in Indonesia that are under pressure from the conversion of paddy fields into settlements as a result of urbanization. The heterogeneity of vegetation cover types on land adjacent to paddy fields may be reduced as a result of this circumstance in Bandung's rice agroecosystem.

Heong [8] found that the structure of the arthropod community is impacted by the use of pesticides. A pesticide "tsunami" has resulted from the increased use of pesticides, particularly insecticides, which has caused the environmental calamity. The pesticide tsunami reduced the diversity of

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arthropods by causing the loss of numerous biota. Ngin [9] claims that decreased arthropod diversity caused an expansion in populations of herbivorous arthropods that have the potential to harm rice plants. The majority of rice farmers in Bandung, West Java, still use chemical insecticides to control arthropod pests. Thus, both landcover heterogeneity and insecticide use have the potential to affect the structure of the arthropod community in Bandung, West Java.

Several studies have examined how the structure of arthropod communities is impacted by the variability of land cover heterogeneity and insecticide use. A recent study by Sattler [4, 10] described the relationship between land cover heterogeneity and pesticide used with arthropod communities during the dry season in Vietnam, while there has been no study of how these two factors affect an arthropod community structure in the rainy season. According to Holmquist [11], the climatic changes between the dry and wet seasons might lead to diverse arthropod community patterns. Additionally, insect pest attacks on rice agroecosystems tend to be more frequent during the rainy season in tropical nations like Indonesia (October – March).

The association between land cover heterogeneity and insecticide usage with arthropod community patterns in rice agroecosystems in the city and district of Bandung during the rainy season must be studied in light of the aforementioned description. The sustainability of the rice agroecosystem is significantly influenced by the structure of the arthropod community, which is characterized by the value of diversity. The high diversity of arthropods indicates the existence of intricate interaction systems that enable the management of the population of arthropods that can become pests for rice.

The investigations on the community structure of organisms require analysis at several spatial scales [12,13]. A thorough understanding of the distribution of species in a region and the dynamics of interactions within a community is made possible by an understanding of community structure at different geographical scales. Whittaker [14] calculated the value of diversity at various spatial scales known as the diversity of the alpha, beta, and gamma dimensions to start the investigation of community structure. Currently, this method is still relevant and useful.

Additionally, Sattler [4] claim that taxonomic diversity, which is the diversity value computed based on abundance and species richness, is insufficient to explain the complexity of the arthropod community structure. The functional diversity of arthropod was also considered in some studies based on their functional categories in the environment [15-17]. Functional diversity is a term used to describe the diversity of organisms through their function in the ecosystem. Dominik [18] showed that the importance of functional diversity may accurately capture the close connection between organisms and processes in ecosystems. Studying the taxonomic and functional diversity of arthropods is thus important to fully comprehend the organization of the arthropod community. Therefore, the objective of this study was to analyze the relationship between land cover heterogeneity and insecticide use with the taxonomic and functional diversity of arthropods on the alpha, beta, and gamma dimensions of rice agroecosystems in the Bandung region of West Java.

2. Methodology

2.1. Study Area

This study was conducted in several rice agroecosystems in Bandung City and Bandung Regency, West Java, Indonesia, during the rainy season planting period (October 2021–March 2022). Four rice agroecosystem sites were selected for this study, i.e., paddy field A (located in the area of Resort Raya Street, Cimenyan District, Bandung Regency), paddy field B and paddy field C (both located in the area of Cigadung Wetan, Cibeunying Kaler District, Bandung City), and paddy field D (located in the area of Binong, Batununggal District, Bandung City). The relative position of the four sites is shown in Figure 1. The four sites are generally similar but differed in the heterogeneity of the land cover in the area bordering the edges of the paddy fields, the use of insecticides, and the age of the plants in each field, which was adjusted to the timeline of data collection. Because they are managed by different farmers, the four sites have different styles of land management. Table 1 illustrates the characteristics and types of management based on the results of interviews with farmers at each site. At each study site, data were gathered 30 and 50 days after planting (DAP).

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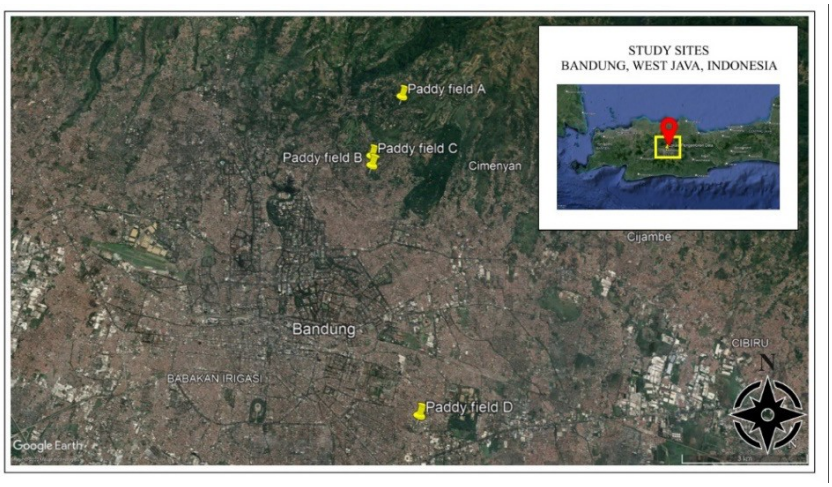

Figure 1. Location of study sites within the City and District of Bandung, West Java.

Table 1. Rice characteristics.

Parameters	Paddy field A	Paddy field B	Paddy field C	Paddy field D
Land cover type	Trees, vegetable plantation, river	Trees, settlements, vegetable plantations, grass field	Trees, settlements, vegetable plantation	Settlements, river
Paddy field area	6.626 m^2	6.905 m^2	5.449 m^2	8.550 m^2
Pesticide use	Insecticide (Decis: Active ingredient Deltamethrin)	Herbicide, insecticide (Decis: Active ingredient Deltamethrin)	Herbicide	Insecticide (Diazinon)
Intensity of insecticide use	1	2	0	2
Concentration of insecticide use	0,11 mL/m^2	0,12 mL/m^2	0 mL/m^2	0,02 mL/m^2
Location altitude	837 – 847 masl	747 – 759 masl	750 – 756 masl	676 – 678 masl

2.2. Arthropod Sampling

Arthropod samples were collected at 30 days after planting (DAP) when the rice plants were in the early vegetative phase, and at 50 DAP when they were in the late vegetative phase. Arthropods were sampled using active and passive methods [19]. The active method used direct retrieval using a sweeping net aimed to obtain arthropods around rice plants, conduct- ed by walking at a speed of 0.5 meters per second with 30 sweeping net swings on each plot measuring 10 x 10 meters [4]. In each rice agroecosystem, eight plots were determined randomly. The passive methods used in this study were the pitfall trap method aimed to obtain arthropods that live on the ground surface, and the malaise trap sampling method which aimed to obtain insects that tend to fly over rice plants. In each site, eight pitfall traps were installed randomly. Sampling with a malaise trap was conducted by installing a malaise trap at the midpoint of the paddy field for 24 hours over three nights to obtain three-time repetition data [20].

Arthropod samples obtained through the sweeping net method were killed using a killing jar and preserved [4, 21]. Samples from malaise traps were collected and preserved in bottles [20]. Pitfall trap samples were rinsed with running water and preserved. All samples were preserved in 70% alcohol and stored in bottles before identification based on location and time of collection [21]. The arthropod samples have been identified at the family taxonomic level using various literature sources and identification manuals [22-27]. Each family was documented, and samples were stored as specimens in bottles with preservative liquid for reference during the study. All samples and family specimens were disposed of after the research was completed.

2.3. Predictor Variable

2.3.1 Land cover heterogeneity

In this study, land cover heterogeneity is determined as the value of land cover diversity at a radius of 120 meters from the midpoint of the rice field. The land cover includes paddy field cover, vegetation-type cover, non-natural land cover types formed due to human activities, and water body cover types. The types and characteristics of the land cover used are described in Table 2.

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Table 2. Description of land cover types.

No.	Land cover types	Description
1	Paddy field	permanently irrigated paddy fields
2	Forest	principally trees, also shrubs, bushes, and storey
3	Fruit plantation	fruit trees, banana plantations, coconut trees, etc.
4	Vegetable plantation	cabbage, eggplant, pepper, etc. plantations
5	Grass	grass cover mainly for grazing
6	Other crops	agricultural areas not covered by types 1-5
7	Water	lakes, rivers, and ponds
8	Bare soil	bare rock, sand, etc.
9	Compacted surface	unpaved roads compacted soil surface
10	Sealed surface	houses and other buildings, streets, etc.

The land cover heterogeneity value was calculated using the Shannon-Wiener formula [4]. Land cover heterogeneity:

$$\text{Shannon Wiener index (H')} = \Sigma \text{ pi ln pi}$$

The pi value is the percentage of land cover area compared to total land area. Measurements were carried out using the digital mapping method using high-quality satellite imagery data. Digital mapping was carried out using the latest satellite imagery data (2021–2022) from Google Earth Pro, which was then processed using Quantum GIS (QGIS) software [28].

2.3.2 Insecticide Use

Information on the use of insecticides was obtained based on direct field observations and interviews with farmers in charge of managing the paddy fields. Insecticides were applied between 30 and 50 DAP. The value of insecticide use was determined by calculating the weight of the active ingredient (w) in each package using the formula below [10]:

$$w\ (g/m^2) = nAI \times vw \times c$$

The weight of the active ingredient is calculated based on the amount of active substance (nAI), the volume of insecticide used per 1 square meter (vw), and the concentration of the active substance (c).

2.3. Data Analysis

Statistical analysis of data was carried out in the form of normality tests, significance tests with ANOVA, correlation tests with Pearson's correlation, and linear regression. The linear regression model was used to see the relationship between land cover heterogeneity and arthropod diversity, and insecticide use with arthropod diversity. The linear regression model uses a confidence value of 0.95. The multiple linear regression model was used to obtain the coefficient values of the multiple linear regression equation with two independent variables, namely land cover heterogeneity and insecticide use, while the independent variable was the value of arthropod diversity. Statistical analysis was performed with Paleontological Statistics (PAST) software and R studio for multiple linear regression models using the package 'lm' [4,10,18].

3. Result and Discussion

3.1. Arthropod Sampling Result

A total of 12,619 individuals of arthropods from 78 different families were found in four paddy field agroecosystems in Bandung, West Java. Ephydridae (3,351 individuals) and Acrididae (2,393 individuals) were the two families with the greatest number of individuals. Herbivorous arthropods accounted for 82.5% of the total arthropod community structure, while decomposers (0.4%) and indifferent arthropods (0.2%) had the lowest abundances. According to the total abundance value, there were more arthropods (7,922 individuals) at 50 DAP than at 30 DAP (4,697 individuals). The average values of the alpha and beta dimensions of arthropod diversity, as well as the gamma values obtained from this study, are shown in Table 3.

Table 3. Average values of the alpha, beta and gamma dimensions of arthropod diversity at four study sites.

Location	Land cover heterogeneity (H)	Insecticide use (g/m^2)	Taxonomy diversity			Functional diversity		
			Dα	b_w	Dγ	Dα	bw	Dγ
A 30 DAP	1.30	2.75×10^{-3}	4.67 ± 1.39	0.50 ± 0.15	7.52	1.93 ± 0.35	0.15 ± 0.11	2.10
A 50 DAP			5.54 ± 1.22	0.37 ± 0.14	7.66	1.67 ± 0.37	0.12 ± 0.14	1.70
B 30 DAP	1.58	5.95×10^{-3}	7.11 ± 2.82	0.35 ± 0.11	8.65	1.81 ± 0.24	0.11 ± 0.10	1.86
B 50 DAP			3.90 ±0.99	0.33 ± 0.09	5.53	1.29 ± 0.10	0.05 ± 0.08	1.27
C 30 DAP	1.28	0	8.12 ± 1.71	0.40 ± 0.14	11.82	2.10 ± 0.25	0.14 ± 0.11	2.26
C 50 DAP			6.78 ± 2.08	0.32 ± 0.07	7.30	1.90 ± 0.48	0.05 ± 0.08	1.77
D 30 DAP	1.19	8×10^{-3}	1.71 ± 1.34	0.93 ± 0.26	8.17	1.19 ± 0.84	0.73 ± 0.37	2.46
D 50 DAP			4.09 ± 1.53	0.66 ± 0.23	10.20	1.76 ± 0.33	0.08 ± 0.15	1.97

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3.2. Land Cover Heterogeneity

According to the results shown in Table 3, the four paddy agroecosystems have various levels of land cover heterogeneity. From highest to lowest in terms of land cover heterogeneity, the rice agroecosystems showed the following sequence: Paddy field B (1.58), Paddy field A (1.30), Paddy field C (1.28), and Paddy field D (1.19). The land cover heterogeneity mapping and values are shown in Figure 2. The results of correlation analysis using linear regression between arthropod taxonomic diversity on the alpha dimension (Dα) and land cover heterogeneity (H') is shown in Figures 3, 4, and 5.

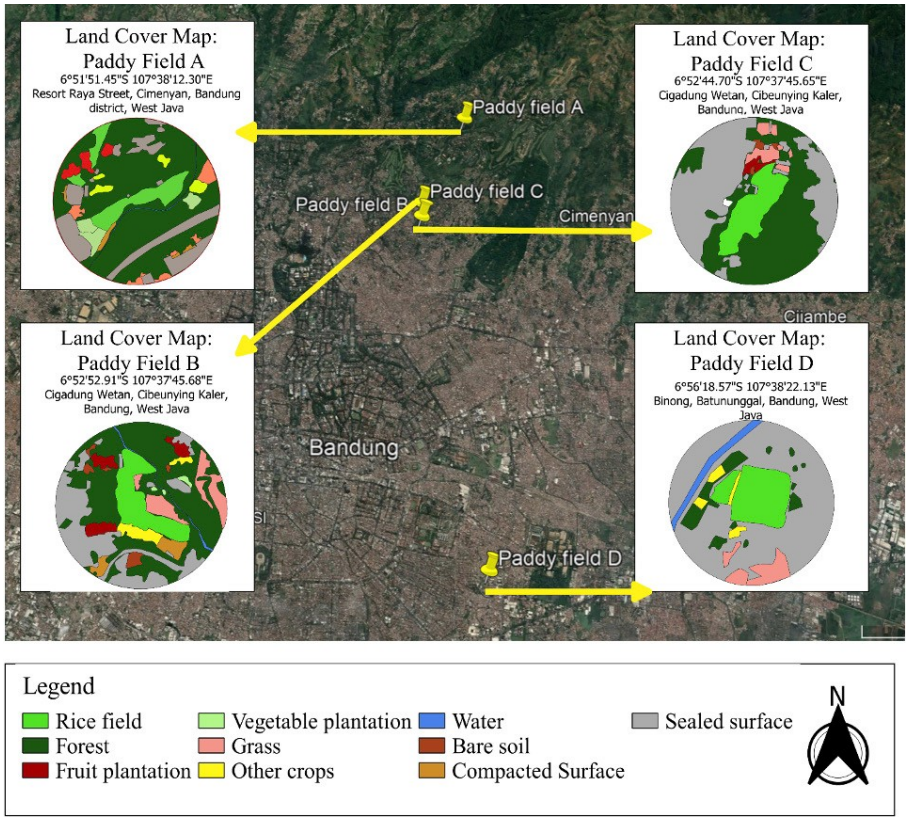

Figure 2. Land cover heterogeneity map of study sites.

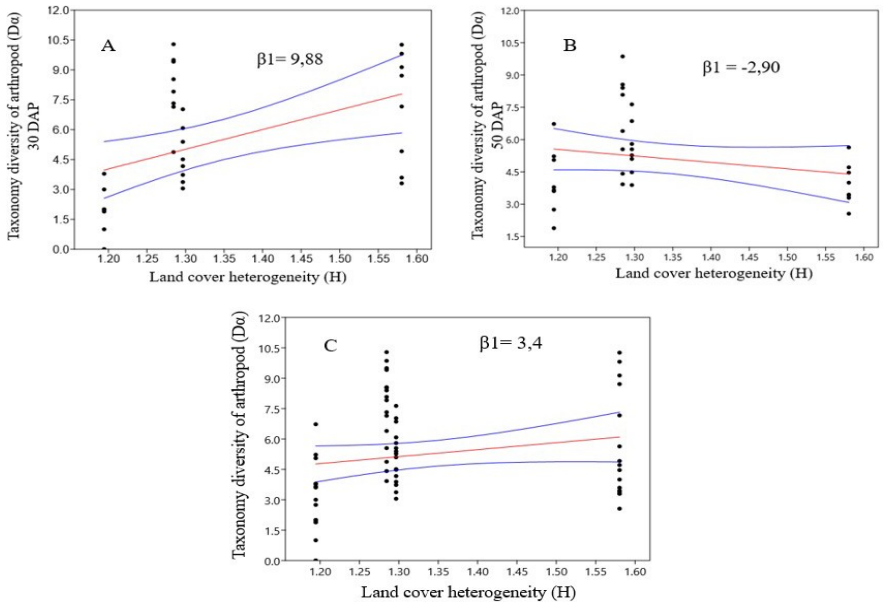

Figure 3. Correlation between land cover heterogeneity and arthropod taxonomic diversity on the alpha dimension at 30 DAP (A), 50 DAP (B), and 30 and 50 DAP data combined (C). The red line shows the linear regression, while the blue line shows the area with a 95% confidence index.

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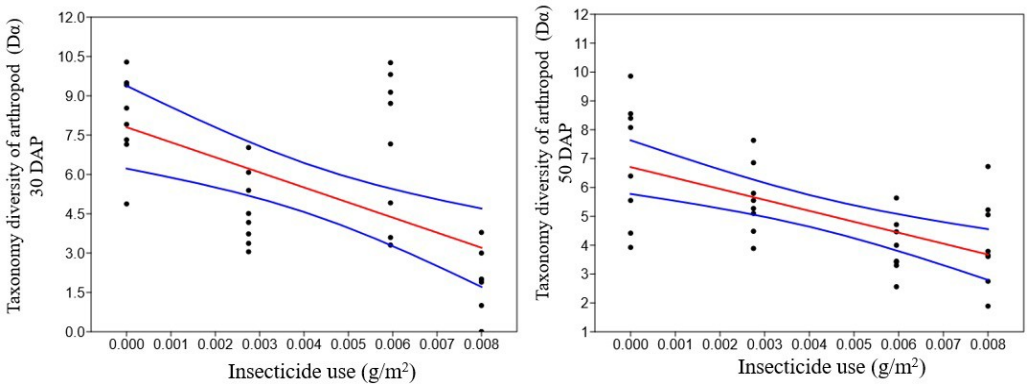

Figure 4. Correlation between insecticide use and arthropod taxonomic diversity at 30 DAP (A) and 50 DAP (B). The red line shows the linear regression, while the blue line shows the area with a 95% confidence index.

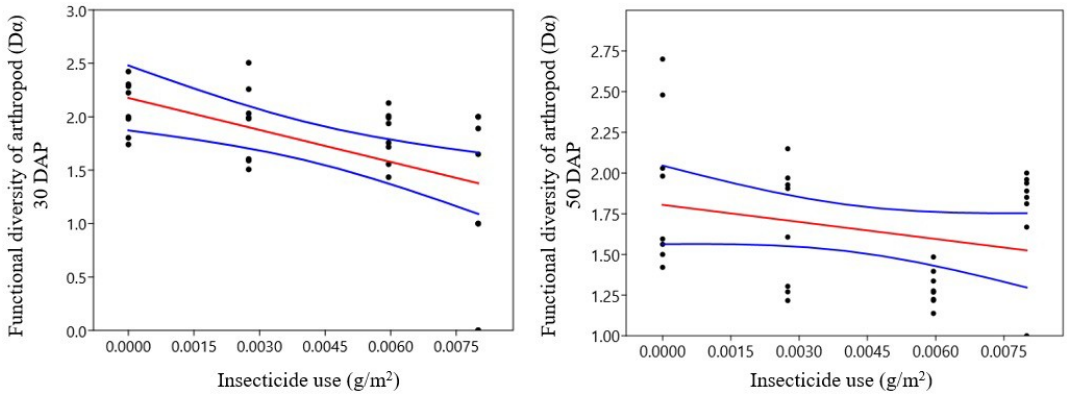

Figure 5. Correlation between insecticide use and arthropod functional diversity at 30 DAP (A) and 50 DAP (B). The red line shows the linear regression, while the blue line shows the area with a 95% confidence index.

At 30 DAP the gradient value (β1) of the linear regression equation between land cover heterogeneity and arthropod taxonomic diversity is 9.88. These findings suggest that the diversity of arthropod taxa is positively correlated with land cover heterogeneity. Nevertheless, a negative association between land cover heterogeneity and arthropod taxonomic diversity was found at 50 DAP, although it had a gradient value that was lower than 30 DAP, specifically -2.90. It is known that the linear regression model exhibits a positive correlation between land cover heterogeneity and arthropod taxonomic diversity with a gradient value of 3.4 when the data from the two sampling times are combined. This result suggests that, in general, the taxonomic diversity of arthropods tends to be positively correlated with land cover heterogeneity. Moreover, by Pearson correlation equation both 30 and 50 DAP have p-value>0.005 which indicates a non-statistically significant correlation between heterogeneity and arthropod taxonomic diversity. Additionally, the trend of the correlation between land cover heterogeneity and arthropod functional diversity is similar to that of the correlation between arthropod taxonomic diversity and land cover heterogeneity, which is positive at 30 DAP (β1= 0.83) and negative at 50 DAP (β1= -1.40). However, a significant correlation (Pearson's correlation) was found between land cover heterogeneity and functional diversity at 50 DAP.

According to Pearson's correlation test, there was a fair amount of association between the taxonomic and functional diversity of arthropods (r = 0.69). This indicates that the patterns of the two variables exhibit the same tendency. As a result, in the analysis of arthropod diversity in beta and gamma dimensions, the relationship between land cover heterogeneity was only observed with the value of arthropod taxonomic diversity.

The value of the beta and gamma dimension of arthropod diversity is shown in Table 3. It can be observed that the value of arthropod diversity in the beta dimension (bw) in the range of 1.28 to 1.58 has a value that tends to be similar, however, in the lowest land cover heterogeneity (1.19), beta diversity tends to be higher than the others. The value of arthropod diversity in the gamma dimension (Dγ) tends to increase as land cover heterogeneity increases at 30 DAP. Although, at 50 DAP, the diversity of arthropods tended to decrease as land cover heterogeneity increased. These results are in line with the value of arthropod diversity in the alpha dimension.

3.3. Insecticide Use

The four rice agroecosystem sites in this study utilized different amounts of insecticides. Based on the calculation of the

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weight of the insecticide active ingredient (w), the order of the highest value to the lowest is as follows: Paddy field D (0.008 g/m^2), paddy field B (0.006 g/m^2), paddy field A (0.003 g/m^2), and paddy field C (0.00g/m^2). Figures 5 and 6 show that at 30 and 50 DAP, the correlation between insecticide use and arthropod taxonomic and functional diversity is negatively correlated, suggesting that the value of arthropod diversity tends to decrease the more insecticidal active ingredient is applied.

Table 3 shows the mean value of beta diversity of arthropods. Based on the study of Sattler [4] a beta diversity value below 1.54 is considered a low diversity value; which means that the arthropod composition tends to be similar in each local site. However, at the highest use of insecticides (0.008g/m^2), the beta dimension of arthropod diversity values tends to be higher than in other paddy fields. The paddy fields with the highest use of insecticides are paddy field D. Paddy field D is a paddy field with the lowest land cover heterogeneity and the highest use of insecticides. The difference in beta diversity values in paddy D compared to other locations is thought to be related to the abundance of arthropods in paddy D, which is much lower than in other paddy fields (Table 2). According to Marathe [13], at locations with the same amount of species richness but different abundances, locations with lower abundances tend to have higher beta diversity values.

3.4. Discussion

The results of this study are consistent with several studies [4,18] that found that arthropod diversity in rice agroecosystems tends to be positively correlated with land cover heterogeneity. In addition, this study supports some studies [4, 6, 29, 30] that found a negative relationship between arthropod diversity in rice agroecosystems and insecticide use. According to a study by Sattler [4] conducted in Vietnam during the dry season, the insecticide use variable had a larger impact on the structure of the arthropod community than the land cover heterogeneity variable. This study attempts to understand how these two variables affect the arthropod community structure during the rainy season in Bandung, West Java, Indonesia. The analysis of multiple linear regression equations between land cover heterogeneity and insecticide use with arthropod diversity is shown in Table 4.

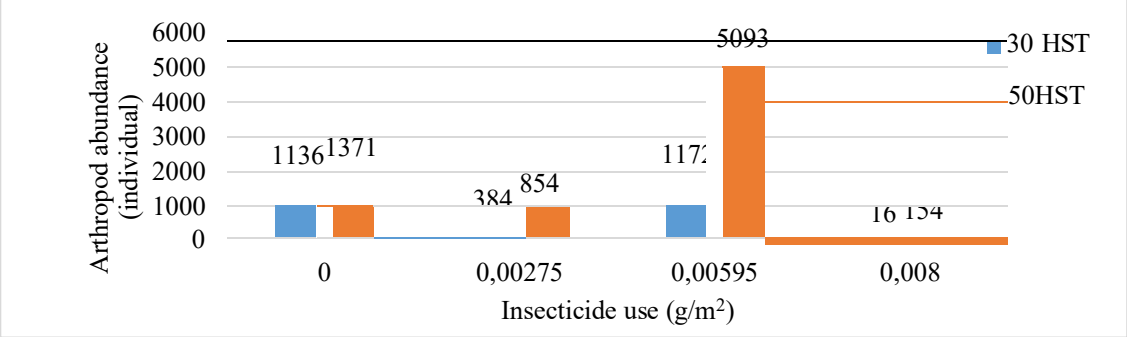

Figure 6. Association of insecticide use and arthropod abundance.

Table 4. The result of multiple linear regression between land cover heterogeneity and insecticide use with arthropod diversity

Variable	Respond variable	Coefficient value	p-value
Land cover heterogeneity	Taxonomy Alpha diversity	4.42	0.014**
	Functional Alpha diversity	0.15	0.08
Insecticide use	Taxonomy Alpha diversity	-496.19*	1.39 x 10^{-7}**
	Functional Alpha diversity	-66.72*	7.78 x 10^{-4}**

*The negative value in the coefficient indicates a negative correlation between variables
** P value <0.05 indicate the correlation between taxonomy and Functional diversity is statistically significant

Based on the analysis of multiple linear regression equations, the coefficient value of the insecticide use variable on the taxonomic diversity and functional diversity of arthropods shows a higher number than the coefficient on the land cover heterogeneity variable. These results indicate that during the rainy season in the Bandung area, the use of insecticides has a greater influence on the characteristics of the arthropod community structure than the heterogeneity of land cover. As a result, this study supports the statement that the use of insecticides is one of the factors that have a major impact on the structure of the arthropod community, both in the dry season and the rainy season. High-intensity rainfall that occurs

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shortly after insecticide application can cause insecticides to be lost to non-target sites through surface runoff and leaching. This can result in less insecticide being available in the soil for plant uptake, which may not be sufficient to kill the target organisms. Consequently, the value of arthropod diversity in the rice ecosystem may decrease, as evidenced primarily by a decline in arthropod functional diversity. The decline in arthropod diversity, particularly among predators and parasitoids, leads to an increase in herbivorous arthropods that can negatively impact rice fields. This poses a threat to the success and sustainability of rice agroecosystems [31].

According to a study by Heong [8], in Asian countries including Indonesia, there is a misuse of insecticides, which results in the ineffective use of insecticides and the emergence of various environmental problems. Some common ways insecticides are misused include inaccuracies in determining the type of insecticide, the concentration of the insecticide used, and the time of application of the insecticide. In this study, the types of active ingredients used by farmers were deltamethrin and diazinon. Both of these active ingredients are included in broad-spectrum insecticides, which are not recommended because they can have negative effects on non-target organisms [32-34]. Even though the amount of insecticide used is far from a lethal dose for high-level organisms, continuous exposure to insecticides causes accumulation in organisms and causes biomagnification effects that are harmful to all organisms in the ecosystem [8, 35].

The incompatibility of insecticide concentrations with the characteristics of the arthropod community structure is another issue that frequently arises when using insecticides. The findings of this study concur with those of Ali [36], who found that controlling arthropod pest populations by boosting insecticide concentrations is often not effective. Due to the loss of controlling arthropods, the increase in improper insecticide concentrations causes an explosion in the population of pest arthropods.

Another crucial element in the application of pesticides is the time of the spraying. Insecticide should not be applied to rice plants during the vegetative phase, which is the rice age range of 20-44 DAP [8]. However, in this study, pesticides were sprayed in the range of 20-50 DAP. The vulnerability of parasitoids and predatory arthropods in groups is increased when insecticides are applied to rice plants during their vegetative phase. This study showed that the abundance of herbivorous arthropods tended to be better under control in paddy fields without insecticides than in paddy fields with insecticides. The study by Ali [36] found that the natural control provided by the natural enemies of herbivorous arthropods is sufficient to avoid outbreaks of these insects in a relatively small rice agroecosystem, negating the need for insecticide use.

In this study, an explosion in herbivorous arthropod populations was observed with the use of an insecticide active ingredient of 0.06 g/m^2, in paddy B. In the late vegetative phase (50 DAP), 79% of paddy B arthropods were herbivore arthropod, which had the potential to become pests of rice plants. Meanwhile, the abundance of controlling arthropods such as predators (18%) and parasitoids (3%) is much lower. Herbivore arthropods that experienced a relatively high increase in abundance between 30 and 50 DAP are the families *Ephydridae* (2,257 individuals), *Erebidae* (712 individuals), *Cicadellidae* (411 individuals), and *Acrididae* (281 individuals).

The family *Ephydridae* (Order: Diptera) is known as the rice whorl maggot. The larvae of this group of arthropods attack rice plants by making holes in the stems and leaves; they use the mesophyll tissue as a nutrient source and refuge. The life span of the *Ephydridae* family from egg to adult is generally 25–28 days. The relatively short life span, the low exposure to insecticides in larva stage, and the reduced abundance of predators due to insecticides are thought to be closely related to the increase in the arthropod population [37]. Similar to *Ephydridae*, the *Erebidae* family (Order: Lepidoptera) attacks rice plants in the larval stage. According to Kurmi [38], the *Erebidae* family is one of the predominant families commonly found in rice agroecosystems, so it is common to find them in high numbers in a rice agroecosystem. The existence of these two families threatens the growth of rice plants. The plants will tend to be stunted, delays in the early reproductive and seed maturation phases [39].

Acrididae is one of the families with the highest abundance based on the results of this study. Both the *Acrididae* and *Cicadellidae* families are arthropods that attack directly by consuming nutrients from rice plants and causing rice plants to become unproductive. In addition, arthropods in the *Acrididae* and *Cicadellidae* families act as vectors for fungal, bacterial, and viral diseases, i.e., *Nephotettix* sp., which causes turgor disease, which is harmful to rice plants [39].

In contrast, the usage of insecticides in this study threatens the families of predatory arthropods *Formicidae* and *Miridae* in danger. Both are capable of managing herbivorous arthropods that may harm rice plants by functioning as broad-range predators. Nevertheless, predatory arthropods from both groups are more vulnerable to insecticide usage than arthropods that feed on plants [39]. As a result, adjusting the application of insecticide to the specific arthropod pest is the most effective strategy for preventing pest attacks on the rice agroecosystem. Information on their abundance and the severity of the harm they cause is used to identify pest arthropods in agroecosystems.

The heterogeneity of land cover in each rice agroecosystem is one of several environmental factors that must be taken into account when adjusting the timing of application and the dose of insecticides used. In order to model arthropod population patterns and to develop preventive measures that can maintain the sustainability of rice agroecosystems, including the use of ecological engineering, further studies and research

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involving modelling the dynamics of the arthropod community structure are required.

4. Conclusion

In general, the more diverse the land cover of the border zone in the rice agroecosystem, the more complex the arthropod community structure formed, which is characterized by higher taxonomic and functional diversity values, particularly during the early vegetative phase of rice plants (30HST). In contrast, the greater the use of insecticides, the less diverse the arthropod community. Moreover, there are indications that during the rainy season, the use of insecticides has a greater influence on the structure of the arthropod community than land cover heterogeneity, which is similar to the results of a study by Sattler [4, 10] in the dry season in Vietnam.

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Optimizing the Wet Fermentation of Ateng Coffee (Arabica) with the Addition of Yeast R1-TKSU and LAB (*Leuconostoc suionicum*) Inoculum

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Abstract

Ateng coffee is an Arabica coffee that generally has a high selling value because of its better taste compared to other coffees. However, ateng coffee has poor post-harvest processing, resulting in low coffee quality and an impact on low coffee selling prices. In this study, optimization of wet fermentation using yeast R1-TKSU and LAB (*Leuconostoc suionicum*) inoculums was used to improve the quality of ateng coffee. Treatment variations were fermentation with the addition of yeast, LAB, yeast : LAB 1:1, and there is also a control without the addition of inoculum. Sensory assessment showed that the best cupping score was the sample added by LAB with a total score of 86.5. The contents of malic acid, citric acid, lactic acid, sucrose, fructose, and glucose and ethanol which were analyzed by HPLC were found to be relatively stable in green coffee beans. Fermentation added inoculum affects the concentration of acetic acid. Amino acid analysed showed that overall the highest concentration of amino acids in green coffee beans was in the yeast : LAB 1:1 treatment. It can be concluded that controlled wet fermentation with the addition of yeast R1-TKSU and LAB (*Leuconsotoc suionicum*) inoculums can improve the quality of ateng coffee.

Keywords: fermentation, coffee, optimization, yeast, lactic acid bacteria, metabolites

1. Introduction

Indonesia is the fourth largest coffee exporting country in the world [1] however, the coffee produced is of low quality when compared to the other 3 largest exporting countries, namely Colombia, Vietnam and Brazil [2]. This is caused by poor post-harvest applications [3].

One of the coffees with poor post-harvest implementation is ateng coffee. Ateng coffee is a type of Arabica coffee originating from Kec. Sumbul, Kab. Dairy, Prov. North Sumatra. Farmers in the area carry out wet fermentation of ateng coffee traditionally without controlling the temperature, causing the fermentation to not go well because the growth of fermenting microorganisms is highly dependent on temperature [4–6]. This traditional fermentation occurs for all-night (±12 hours), then the farmers dried using sunlight for 2 – 4 hours at a temperature of ± 27°C (depending on the weather), then the farmers sell the coffee to coffee agents at a low price. With a drying time of 2-4 hours, the water content of green coffee beans is still too high. High water content can facilitate the growth of fungi and can produce mycotoxins which are harmful to human health [7], for this reason coffee should be dried for 7-14 days to reach a standard water content range between 8.0% and 12.5% [8–10]. Subsequent coffee processing is carried out by coffee agents, but the initial processing by farmers will certainly affect the quality of the subsequent coffee.

One of the most important stages in improving coffee quality is coffee fermentation [11, 12]. Coffee fermentation occurs naturally, microorganisms are present by utilizing various compounds in the pulp and mucilage as nutrients [12]. These microorganisms can produce metabolites that affect the taste of coffee drinks [13], yeast and bacteria play an important role in producing coffee flavor precursors in the form of esters, organic acids, ethanol, amino acids, volatile compounds [11, 14–17].

Many studies have been conducted on coffee fermentation with the addition of yeast and bacteria inoculums [18– 22]. Pereira et al., (2016) in his research fermenting Arabica coffee with *Lactobacillus plantarum* LPBR01, the results showed that the inoculum was able to increase the formation of aroma compounds so that the quality of the coffee was increased compared to conventional methods [16]. C. Wang et al., (2020) stated that coffee fermentation with the addition of yeast inoculum can increase the content of ester compounds in coffee beans which act as flavor precursors [15].

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In this study, efforts to improve the quality of ateng coffee were optimizing the wet fermentation of ateng coffee by adding inoculums, specifically yeast R1-TKSU and Lactic Acid Bacteria (LAB) *Leuconostoc suionicum*. These two isolates, derived from earlier studies, demonstrated specific enzymatic capabilities: yeast R1-TKSU was able to produce pectinase, and LAB (*Leuconostoc suionicum*) was able to produce amylase, cellulase and protease [23, 24]. The enzymatic activities of these microorganisms are crucial because they break down polysaccharides (pectin), cellulose, and starch in coffee mucilage [12], which are a source of nutrition for microorganisms [13]. This fermentation is expected to be more optimal, so that the microorganisms produce metabolites/flavor precursors such as organic acids, amino acids, volatile compounds which can diffuse into the green coffee beans thereby affecting the taste of coffee [13, 16, 17]. Yeast R1-TKSU has been used in the fermentation of Arabica coffee originating from West Java. The fermentation results show that the addition of yeast R1-TKSU can improve the taste of coffee [23]. Meanwhile, the LAB (*Leuconostoc suionicum*) has been used in the fermentation of robusta coffee originating from West Java. The fermentation results show that the addition of LAB (*Leuconostoc suionicum*) is able to eliminate the unpleasant taste of coffee [24]. This study aims to improve the quality of ateng coffee from Sumbul, Dairi, North Sumatra by optimizing wet fermentation using yeast R1-TKSU and LAB (*Leuconostoc suionicum*).

2. Methodology

2.1. Materials

Ateng coffee cherries originating from Sumbul, Dairi - North Sumatra, green coffee beans, yeast R1-TKSU dan LAB (*Leuconostoc suionicum*) Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), Nutrient Broth (NB), Nutrient Agar (NA), de Man Rogosa Sharpe Broth (MRSB), de Man Rogosa Sharpe Agar (MRSA), NaCl,Aquadest, 70% alcohol, Pro-analytical alcohol, rubbing alcohol, acetonitrile, methanol grade HPLC, and deionized water.

2.2. Sample Preparation and Fermentation

The selected ateng coffee cherries are mechanically pulped using a local farmer's pulper machine. Two kg of peeled coffee was fermented with the addition of yeast R1-TKSU inoculum, LAB (*Leuconostoc suionicum*), and yeast R1-TKSU : LAB (*Leuconsotoc suionicum*) 1:1, there was also a control without the addition of inoculum. Henceforth, treatment with the addition of yeast R1-TKSU inoculum will be referred to as "yeast treatment", treatment with the addition of LAB (*Leuconostoc suionicum*) inoculum will be referred to as "LAB treatment", treatment with the addition of yeast : LAB - 1:1 inoculum will be referred to as "yeast : LAB – 1:1". The inoculum concentration used was 10% (v/w), with cell density of 10^8 CFU/mL. Fermentation occurred for 12 hours, at 30ºC. During fermentation, samples were taken every 4 hours to determine the dynamics of the microbial population and the pH value. After fermentation, the coffee beans are washed thoroughly, then dried in the sun until the water content reach standard water content range between 8.0% and 12.5% [8– 10]. Then the parchment skin / silver skin is peeled to obtain the green coffee beans.

2.3. Analysis of Organic Acids

The organic acid analysis refers to Figueroa Campos et al., (2020), 100 mg of ground coffee beans are dissolved in 10 mL of deionized water. Extraction was performed at room temperature under shaking conditions for 30 min. Subsequently, the suspension was centrifuged at 9300× g for 10 minutes. After the pellet and supernatant were separated, the supernatant was collected and then stored at 4ºC for further quantification. Organic acids content was determined using a HPLC system (Shimadzu UFLC HPLC system). Analyses was performed with column C18 (4.6 x 250 cm, 5 um), mobile phase of aceto- nitrile, 0.1% H3PO4. Analysis was performed for lactic acid, acetic acid, citric acid and malic acid. Standard solutions were used to determine the concentration of the organic acids being analyzed.

2.4. Analysis of Sugar and Ethanol

For the sugar and ethanol analysis refers to the method of Con- stantino et al., (2020), 500 mg of green coffee beans that have been mashed are dissolved in 20 mL of deionized water, then shaken for 90 minutes at 60ºC. The suspension was centri- fuged at 1048 x g for 10 minutes, then the supernatant was transferred to a new small glass tube and then stored in the refrigerator at 4ºC for further quantification. Sugars and ethanol analysis were determined by HPLC system (Shimadzu UFLC HPLC System), with a column Shimadzu SCR 101-C, deion mobile phase. Analysis was performed for sucrose, glucose, fructose, and ethanol. Standard solutions were used to determine the concentrations of the sugars and ethanol analyzed.

2.5. Analysis of Amino Acid

Amino acid analysis was carried out at PT. Saraswanti Indo Genetech, Bogor using Ultra-performance Liquid Chromatography (UPLC). The analysis followed the rules that apply in the laboratory. A sample of 0.1 – 1 gram of green coffee bean was placed into a 20 mL headspace vial, then the coffee beans were hydrolyzed with HCL. The hydrolysis results were transferred into a 50 mL measuring flask. In the hydrolysis results that have been transferred, aquabidest was added up to the tera mark, then homogenized. The sample solution was filtered with a 0.2 µm filter syringe, then the filtrate was collected. The process the continues to the derivatization stage, which the solution was injected into the UPLC system. The column used was C18, the mobile phase was Eluent Accq.

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Ultra Tags; Aquabidest, with a gradient pump system, column temperature 49°C, the detector used was PDA.

2.6. Cupping Test

The cupping test was carried out by Q-grader at the Coffee and Cocoa Research Center, Jember. Cupping test on fermented green coffee beans using the Specialty Coffee Association of America method. The parameters tested were Fragrance/Aroma, Flavor, Aftertaste, Acidity, Body, Uniformity, Balance, Clean cup, Sweetness, Overall. Parameter scale from 1 – 10 points. The results of the evaluation of each parameter are summarized as an explanation of the final coffee score [27].

2.7. Statistic Analysis

Statistical analysis was performed using SPSS Statistics 21, with a 95% confidence level. One-way analysis of variance (ANOVA) was performed and the statistical significance of the difference ($p<0.05$) was evaluated followed by Dun- can's multiple range test (DMRT).

3. Result and Discussion

3.1. Dynamics of Microorganisms Population

In the control treatment, yeast was the predominant microorganism, followed by total bacteria, while LAB had slower growth compared to yeast and total bacteria (Figure 1a). This is also shown by the growth rate of each microorganism: LAB (0.018/hour) grew slower than yeast (0.026/hour), and total bacteria (0.029/hour).

3.1.1. Fermentation with Yeast Treatment

In fermentation with yeast treatment, the yeast population dominates the microbial community (Figure 1b). The addition of yeast did not seem to affect the condition of the LAB, because the growth of LAB was observed to be the same as LAB in control treatment (Figure 1.b). The growth rate of LAB in the control and LAB with yeast addition showed similar figures: LAB in control treatment was 0.018/hour, while LAB in yeast treatment was 0.015/hour. However, it is hypothesized that the addition of yeast affects the number of total bacteria so that the number of total bacteria is less than yeast (compare yeast in control conditions and yeast in the addition of yeast treatment) (Figure 1b). This may occur due to nutritional competition, as both yeast and total bacteria use the same nutrient such as glucose [28–30], while indigenous LAB can use fructose [31].

In yeast treatment, a decrease in number of yeast cells was observed at the 8th hour of fermentation. This decrease could be caused by the high accumulation of ethanol and other alcohol-derived compounds produced by yeast metabolism [32]. In this treatment, the number of yeast cells at 0 hour was significantly higher compared to the number of LAB cells and total bacteria (yeast: $2x10^8$ CFU/mL, LAB: $1x10^7$ CFU/mL, Total Bacteria: $3x10^7$ CFU/mL) (Figure 1.b), resulting in yeast dominating the microbial community. This yeast dominance was clearly visible until the 4th hour of fermentation (yeast: $2x10^9$ CFU/mL, LAB: $3x10^7$ CFU/mL, Total Bacteria: $3x10^7$ CFU/mL) (Figure 1.b). Although the number of yeast cells in this treatment did not exceed the number of yeast cells in the control treatment, yeast dominance is thought to have caused the high accumulation of ethanol (38,2 mg/g) (Figure 3d). However, the high accumulation of ethanol compounds, produced by yeast metabolism is thought to have caused the decrease in the number of yeast cells in the 8th hour of fermentation. The high accumulation of ethanol compounds and other alcohol derivatives is supported by the dominance of yeast cells at the beginning of fermentation (0 to 4th hour of fermentation) (Figure 1b).

3.1.2. Fermentation with LAB Treatment

The addition of LAB was able to increase the amount of LAB when compared to LAB in the control and in yeast treatment (Figure 1c). Microorganisms' growth conditions in the LAB treatment at the start of fermentation (0 hour) showed that LAB fermentation had higher cell count compared to LAB in the control and LAB in yeast treatment (Figure 1a, 1b, 1c). At the 12th hour, LAB in fermentation with LAB treatment also still had a greater cell number compared to LAB in the control and in yeast treatment. This indicates that the addition of LAB inoculum was able to increase the number of LAB when compared to LAB in control and in yeast treatment. In the LAB treatment, at 8th hour, the number of yeast cells remained constant. This is thought to be related to the condition of the LAB cells during fermentation, where in the LAB treatment the number of LAB cells was in large numbers (the number of LAB cells in the LAB treatment had larger numbers than LAB in the control treatment, and LAB in yeast treatment, respectively in CFU/mL: $3x10^9$, $7x10^8$, $9x10^7$, Figure 1).

He et al., (2021) in their research stated that LAB can inhibit yeast growth by releasing organic acids or competing for nutrients and physical space. However, the presence of LAB may provide benefits for yeast growth such as promoting or inhibiting various metabolic processes in yeast cells: metabolism of trehalose, ergosterol, certain amino acids, proton pumps, stress response transcription activators. Additionally, LAB presence is also able to increase yeast tolerance to ethanol [33]. This suggests that there is a complex relationship between yeast and LAB.

Therefore, based on the explanation above, there are two hypotheses regarding the cause of the constant number of yeast cells in the 8th hour of fermentation. The first hypothesis is that LAB inhibits yeast growth by releasing organic acid compounds, or through competition for nutrients so that yeast cell growth does not increase, but remains constant.

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The second hypothesis is that the presence of LAB increases yeast tolerance to ethanol so that yeast growth does not decrease, but remains constant.

On the other hand, the condition of the constant number of yeast cells at the 8th hour of fermentation is thought to have an influence on the condition of the total bacteria which is also constant at the 8th hour of fermentation. Based on previous hypothesis about yeast condition, the presence of LAB can increase yeast tolerance to ethanol, but the yeast still produces ethanol. Ethanol production by yeast can lower oxygen levels in the fermentation environment. This situation is less favorable for the growth of total bacteria because the total bacteria which is likely consists of aerobic bacteria. This suggests that aerobic bacteria cannot grow, leading to no increase in total bacterial growth. In addition, based on previous hypothesis, there is inhibition of yeast cell growth by LAB. Inhibition yeast growth may reduce the concentration of ethanol in the fermentation environment [34, 35]. This ensures the fermentation environment have sufficient oxygen concentration so that total bacterial growth did not decrease, but remains constant. At the 12th hour of fermentation, there was an in- crease in yeast cells and total bacteria. This could be due to reduced ethanol concentration from the fermentation environment because ethanol had diffused into the coffee beans [36], or evaporated [37]. This evaporation can be supported by stirring during sampling so that the ethanol exits the fer- mentation environment. Stirring also facilitates the addition of oxygen into the fermentation environment, supporting the growth of total bacteria.

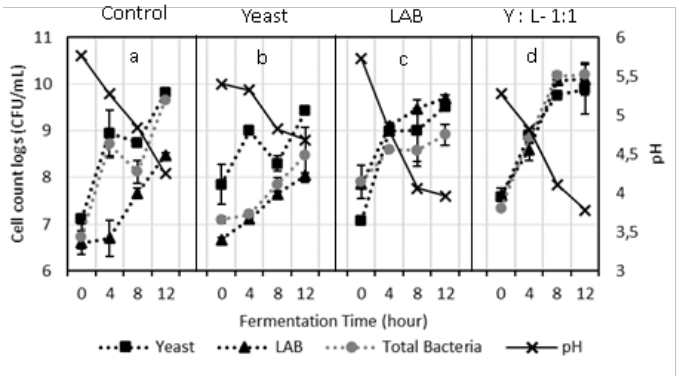

Figure 1. Population Dynamics of Microorganisms, and pH in All Fermentation Treatments. The fermentation scale was 2 kg of coffee beans, 10% inoculum concentration (v/w), with an initial cell density of (b) Yeast 1 x 10^8 CFU/mL, (c) LAB 1.1 x 10^8 CFU/mL, (d) Yeast 1.25 x 10^8 CFU/mL; LAB 1.35 x 10^8 CFU/mL.

3.1.3. Fermentation with Yeast : LAB – 1:1 Treatment

In the yeast : LAB - 1:1 treatment, the growth of microorganisms had a different pattern compared to the growth of microorganisms in other treatments. In other treatments, at the 8th hour of fermentation, there was a decrease in yeast cell growth (as seen in the control treatment and yeast treatment) and there was constant yeast cell growth (as seen in the LAB treatment). However, in the yeast : LAB - 1 : 1 treatment, yeast growth continued to increase (Figure 1). Similarly, at the 8th hour of fermentation, there was a decrease in total bacterial cell growth (in the control treatment) and there was constant total bacterial cell growth (in LAB treatment), while in the yeast : LAB - 1:1 treatment, total bacterial growth continued to increase (Figure 1).

Apart from that, in the treatment yeast : LAB - 1:1, the growth of yeast, LAB, and total bacteria at each sampling hour relatively consistent (Figure 1d). The growth of microorganisms in the yeast : LAB - 1:1 treatment can be caused by positive interactions between the added inoculum, that are yeast and LAB. As previously explained by He et al., (2021), LAB can inhibit yeast growth, but on the other hand, it can provide benefits for yeast growth. This is a complex relationship between yeast and LAB. Another study by Pregolini et al., (2021) states that there is a positive interaction between yeast and LAB, that yeast autolysis will release nutrients such as polysaccharides, riboflavin, and amino acids for the growth of LAB. The growth of LAB will make the fermentation medium acidic and create a favorable environment for yeast development [38]. There is a hypothesis that this positive interaction between yeast and LAB occurs because the amount of inoculum inoculated is the same. So that the same growth between yeast and LAB at this starting point mutually supports the growth of yeast and LAB. Apart from that, Canon et al., (2020) also stated that co-inoculum treatment can increase the content of peptides, amino acids, organic acids, volatile compounds which can better support microbial growth compared to single inoculum treatment. Based on this, it is hypothesized that the total growth of bacteria which continues to increase in the 8th hour of fermentation is supported by the growth of yeast and LAB which have a positive interaction so that the organic acid content, volatile compounds and peptide content are able to support the total growth of bacteria.

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3.1.4. pH Value

The pH value during the fermentation treatment decreased. The decrease in pH value is caused by the consumption of carbon compounds by microorganisms that produce organic acids such as lactic acid or acetic acid [32], as well as organic acids found in coffee mucilage such as citric acid [40]. In all treatments with the addition of LAB inoculum, that are the LAB treatment and the yeast : LAB-1:1 treatment, the final pH had a lower value compared to the other treatments (final pH value of each treatment: control: 4.25, yeast : 4.68, LAB : 3.97, yeast : LAB - 1:1 : 3.87) (Figure 1). This is due to the large number of LAB cells in this treatment (Figure 1c, d) which produce lactic acid or acetic acid, because the inoculated LAB (*Leuconsotoc suionicum*) is a heterofermentative type of LAB [41]. Meanwhile, the final pH value in the yeast treatment was the highest among all treatments (Figure 1). This can be caused by the growth conditions of microorganisms in this treatment, where the growth of microorganisms is dominated by yeast or the growth of LAB is not higher than yeast (Figure 1b).

3.2. Organic Acids

Citric acid and malic acid are naturally present in green coffee beans [42, 43]. The concentrations of malic acid and citric acid in green coffee beans in all fermentation treatments did not have a significant difference (p-value > 0.05) (Figure 2a, 2b). The content of malic acid and citric acid can contribute to the citric and herbaceous taste after roasting [43]. In addition, malic acid can also provide apple flavor [44], but it did not appear in this study.

The presence of lactic acid and acetic acid in green coffee beans is the result of microorganisms metabolism during fermentation [45]. Lactic acid can give a sour, astringent, and acrid or sharp taste [43]. Lactic acid can also give a buttery taste [44], but this taste did not appear in this study. Meanwhile, acetic acid can give a fruity taste when it is at low concentrations [46]. The presence of acetic acid at low concentrations is desirable because high concentrations of acetic acid will give an unpleasant taste. The highest concentrations of lactic acid and acetic acid were in green coffee beans treated by the yeast : LAB – 1:1, with lactic acid 264,88 mg/g and acetic acid at 19,84 mg/g (Figure 2c, 2d). The high concentration of lactic acid and acetic acid in this treatment was caused by lactic acid bacteria has high growth (12th hour of fermentation number of cells reach 3x10^{10} CFU/mL), in which the inoculated LAB (*Leuconsotoc suionicum*) is a heterofermentative bacteria that converts carbon compounds into lactic acid, acetic acid, CO_2, and ethanol, through phosphoketolase or pentose phosphate pathway [47]. In addition, yeast : LAB – 1:1 addition also had the lowest pH value compared to other treatments. The decrease in pH value is caused by organic acids resulting from the metabolism of fermented microbes [32].

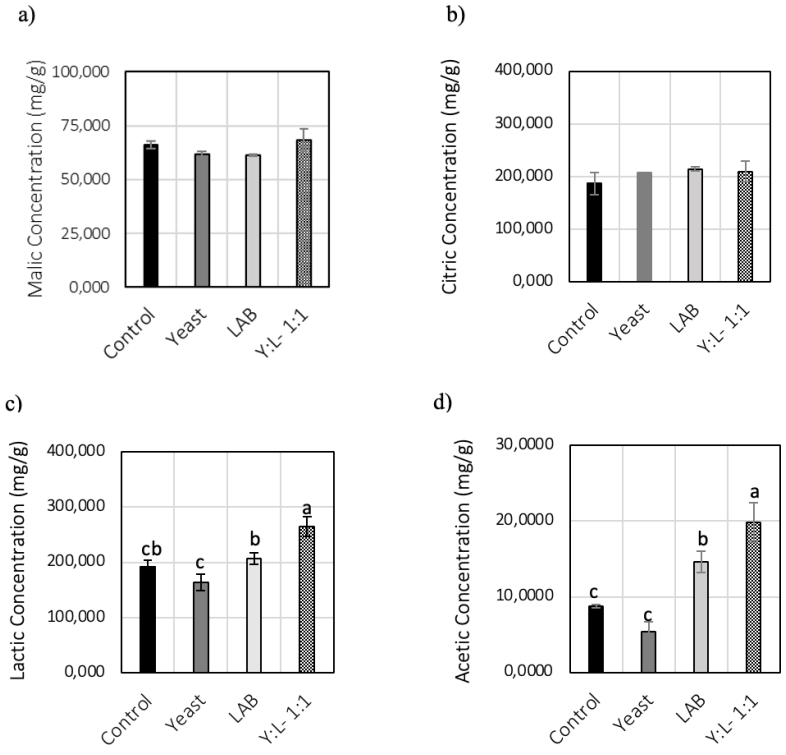

Figure 2. Content of (a) malic acid, (b) citric acid, (c) lactic acid (d) acetic acid in green coffee beans from all fermentation treatments. The vertical bar represents the standard deviation. Different letters represent significant differences on the Duncan Multiple Range Test at a significant level of 0.05.

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3.3. Sugar and Ethanol

In this study, the sucrose content was much higher than glucose and fructose (Figure 3a, 3b, 3c). According to Knopp et al., (2006), the concentration of fructose and glucose in green coffee beans was 20 times lower than sucrose. This difference was due to the abundant presence of sucrose in the beans, while glucose and fructose were abundant in the coffee pulp and mucilage [48]. In this study, the sucrose, glucose, and fructose content of green coffee beans from all fermentation treatments did not differ significantly from one another (p-value > 0.05), indicating that variations in fermentation treatments did not have a large effect on the sucrose, glucose, and fructose content of coffee.

The low concentration of glucose and fructose in green coffee beans observed in this study is related to the wet processing process [49], which is pulp stripping process where glucose and fructose are abundant in pulp and mucus [48]. In contrast, glucose and fructose will be found in higher level in coffee beans processed using dry method [49].

Sucrose, fructose, and glucose are thought to play an important role in the organoleptic quality of coffee. Sucrose can decompose during roasting to release several aroma and taste precursors [50], while fructose and glucose are also precursors in the formation of coffee flavors [51]. Sugar as a precursor will produce volatile and non-volatile compounds, such as furan, pyrazine, aliphatic acid, and hydroxymethyl furfural which affect the taste of coffee [52]. Higher sugar content is associated with better cup quality [53].

The ethanol content in green coffee beans did not only come from yeast [54], but also *Leuconostoc suionicum*, a type of heterofermentative LAB that produces ethanol [41]. The highest concentration of ethanol in the green coffee beans was resulting from the yeast treatment, while the ethanol concentration of green coffee beans from the other treatments was lower and not much different from one another (Figure 3d). The high concentration of ethanol in green coffee beans treated with yeast was supported by a decrease in the number of yeast cells at the 8th hour during fermentation. As mentioned above, the decrease in the number of many cells is caused by the accumulation of high concentration of ethanol and other alcohol-derived compounds resulting yeast cells cannot survive and their numbers decrease.

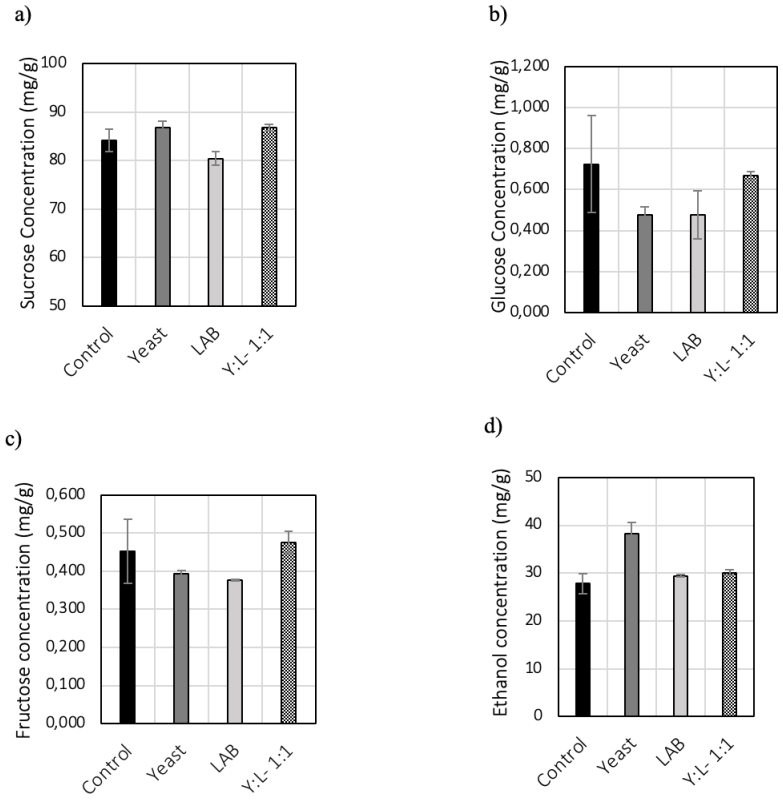

Figure 3. Contents of (a) sucrose, (b) glucose, (c) fructose, and (d) ethanol in green coffee beans in all fermentation treatments. The vertical bar represents the standard deviation.

3.4. Amino Acid

Amino acids are flavor precursors in coffee [55, 56]. When green coffee beans are roasted, these precursors play a role in Maillard and Strecker degradation reactions forming aldehydes, furans, pyrazines, pyridines, oxazoles, ketones, phenols, pyrroles [57]. The amino acid content in green coffee beans is affected by protein or peptide catabolism by fermenting microorganism [58, 59], metabolism of microorganisms

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during fermentation [60], or through the germination process that occurs in coffee beans during processing [42].

The amino acid concentrations in this study can be seen in Table 1. Green coffee beans resulting from the yeast and LAB treatment have 5 amino acids with slightly higher concentrations than the control. These amino acids (respectively for control, yeast, and LAB) are phenylalanine (6.43 - 7.15 - 7.13 mg/g), arginine (6.86 - 7.30 - 7.28 mg/g), aspartic acid (7,45 - 7,66 - 7,76 mg/g), threonine (5.10 - 5.29 - 5.16 mg/g) histidine (2, 82 - 3.009 - 3.01 mg/g). Meanwhile, green coffee beans treated with yeast : LAB-1:1 exhibit higher concentrations of four specific amino acids compare to those treated with yeast and LAB. These amino acids and their concentrations are: phenylalanine (7.71 mg/g), arginine (8.22 mg/g), tyrosine (4.14 mg/g), histidine (3.31 mg/g). Apart from that, in the yeast : LAB 1:1 treatment there were also 6 amino acids with higher concentrations compared to other treatments: serine (7.35 mg/g), isoleucine (4.18 mg/g), valine (5.31 mg/g), alanine (5.14 mg/g), glycine (8.55 mg/g), leucine (5.58 mg/g), so there were 10 amino acids with higher concentrations in green coffee beans treated with yeast : LAB 1:1 compared to control. This indicates that the yeast and LAB treatment were able to increase the concentration of amino acids over the control, but the treatment of yeast : LAB-1:1 resulted in even greater increases the amino acid concentrations compared to treatment with yeast and LAB alone.

The high concentration of amino acids in green beans coffee resulting from the treatment of yeast : LAB- 1:1 is in accordance with research by Canon et al., (2020) which states that co-culture treatment can increase the concentration of amino acids in food fermentation. The high concentration of this amino acids also indicates that there is a positive interaction between yeast and LAB, which Pregolini et al., (2021) in their research stated that co-culture treatment can increase metabolite production.

Table 1. Amino acid content of green coffee beans in all fermentation treatments.

Amino Acid	Treatment			
	Control	YEAST	LAB	Y:L- 1:1
	(mg/g)	(mg/g)	(mg/g)	(mg/g)
L-Serine	6,88 ± 0,018	6,99 ± 0,024	6,88 ± 0,013	7,35 ± 0,027
L-Glutamic Acid	12,27 ± 0,045	11,50 ± 0,034	12,07 ± 0,057	12,14 ± 0,009
L-Phenylalanine	6,43 ± 0,019	7,15 ± 0,017	7,13 ± 0,027	7,71 ± 0,006
L-Isoleucine	3,99 ± 0,002	3,99 ± 0,013	3,88 ± 0,008	4,18 ± 0,002
L-Valin	5,05 ± 0,021	4,91 ± 0,010	4,95 ± 0,009	5,31 ± 0,005
L-Alanine	5,004 ± 0,019	5,05 ± 0,008	5,04 ± 0,010	5,14 ± 0,006
L-Arginine	6,86 ± 0,037	7,30 ± 0,027	7,28 ± 0,016	8,22 ± 0,017
L-Glycine	7,99 ± 0,042	8,07 ± 0,028	8,07 ± 0,028	8,55 ± 0,011
L-Lysine	4,86 ± 0,015	4,44 ± 0,011	4,69 ± 0,014	4,64 ± 0,002
L-Aspartic Acid	7,45 ± 0,028	7,66 ± 0,014	7,76 ± 0,025	7,67 ± 0,013
L-Leucine	8,38 ± 0,031	8,13 ± 0,009	8,05 ± 0,020	8,58 ± 0,008
L-Tyrosine	3,38 ± 0,008	3,68 ± 0,002	3,59 ± 0,010	4,14 ± 0,000
L-Proline	5,63 ± 0,014	5,50 ± 0,009	5,43 ± 0,020	5,66 ± 0,007
L-Threonine	5,10 ± 0,023	5,29 ± 0,020	5,16 ± 0,017	5,60 ± 0,012
L-Histidine	2,82 ± 0,007	3,009 ± 0,00	3,01 ± 0,002	3,31 ± 0,004

*Values represent mean ± SD (n=2).

3.5. Cupping Test

The cupping test results from fermentation optimization, which consist of 10 characteristics with the final score, are shown in Table 2. All samples have a cupping score which is classified as specialty coffee because the total value produced is >80 [27]. However, the sample that underwent fermentation with the addition of inoculum had a higher cupping score than the control.

The control and yeast treatments shared the same flavor notes: brown sugar, nutty, flowery, honeyed, spicy-coriander seed like, and rather woody. The LAB treatment has notes of honeyed, flowery-coffee blossom, nutty, and spicy-chili like. The yeast : LAB- 1:1 treatment has notes of honeyed, flowery-coffee blossom, and lemony.

The same flavor notes of control and yeast treatment can occur because of the similarity in the pattern of microbial growth, which are both dominated by yeast. However, the yeast treatment has a higher cupping score than control treatment (86.00 > 84.50) (Table 2). This could be due to the presence of amino acids with higher concentrations than the control, such as phenylalanine, arginine, aspartate, threonine, histidine. Yeast can produce flavor precursors such as aldehydes, ketones and fatty acid esters [61]. It is hypothesized that in yeast treatment, the dominance of the yeast population produces flavor precursors in higher concentrations than in the control treatment, thus this supports the cupping score yeast treatment was higher than the control treatment. In addition, green coffee beans treated with addition of yeast had the high-

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est concentration of ethanol compared to other treatments. Ethanol produced by yeast is known to also act as an aroma precursor [37]. Ruta & Farcasanu, (2021) in their research stated that alcohol produced by yeast goes through the ester formation stage, in which ester formation contributes to flowery and fruity notes in coffee. The high concentration of esters can have a positive impact on coffee quality [17].

The LAB treatment had the highest cupping score of all treatments that is 86,5 (Table 2). LAB is able to produce lactic acid and acetic acid which play a role in the taste of coffee, LAB is also able to produce 4-carbon compounds, active flavor compounds, including diacetyl, acetoin, and 2,3-butanediol by metabolizing citric acid [14], LAB is also able to produce mannitol (sugar alcohol) as a result of fructose reduction [13, 62, 63]. In the fermentation process, LAB is able to break down amino acids which has implications for the formation of low molecular weight compounds, such as aldehydes, esters, carboxylic acids, and alcohols which can give flavor to coffee [14]. The high number of LAB cells in this treatment indicated the possibility of the presence of the metabolites mentioned above in high concentrations as well, thus being the cause of the high cupping score. In addition, in this treatment, the number of yeast cells is also high, as has been stated that yeast is able to produce ethanol [37], aldehydes, esters [61] which contribute to the taste of coffee.

Table 2. Cupping Test results in all fermentation treatments.

Characteristic	Treatment			
	Control	YEAST	LAB	Y:B-1:1
Fragrance/aroma	8,00	8,50	8,50	8,00
Flavor	8,00	8,00	8,00	8,00
Aftertaste	7,50	8,00	8,00	7,75
Acidity	8,00	7,75	8,00	8,00
Body	7,75	8,00	8,00	7,75
Uniformity	10,00	10,00	10,00	10,00
Balance	7,75	7,75	8,00	7,75
Clean cup	10,00	10,00	10,00	10,00
Sweetness	10,00	10,00	10,00	10,00
Overall	7,50	8,00	8,00	7,75
Final Score	84,50	86,00	86,50	85,00

As mentioned above, there is a hypothesized that yeast and LAB that grow in yeast : LAB – 1:1 treatment had a positive interaction which able to increase metabolite production [38, 39]. This indicates that there is a high probability of the presence of metabolites that affect the taste in high concentrations such as aldehydes, esters, ethanol compounds. In addition, amino acid testing also proved that the concentration of amino acids (role as flavor precursors) was higher in green coffee beans treated with yeast : LAB-1:1 than other treatments (Table 1). However, the cupping score in this treatment did not exceed the cupping score in the yeast treatment and LAB treatment. By comparing the pattern of microbial growth during fermentation, there is one difference between yeast, and LAB treatment, and the yeast : LAB treatment, the difference is in the growth pattern of total bacteria.

In yeast treatment and LAB treatment, total bacteria grew in lower abundance than yeast and LAB. Meanwhile, in the yeast : LAB-1:1 treatment, the total bacteria grew well, showing growth similar to that of yeast and LAB (Figure 1). This can be caused by the positive interaction between yeast and LAB which produces many metabolites such as peptides, amino acids, organic acids so that they can support total bacterial growth [39]. In this study, it was not carried out to check the types of bacteria that were present during fermentation, but this can be learned from other studies, in which there is one type of microbial family that is generally present in coffee fermentation, that is Enterobacteriaceae [12, 64–66]. The Enterobacteriaceae family is generally present in coffee because it can come from water or from the coffee fruit itself [38]. Research in Indonesia also states that Enterobacteriaceae is a microbe that plays a role in the fermentation of Arabica coffee, Jember, East Java [67]. Pregolini et al., (2021) stated that during coffee fermentation, Enterobacteriaceae was able to be present in high numbers until the 36th hour of fermentation even though there were lactic acid bacteria in high cell numbers. Enterobacteriaceae is one of the bacteria whose presence is undesirable because it is not related to the preferred flavor precursors in coffee, and these bacteria can produce off-flavor metabolites such as 3-isopropyl-2-methoxy-5-methylpyra- zine, 2,3-butanediol and butyric acid. Based on this, it does not rule out the role of Enterobacteriaceae in the fermentation of ateng coffee. Although in the yeast : LAB 1:1 treatment showed no defects (distorted taste), the compounds produced by total bacteria (presumably one of the species is Enterobacteriaceae) can cause other processes that contribute to the taste of coffee.

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This suggests that despite the pattern of microbial growth in yeast : LAB 1:1 treatment was better than yeast treatment, and LAB treatment; and the concentration of amino acids in the yeast : LAB 1:1 treatment higher than the addition of yeast, and addition of LAB treatments, but the presence of high total bacteria resulted in a cupping score that did not exceed the yeast, and LAB treatment.

3.6. Relationship of Organic Compounds with Coffee Flavors Formed

The role of amino acids, sugars, organic acids in forming flavors occurs when green coffee beans are roasted. Reactions that can form include Maillard and Strecker degradation which can produce flavor compounds [68]. The Maillard reaction is a non-enzymatic browning reaction that occurs between amino acids and sugars [69]. Strecker degradation is the reaction of free amino acids with carbonyl group derivatives from the Maillard reaction which causes the degradation of amino acids into aldehydes which contribute to the aroma of coffee. Maillard reactions and Strecker degradation can produce flavor compounds such as pyrazines, alcohols, esters, aldehydes, ketones, furans, thiazoles, pyrones, acids, imines, amines, oxazoles, pyrroles, and ethers [70].

The relationship of organic compounds with coffee flavors formed shown in Table 3. In all treatments there is a honeyed flavor. The honeyed flavor is formed through a Strecker degradation reaction, the compound that plays a role is phenylacetaldehyde which is a phenylalanine derivative [71,72]. This shows the important role of phenylalanine as a honeyed flavor precursor. The concentration of phenylalanine in green coffee beans treated with control, yeast treatment, LAB treatment, and yeast : LAB - 1:1 treatment increased sequentially.

Table 3. The relationship between amino acids and coffee flavor formed in all treatments.

Treatment	Amino Acid	Flavour	Reaction
Control	L-Fenilalanin	Honeyed	Strecker degradation
	L-Alanine L-Serin L-Threonine	Nutty	Maillard
	L-Fenilalanin L-Tyrosine	Flowery	Maillard
	L-Leucine	Brown sugar	Maillard
YEAST (R1-TKSU)	L-Fenilalanin ○	Honeyed	Strecker degradation
	L-Alanine ○ L-Serin ○ L-Threonine ○	Nutty	Maillard
	L-Fenilalanin ○ L-Tyrosine ○	Flowery	Maillard
	L-Leucine ○	Brown sugar	Maillard
LAB (*Leuconostoc suionicum*)	L-Fenilalanin ○	Honeyed	Strecker degradation
	L-Alanine ○ L-Serin ○ L-Threonine ○	Nutty	Maillard
	L-Fenilalanin ○ L-Tyrosine ○	Flowery-Coffee Blossom	Maillard
YEAST : LAB (1:1)	L-Fenilalanin ●	Honeyed	Strecker degradation
	L-Histidine ●	Lemony	Maillard
	L-Phenylalanine ●	Flowery-Coffee Blossom	Maillard

*Note: ●: the concentration of the amino acid is the highest in this treatment compared to other treatments. ; ○ The concentration of these amino acids was higher in this treatment than in the control.

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The nutty flavor is possessed by control, yeast, and LAB treatment. Compounds that play a role in the nutty flavor are alkylpyrazine [71] which are formed through the Maillard reaction [57], the amino acids that play a role are alanine, serine, and threonine [51]. The nutty flavor did not appear in coffee produced by yeast : LAB 1:1 treatment, presumably because there were other types of amino acids that were more dominant or the influence of other compounds [73].

All treatments exhibit flowery flavor, which could be related to the Maillard reaction, where amino acids such as glycine, proline, phenylalanine, and tyrosine contribute to this flavor [74]. Apart from going through the Maillard reaction, a flowery flavor can also appear due to the ester content in the green coffee beans, which is indicated by the presence of ethanol content in the green coffee beans. According to Ruta & Farcasanu (2021), alcohol produced by yeast goes through the stage of ester formation. Formation of the ester contributes to the floral sensory note [17].

Brown sugar flavor is a sweet chocolate flavor, this flavor is formed through the Maillard reaction in which the amino acid that plays a role is leucine [75].

The lemony flavor is classified into fruity, citrus flavors with a taste description of citric, sour, astringent, slightly sweet, flaky, and slightly floral aroma associated with lemon [76]. The lemony flavor can be formed through the Maillard reaction with histidine as an amino acid that plays a role [74]. In the yeast : LAB- 1:1 treatment, the lemony flavor formed was thought to be related to histidine which was present in the highest amount compared to other treatments. In addition to amino acids, the lemony flavor is associated with citric acid [77, 78]. The results of the citric acid test for all treatments did not show a significant difference with one another (p-val- ue > 0.05). However, because the concentration of citric acid was supported by the high concentration of histidine in the yeast : LAB1:1 treatment, enhancing the flavor. As previously mentioned, the taste of coffee is not only formed by one compound, but can be caused by several compounds [73].

Spicy and woody flavors are related to phenol compounds in green coffee beans [71, 79]. There is an assumption that the green coffee beans resulting from the control, the yeast, and LAB treatments have a high content of phenolic compounds that support the formation of spicy and woody flavors.

4. Conclusion

Optimization of the wet fermentation of ateng coffee with the addition of yeast R1-TKSU and LAB (*Leuconostoc suionicum*) can improve the quality of ateng coffee and compounds related to coffee flavor such as amino acids and organic acids. However, it is necessary to test the volatile compounds of green coffee beans which play an important role in the taste of coffee in order to further refine the knowledge of the influence of microorganisms on the formation of coffee flavor precursors.

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