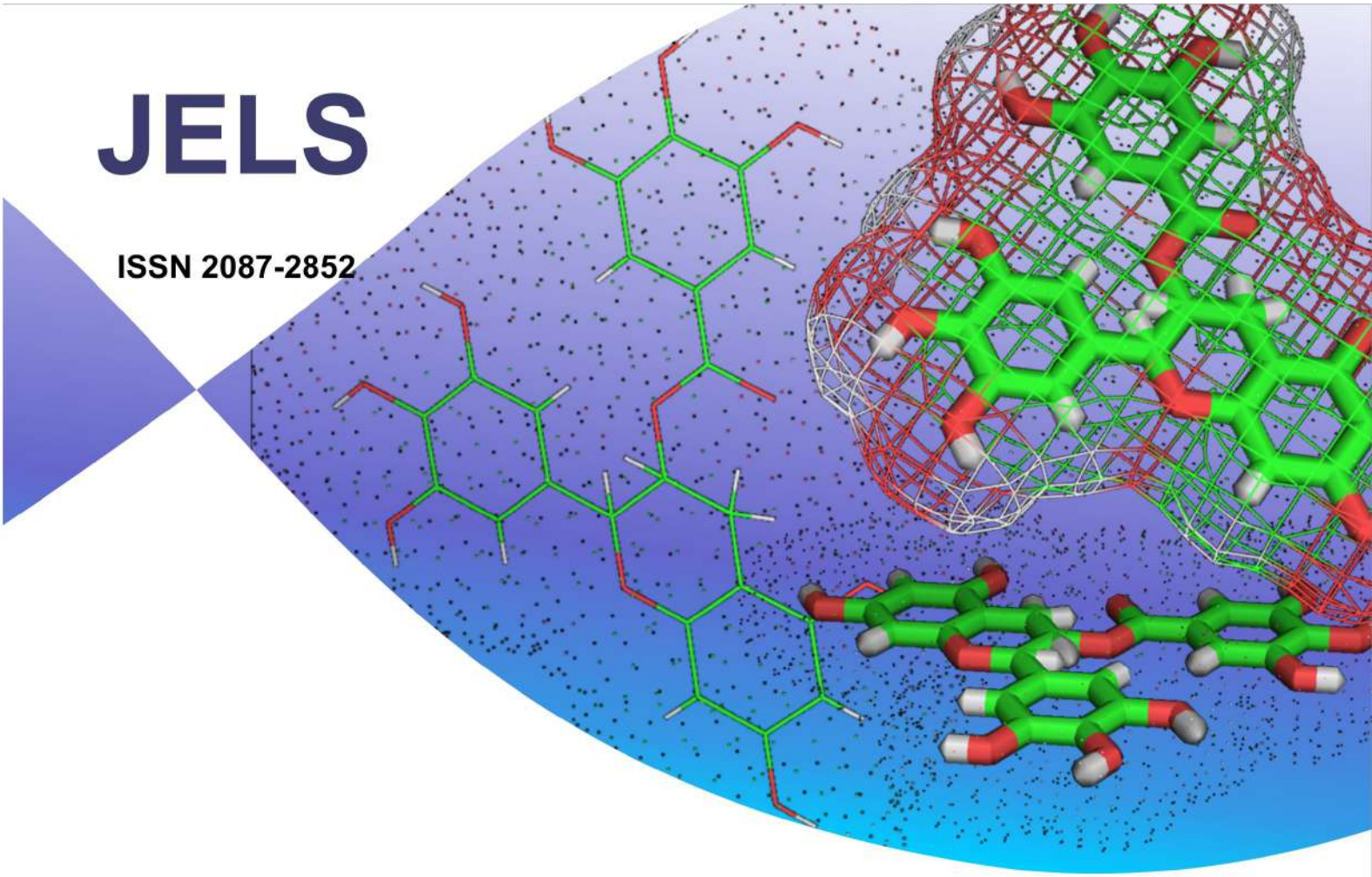

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Isolation and Identification of Nitrogen-Fixing Rhizobacteria associated with Cocoa plantation (*Theobroma cacao* L) as Biofertilizer Agent

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Abstract

Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency, is one of the centers for cocoa plantations using an organic farming system. However, over time this organic farming system experienced a decrease in fruit production, possibly from soil fertility and biofertilizers that were less available in the soil. This study aims to analyze the nitrogen-fixing ability and identify rhizosphere isolates that excel in nitrogen-fixing obtained from the rhizosphere of cacao (*Theobroma cacao* L) plant. Bacteria were isolated from the soil surrounding cocoa plant roots and grown on Nfb (*Nitrogen free Bromothymol Blue*) agar media. The nitrogen-fixing bacteria were analyzed with quantitative and qualitative methods. Six potential nitrogen-fixing isolates were identified based on the 16S rDNA sequence. The total number of isolates obtained from nitrogen-fixing isolation was six isolates. The R3.FN1 isolate showed the highest ammonia index at 0.52 µg.L^{-1} and was identified as *Stenotrophomonas maltophilia* KB13 with 99.87% similarity to the 16S rDNA sequence.

Keywords: Cocoa, Nitrogen-fixing**,** Rhizosphere, *Stenotrophomonas maltophilia.*

INTRODUCTION

The cocoa plant (*Theobroma cacao* L.) is a member of the Sterculiaceae family of the *Theobroma* genus, originating from the Amazon and other tropical areas in Central and South America. In Indonesia, the cocoa plant was first introduced by the Spanish around the XV century [1]. Indonesia's cocoa bean production is produced by 95% from independent smallholdings, 3% by Private Large Plantations, and 2% from State Large Plantations [2]. Indonesia is the 3rd largest cocoa bean-producing country in the world, after Ghana and Côte d'Ivoire. Indonesia accounted for 14% of the world's cocoa bean production in 2017, 657.000 tons from a total of 4.744.000 tons [3]. Most of the residents of Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency work as planters. Some of the commodities that are cultivated on farmers' land are sugar cane, cocoa, and coffee. In the history of Ringinkembar Village, the potential as the largest cocoa producer at that time was in Sumbermanjing Wetan District, with an area of more than 1000 hectares. However, over time it decreased to 125 hectares [4].

Biofertilizers are more environmentally friendly in promoting plant growth than synthetic fertilizers commonly used. Previous studies have shown that biofertilizers increase yields by 20-30% and can replace synthetic N and P fertilizers by 25%. Plant growth promoter rhizobacteria (PGPR) is one of the most widely used biofertilizers in agriculture. PGPR agents that play a role include *Azotobacter* sp., *Pseudomonas* sp., and *Bacillus* sp. [5]. Biofertilizer is a fertilizer that contains microbes, and these microbes help provide nutrients in the soil so that they can be used directly by plants [6]. The periodic provision of biofertilizers can help the plant's immune system by improving the balance of nutrients through the soil so that plants become more productive.

Efforts to obtain plant growth promoter bacteria from the rhizosphere of cocoa plant in Indonesia have not been done much. This research was conducted to obtain rhizosphere bacteria that can fix nitrogen. These bacteria were isolated from cocoa plantation soil in Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency, as a biofertilizer to replace synthetic pesticides or chemical fertilizers.

MATERIAL AND METHOD

Rhizosphere Soil Sampling

The sampling location is one of the organically managed cocoa plantations in Ringinkembar Village, Sumbermanjing Wetan District, Malang Regency. Three selected plants with coordinate locations, plant 1: S"08°16'14.3" E"112°43'.0"; plant 2: S"08°16'15.0" E"112°43'42.9" and plant 3: S"08°16'14.5" E"112°43'43.2". Next, for each plant, samples of

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the rhizosphere soil were taken from five points. The five samples were composited. Thus, a total of three rhizosphere soil samples were obtained. Samples were stored in an isothermic box at 4-10° C during the trip to the laboratory.

Isolation of nitrogen-fixing bacteria

Twenty five grams of rhizosphere soil sample was suspended in 225 mL of 0.85% NaCl solution to obtain a dilution of 10^{-1}. The next step is to make a dilution series of 10^{-2} to 10^{-6} in 9 mL of 0.85% NaCl solution. The suspension of each dilution was taken at 0.1 mL and inoculated using the *spread plate* method on Nfb Agar media. With the composition ($g.L^{-1}$): KH_2PO_4 0.5; $FeCl_3.6H_2O$ 0.015; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; DL-Malic Acid 5; KOH 4.8; Yeast exstract 0.05; Agar 15 in 1 liter aqua dest [7].

The cultures were incubated at 28°C for seven days. All bacterial colonies are considered nitrogen-fixing bacteria. Bacterial cell density per gram of sample was calculated by TPC (*Total Plate Count*). The different colony morphology was characterized, and the bacterial diversity index was calculated using the Simpson diversity index. The bacteria were then purified using the spread plate method and confirmed their purity by Gram staining. The single colonies obtained were stored as culture stock in NA medium, which was incubated at 28°C for 24 hours [8].

Quantification of nitigen fixing rhizobacteria

Twenty-one isolates were quantified using the qualitative method. Qualitative tests were carried out by inoculating two loops of culture suspension into 5 mL of semi-solid NFb medium by adding 0.3% agar concentration in a test tube. The pellicle was shown, or color changed from green to blue within seven days at 28°C, indicating the activity of nitrogen-fixing rhizobacteria.

Six isolates were tested with a quantitative method using Sera Ammonia Test Kit with a modified procedure. The test was conducted based on a factorial Completely Randomized Design (CRD) with three replications. The independent variables used were the type of isolate that passed the qualitative test screening and incubation time. Five mL culture starter of each isolate 10^7 cells.mL^{-1} were grown in 80 mL of liquid NFb medium without the addition of Bromothymol Blue. Medium without the addition of inoculum was used as a control. Control and test cultures were statically incubated at 28°C for seven days. Ammonia produced by each isolate was measured at incubation at 0, 2, 4, and 8 days. Cultures were taken 5 mL and centrifuged at 6.000 rpm, 10°C, for 10 minutes. Total of 2.5 mL supernatant was diluted with the addition of 2.5 mL of distilled water. The reagents provided by the Sera Ammonia Test Kit were added. An indication of the nitrogen-fixing activity of each isolate was a change in the color of the medium from clear to green or blue. The results obtained were quantified by spectrophotometry at a wavelength 700 nm. Ammonia concentration was calculated based on the standard of ammonia curve equation [9]. The nitrogen-fixing parameter data were analyzed for variance using one-way ANOVA ($p \leq 0.05$). If there is a significant difference, then proceed with the Tukey test.

Identification of Bacteria Based on 16S rDNA

The selected isolate were extracted for their chromosomal DNA according to the *Zymo Research* protocol, Quick-DNATM Fungal/Bacterial Miniprep Kit, USA. Bacterial 16S rDNA sequences were amplified using a Thermocycler Amplitron® with universal primers 27f (5' AGA GTT TGA TCC TGG CTC AG) and 1492r (5' GGT TAC CTT GTT ACG ACT T) with the PCR program: Pre-Denaturation 94°C for 5 minutes, Denaturation 94°C for 30 seconds, Annealing 52°C for 30 seconds, Extension 72°C for 1.5 minutes and final extension 72°C for 10 minutes.

Amplicons were sequenced using the Automatic Sequencer Analyzer ABI 3130, which was done by Firstbase Malaysia. The 16S rDNA sequences were edited using the Sequencer Scanner V.1 program and combined using the CAP Contig Assembly in the BioEdit V.7.2 program. 16S rDNA sequences were BLAST using the NCBI BLASTN program, and nucleotide sequences were determined according to *GenBank* [10]. The isolated 16S rDNA sequences and the reference strain were aligned with the ClustalW Multiple Alignment program MEGA 11. The phylogeny tree was constructed and inferred using the *Nearbor-Joining* (NJ) algorithm, using 1000 times bootstrap [11].

RESULTS AND DISCUSSION

The average nitrogen-fixing bacteria density of the three samples was 1.40×10^4 $cfu.g^{-1}$. According to the result, N-fixing rhizobacteria was influenced by C-organic, N-total, C/N ratio and organic matter. The area around the roots whose chemical, physical and biological properties are influenced by root activity [12]. Chemical parameters of the rhizosphere soil of cocoa plants showed C-organic content of 4.71 ± 0.07%. This value is classified as high.

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Table 1. Physicochemical parameters of cocoa plant soil

Parameter	Average	Description
Organic matter (%)	8.74 ± 0.04	
C- Organic (%)	4.71 ± 0.07	High (3-5)
N- Total (%)	0.44 ± 0.61	Medium (0.21-0.50)
P- Total (mg.kg^{-1})	565.24 ± 0.43	
C/N ratio	10.70	Low (<11)
pH	5.72 ± 0.80	
Air temperature (°C)	25 ± 0	
Light intensity (lux)	2171 ± 1018	
Water content (%)	28.59 ± 0.26	
Bacterial density		
Nitrogen-fixing bacteria (cfu.g^{-1})	1.40 x 10^4	
Diversity Index		
Nitrogen-fixing bacteria	0.50	Medium (0.31-0.60) [13]

The content of organic matter is 8.74 ± 0.04%, which is classified as very high. The nitrogen content (N-total) of 0.44 ± 0.61% was classified as moderate. Based on these data (Table 1), it is known that the organic carbon content in the rhizosphere soil of cocoa plants is higher than the nitrogen content.

The high levels of C-organic in the soil are influenced by high organic matter. The higher the levels of organic matter, the higher the amount of nitrogen it contains so that plant growth will be good. However, if the C-organic is too high, it will affect the life of the organism population and cause a shading effect [14]. Based on the criteria for assessing the chemical properties of the soil research center [15], this research plot was classified as having a moderate total N. It is directly proportional to the organic matter content, where the higher the soil organic matter content, the higher the total N-content of the soil. In other words, every change in organic matter content will change the total N-content. The availability of N in the soil is not only determined by the amount of N-total soil. It is also closely related to the organic matter content of the soil, especially the level of decomposition (C/N) [16,17]. The rhizosphere C/N ratio of cocoa plants was 10.70 in this study, and the C/N ratio of the soil type of cocoa was low. It is because some of the available N is used by microorganisms in the breakdown of organic matter [18].

Bacterial density was influenced by high C-organic, moderate total nitrogen, low C/N ratio, and high organic matter in the rhizosphere samples of cocoa plants (Table 1). Organic matter is essential in the density and pH of microorganisms [19]. The low pH in cocoa plants is caused by the activity of fermenting microorganisms to form organic acids (acetic acid, pyruvic acid, and lactic acid) [20,21]. C and N are macromolecules with structural and functional roles in bacterial cell components [22].

Potential Isolates of Nitrogen-Fixing Bacteria

The results of the qualitative potency test of 21 isolates showed a change in the color of the medium. Based on the qualitative test, it was found that 51% of the total isolates showed a color change in semi-solid NFB media, from green to bluish, six from plant 1, seven from plant 2, and eight from plant 3. The presence of pellicle produced by bacteria in the medium is caused by the absence of excess oxygen in the medium. The rate of oxygen diffusion is in line with the respiration rate of the organism, which is a good condition for the activity of the nitrogenase enzyme, which assisted in the reduction of acetylene to ethylene [23]. The color changed in the medium from green to blue with bromthymol blue as a color indicator. Bromthymol blue changes color if there is an increased pH in the medium due to nitrogenase activity [24]. The medium was able to provide the nutrients needed by nitrogen-fixing bacteria, a blue color change in the medium indicated that there was nitrogenase activity carried out by nitrogen-fixing bacteria [25].

The quantitative nitrogen fixation potential test (Fig. 1) showed the difference in ammonia concentration of each isolate was influenced by different types of isolates and variations in incubation time ($p<0.05$). R3.FN1 has the highest ammonia concentration with 0.52 mg.L^{-1} at two-day incubation. This isolate was able to produce ammonia for up to eight days of incubation but decreased up to 0.22 mg.L^{-1} because the ammonia was used for bacteria [26]. It was found that all isolates experienced a decrease in ammonia concentration at four and eight days of incubation.

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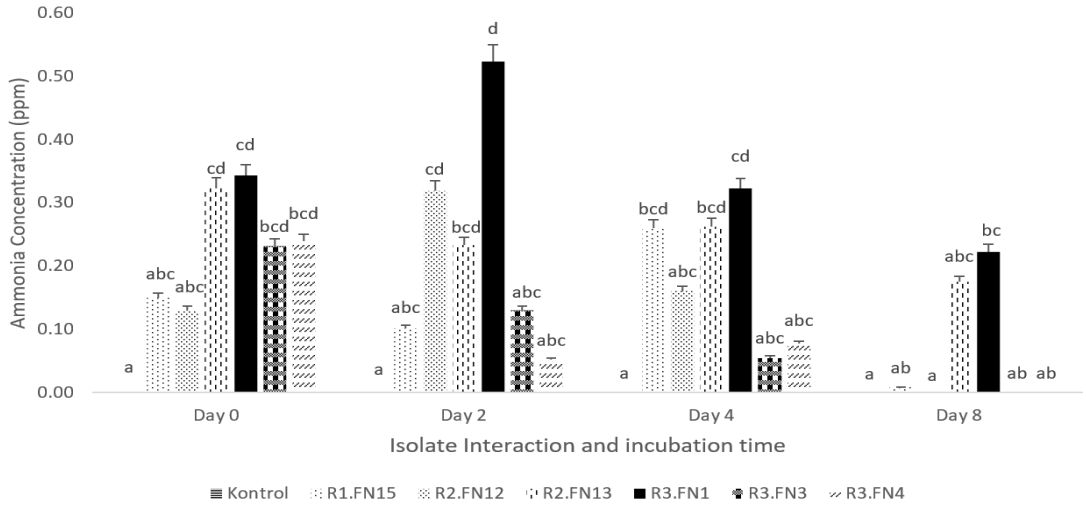

Figure 1. Ammonia concentration from nitrogen fixation activity of rhizosphere bacterial isolates of Cocoa plants with variations in incubation time
*The different notations above the histogram show significant differences between isolates and between isolates' incubation time ($p<0.05$)

The variation in ammonia concentration was due to the different abilities of each isolate to fix nitrogen into ammonia. It was due to the different types of bacterial isolates [27]. Nitrogen-fixing bacteria convert N2 in the air into ammonia using the enzyme nitrogenase. Based on the ability to produce ammonia with stable and highest criteria, the isolate R3.FN1 was selected to be identified.

Molecular Identification of Nitrogen-Fixing Bacteria

Potential nitrogen-fixing bacteria R3.PF1 isolates from Ringinkembar Village were identified phylogenetically based on the similarity of the 16S rDNA sequence. Figure 2 shows that the isolate R3.FN1 was identified as *Stenotrophomonas maltophilia* KB13 with 99.8% similarity to the 16S rDNA sequence (Fig. 2).

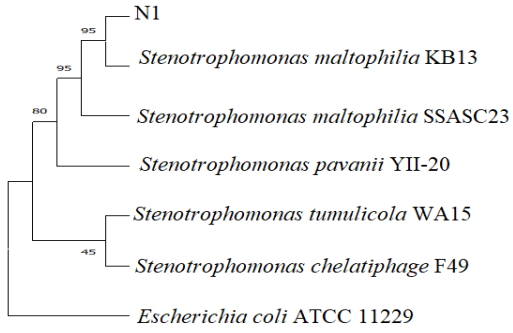

Figure 2. The phylogeny tree showed the relationship of R3.FN1 isolates and references bacterial strains based on 16S rDNA sequence according to the *Neighbor-joining* algorithm

Stenotrophomonas maltophilia is a bacillus, gram-negative, non-fermenting, non-sporulating bacterium with polar flagella 0.5-1.5 in length [28]. It is often found in the rhizosphere or the soil around plant roots. These bacteria can be found widely in the natural environment [29]. The genus *Stenotrophomonas* is the dominant genus in nature (water, soil, and plants), has a wide distribution, and plays an important role in the nitrogen and sulfur cycle [30].

CONCLUSIONS

The rhizosphere isolate of cocoa plants were taken from Ringinkember Sumbermanjing Village, Malang Regency had the highest potential as a nitrogen-fixing activity of 0.52 $mg.L^{-1}$. The isolate R3.FN1 was identified as *Stenotrophomonas macrophilia* KB13 with 99.87% similarity. Therefore, the *S. maltophilia* is a potential bacteria for PGPR and plays an important role in the nitrogen cycle to be used for Cocoa plants.

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Isolation and Characterization of α-Amylase Enzyme on Brown Planthopper (*Nilaparvata lugens* Stal) On Rice

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Abstract

Brown leafhopper (*Nilaparvata lugens* Stal) is an important pest in Indonesia that causes heavy losses. The rice was damaged by *N. Lugens* sucking plant liquid in the form of carbohydrates in the form of starch. Environmentally friendly control methods are needed to reduce the increasingly high use of inorganic pesticides. Control of *N. lugens* with biotechnology can be done by inhibiting the metabolic cycle in these insects. This method utilizes the proteins available in rice seeds to stop the performance of enzymes α-amylase in the digestive system of *N. lugens*. α-amylase is an enzyme that plays a role in the process of starch degradation so that it becomes a simpler form both in microorganisms. The characterization of α-amylase enzymes in insects is an important first step to the determination of appropriate protein inhibitors so that they can be used to produce pest-resistant Genetically Modified Organisms crops. The research was conducted in the Agrotechnology laboratory, University of Jember. The research began with isolation and purification of α enzymes from brown stems and then continued with testing of α-amylase activity. Test parameters include the effect of temperature, pH, and substrate concentration on the activity of the α-amylase enzyme. The results showed that supernatant extracted from brown planthoppers produces a clear zone in the agar medium, which means the activity of enzymes α-amylase in the hydrolysis of starch. The pH value of 6 provides the most optimum conditions for the activity of α enzymes. The α-amylase enzyme is able to work optimally in the temperature range of 30°C - 45°C, and experiences a decrease in activity when the temperature reaches 50°C. The α-amylase enzyme shows the ability to hydrolyze the amylase substrate to a concentration of 0.8 $\mu g.\mu L^{-1}$.

Keywords: α-amylase, *Nilaparvata lugens* Stal, Rice.

INTRODUCTION

Brown planthopper (*Nilaparvata lugens* Stal) is a significant pest occurred in rice cultivation in Indonesia. Brown planthopper attacks cause heavy losses and pose a threat to rice farmers in Indonesia. Data shows that brown stem attacks can cause up to 50% damage to vulnerable varieties such as Cisadane [1]. A fairly high degree of damage can lead to a significant decrease in yield. Baehaki and Mejaya [2] said that brown stem attacks can cause an average loss of 1-2 $tons.ha^{-1}$ in severe attack conditions. It causes dependence on inorganic pesticides to be higher for pest control [3].

Control of brown planthopper utilizing biotechnology can be done by inhibiting the metabolic cycle in these insects. This control method is carried out by stopping the performance of α enzymes using protein inhibitors available in rice seeds. α-amylase is an enzyme that plays a role in the degradation process of starch into a simpler form, both in microorganisms, plants, and humans [4]. α-amylase is included in the enzyme endoamylase, which catalyzes the hydrolysis of starch into shorter oligosaccharides through the division of bonds α-1,4-glycosidic [5]. Bahagiawati in her research explained that enzyme inhibitors α-amylase can suppress the growth of *Callosobruchus maculatus* warehouse pest insects, by extending the larval period in such insects and increasing the percentage of larval mortality [6].

Brown planthopper sucked carbohydrates in the form of starch from rice plants. This process can be inhibited with protein inhibitors α amylase that can be obtained from plants, humans, and microorganisms. This protein inhibitor can be found in several types of food crops such as wheat (*Triticumaestivum*), barley (*Hordeum vulgareum*), sorghum (Sorghum bicolor),rice (*Oryza sativa*), and some legume plants such as gude beans (*Cajanuscajan*), red delinquent beans (*Vignaunguiculata*), and beans (*Phaseolus vulgaris*) [4]. Characterization of α enzymes in insects is an important first step and must be taken to go to the stage of determining appropriate protein inhibitors so that they can be used to produce pest-resistant Genetically Modified Organisms (GMO) crops. Previous research has discussed the influence of various factors applied to the process of characterization of α enzymes in insects. Research by Abdolmaleki

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et al. [7] showed that the activity of the enzyme α-amylase in species *Andralus spinidens* can be influenced by temperature, pH, and concentration conditions of certain elements. Other results were shown in Ravan's study [8], where the enzyme α-amylase in the species *Eurygaster maura* is active at pH 6, and the temperature is 35-40°C. It shows the characteristics of different α enzymes in each insect species, so characterization is needed to support the process of finding suitable inhibitor proteins.

MATERIAL AND METHOD

Isolation and purification of α-amylase

The study was conducted at the Agrotechnology Laboratory, Faculty of Agriculture, University of Jember. Isolation and purification of α-amylase from *N. lugens* Stal are carried out by preparing 2 grams of *N. lugens* Stal stored at -20°C for 30 minutes. The frozen *N. lugens* Stal is crushed using a mortar with 8 mL of sodium phosphate buffer of 20 mM pH 7 and then centrifuged at 10,000 rpm for 20 minutes with a temperature of 4°C. Clean supernatant is used as an α-amylase material [9].

Detection of α-amylase enzyme activity

Detection of alpha-amylase enzyme activity is carried out by the method of Silaban and Simamora [10]. The supernatant obtained through the isolation process is tested to determine the activity of enzymes α-amylase. Testing is carried out by the starch method to plate. Agar medium containing 2% starch is used in this method. The medium is to be hollowed out at 4 points and placed with an enzyme solution at 4 points. Medium to be incubated for 30 minutes at a temperature of 37°C and then added Iodine. Indications of the activity of the enzyme α-amylase are indicated by the formation of a clear zone around the point added to the enzyme of the brown stem leafhopper.

Testing of enzyme activity α-amylase

The activity of α-amylase was measured using Bernfeld's method [11]. A total of 25 µL of enzyme α-amylase from the isolation of brown planthopper was added to 475 µL buffer of 1% amylase reaction. The reaction mixture is then vortex until homogeneous and then incubated at 30°C for 10, 20, and 30 minutes. The reaction ends by adding 500 µL of 3.5-dinitrosalicylic acid reagents, then heated to boiling water for 10 minutes. The reaction mixture is cooled, and subsequently, absorbance is measured using a spectrophotometer at a wavelength of 560 nm.

Testing the effect of pH on α-amylase enzymes

The effect of pH on α-amylase was measured at different pH values. pH was adjusted using a mixture of K-Phospate and aqua dest buffers of 50 mL. The addition of HCl and NaOH was done to set the pH value at 5, 6, 7, and 8. The mixture of 20µL α-amylase and 475 µL buffer of 1% amylase reaction was incubated at 30°C for 10 minutes. The reaction was stopped to add 500 µL regent 3.5-dinitrosalicyliclic. Enzyme activity was measured on a spectrophotometer at a wavelength of 560 nm.

Testing the effect of temperature on enzymes α amylase

The effect of temperature on α-amylase activity is determined by the incubation of 20 µL α-amylase and 475 µL buffers of 1% amylase reaction at different temperatures, namely 30, 35, 40, 45, and 45°C. The pH conditions are adjusted to the optimum results obtained in the previous test. Temperature treatment is carried out with two different incubation intervals, 10 and 20 minutes. The reaction is stopped to add 500 µL regent 3.5-dinitrosalicyliclic. Further enzyme activity is measured on the spectrophotometer at a wavelength of 560 nm.

Testing the effect of substrate concentration on α-amylase enzymes

The effect of substrate concentrations on α-amylase enzymes was measured in several samples with amylum stock solution concentration of 5 $\mu g.\mu L^{-1}$ with volumes of 0, 10, 20, 40, 80, 120, 160 µL. The starch solution, according to the specified amount, is added 20µL extract of the enzyme α-amylase and 475 µL buffer of 1% amylase reaction and aqua dest until it reaches 1 mL resulting in a concentration of 0, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 $\mu g.\mu L^{-1}$. The mixture is incubated for 10 minutes. The pH and temperature conditions are adjusted to the optimum conditions obtained in the previous test. The reaction is stopped to add 500 µL reagent 3.5-dinitrosalicylic. Furthermore, enzyme activity is measured on the spectrophotometer at a wavelength of 560 nm.

Measurement of protein molecular weight

Measurement of the molecular weight of proteins in the sample was done using the Bradford method [12]. Measurements are carried out in several stages, namely the manufacture of BSA (Bovine Serum Albumin) with a concentration of $1\mu g.\mu L^{-1}$ as a standard protein, the manufacture of Bradford reagents with Coomassie Brilliant Blue (CBB) G-250 staining, the

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manufacture of standard curves, and the measurement of protein levels obtained from the calculation of absorbance values entered into linear equations. The measurement of protein levels was carried out with six samples of protein counts of 0, 5, 10, 15, 20, and 30 µL using 5 µL of enzyme extracts in each sample.

RESULT AND DISCUSSION

Enzyme Activity α-amylase

The supernatant obtained through the isolation process of brown planthopper is tested on a medium agar that has been added iodine to detect the presence of enzymes α-amylase. The results of testing on the medium agar, it appears the emergence of clear zones that show the activity of enzymes α-amylase in hydrolysis starch compounds (Fig. 1).

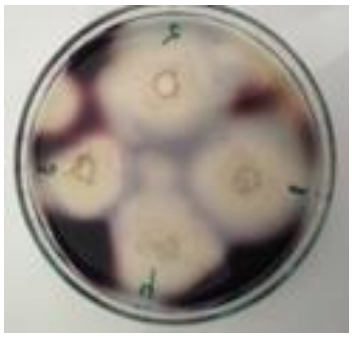

Figure 1. The results of testing the activity of the enzyme a-amylase in agar media

The results of isolation and testing in this study showed the presence of the enzyme α-amylase in the digestive system of brown leafhoppers, which played a role in the starch hydrolysis process. This enzyme has an important role in the digestion of insects, to break down starch in plant tissues into oligosaccharides before being hydrolyzed into glucose by glucosidase [13]. The formation of clear zones in the agar medium that has been added with iodine proves the activity of the enzyme α-amylase in hydrolyzing starch.

The results of reaction testing showed that the enzyme α-amylase had good rection in different incubation intervals. At intervals of 10 minutes, the absorbance value shows a result of 1.267. The reaction also showed an increase directly proportional to the incubation interval at 20 and 30 minutes (Table 1).

Table 1. Test of enzyme reaction α-amylase

Incubation Interval (Minutes)	Absorbance Value
10	1.267
20	1.689
30	1.821

Effect of pH on α Enzymes

Different pH values indicate an influence on the activity of α-amylase enzymes. This is reviewed from the absorbance value and enzyme activity obtained in Table 2.

Table 2. Effect of pH on the activity of α-amylase enzymes

Buffer pH	Incubation Interval (minute)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
5	10	14.138	0.044
6	10	77.472	0.234
7	10	77.138	0.215

A pH value of 6 indicates the most optimal result against the activity of the α-amylase enzyme based on testing. Based on this data, it can be concluded that an increase in pH above the optimum value tends to cause a decrease in enzyme activity α-amylase.

The pH condition plays an important role in ensuring the function of the α-amylase enzyme can work. Changes in pH values can affect the total load of enzyme proteins α-amylase, both through changes in structure and changes in charge in amino acid residues that function in binding substrates [14]. Changes in pH conditions are what can cause an increase or decrease in the activity of α-amylase enzymes. Ravan's research [8] states that the enzyme α-amylase in insects generally tends to be most active under neutral to slightly acidic pH conditions. It is by the data obtained in this study. The enzyme α-amylase from the brown stem leafhopper showed the best activity under pH 6 conditions, and its activity value did not decrease significantly until the neutral pH 7 condition. The more acidic pH condition of 5 and pH 8, which is more alkaline, affect the activity of the enzyme α-amylase, seen from the decrease in the value of its activity.

Data on the influence of pH conditions and optimum values in this study is supported by the results of previous studies that show similar pH ranges provide optimum conditions for enzyme activity α-amylase. Research on *Callosobruchus maculatus* [15] and *Andralus spinidens* [7] mentioned the optimum pH 6 for enzyme activity α-amylase, while research in the species *Oryctes owariensis* mentioned pH 7 [16].

The enzyme α extracted from insects has a different optimum pH range depending on the species. The enzyme α-amylase extraction from the order Hemiptera generally has an optimum pH range ranging from 6 to 7 as found in *Podisus maculiventris, Graphosoma lineatum, Eurygaster maura, Eurygaster integricep*s, and *Aphis fabae*.

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This optimum pH value is a picture of the pH conditions in the lumen of the insect midgut and becomes part of the insect adaptation process to digest nutrients sourced from its host plant [17].

Effect of Temperature on α-amylase Enzymes

The temperature effect on the activity of the enzyme α-amylase was tested under a pH of 6 conditions and showed optimum conditions based on Table 3 data. Temperature exerts a different influence on the activity of enzymes α-amylase extracted from brown planthoppers. It is indicated in the absorbance value and enzyme activity obtained in Table 3.

Table 3. Effect of temperature on enzyme activity α-amylase

Mouth (°C)	Incubation Interval (minute)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
30	10	157.138	0.473
35	10	154.805	0.466
40	10	149.805	0.451
45	10	144.472	0.435
50	10	89.138	0.269

A temperature of 30°C produces optimum conditions for the activity of the enzyme α-amylase. Increasing the temperature to the optimum point can accelerate the rate of enzyme catalysis reaction due to the higher kinetic energy and frequency of collisions between the molecules involved [18]. Data from this study showed a temperature of 30°C provided optimum conditions for the activity of α-amylase enzymes isolated from brown planthopper. The temperature rises to 45°C do not significantly affect the activity of α-amylase enzymes, so it can be concluded that the temperature range of 30°C - 45°C supports the activity of enzymes α-amylase. These results are by research related to the influence of temperature on the activity of α enzymes in other insect species.

Previous research has shown optimum temperature conditions for α-amylase enzyme activity in Eurygaster maura range from 30°C - 40°C [8], 35°C - 40°C in *Aeolesthes holosericea* [19], 35°C in *Plagiodera versicolora* [20], 37°C in *Leptinotarsa decemlineata* [21], and 45°C in *Andralus spinidens* [7]. Insects such as brown leafhoppers are poikilotherm organisms. Their body temperature and physiological processes are influenced by environmental conditions [22]. The optimum temperature range for the activity of this α-amylase enzyme is vital, as an overview of the appropriate environmental conditions for brown planthoppers to attack their hosts.

The temperature of 50°C is the point where the activity of the enzyme α-amylase decreases significantly. High temperature, as a trigger for decreased activity, was also mentioned in the study of Patil *et al.* [19]. There was a significant decrease in the activity of the enzyme α-amylase when the temperature was raised to 50°C. Damage to the constituent components of enzymes due to high temperatures is suspected to be the cause of the decreased activity of enzyme α-amylase. High-temperature conditions cause the kinetic energy of enzyme molecules to b too high, thereby breaking the bonds that maintain the shape of the enzyme and causing the denaturation of hydrophobic residues on the surface of the enzyme [14,23]. These hydrophobic residues include tryptophan and phenylalanine, which play a role in stabilizing the enzyme α-amylase. Based on the data obtained, it can be concluded that the enzyme α-amylase from brown stem leafhoppers can work optimally in the temperature range of 30°C - 45°C and decreases when the temperature reaches 50°C.

Effect of Substrate Concentration on α-amylase Enzymes

The concentration of the added amylase substrate affects the activity of the enzyme α-amylase based on the tests performed. It is reviewed from the absorbance value and the results of the calculation obtained in table 4.

Table 4. Effect of substrate concentration on enzyme activity α-amylase

Substrate Concentration ($\mu g.\mu L^{-1}$)	Number of Enzymes (μL)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
0.00	20	47.472	0.144
0.05	20	63.138	0.191
0.10	20	54.138	0.164
0.20	20	57.805	0.175
0.40	20	68.805	0.208
0.60	20	91.138	0.275
0.80	20	109.805	0.331

The addition of substrate concentrations is related to the increased activity of enzyme α-amylase resulting from isolation from brown planthopper. In general, enzyme activity tends to increase with the addition of the concentration of the amylase substrate.

The Lineweaver-Burk analysis is carried out to find out the kinetic parameters of the enzyme α-amylase at different concentrations of amylase substrates (Fig. 2). The maximal velocity (Vmax) of the enzyme α-amylase brown planthopper s was recorded at 43.47 $U.mg^{-1}$ protein and Km 1%.

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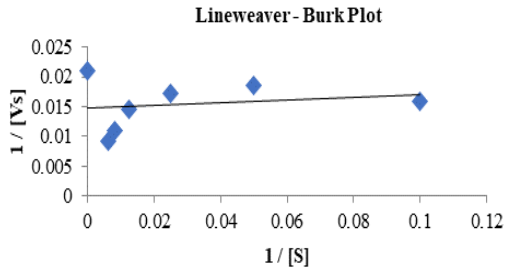

Figure 2. Lineweaver – Burk plot of alpha-amylase enzyme activity brown planthopper at substrate concentrations different.

Dissolved protein levels of the enzyme α-amylase of brown planthopper

The absorbance results of the BSA standard protein solution are used to create the standard curve of the protein (Fig. 3). The protein levels of the enzyme α-amylase brown planthopper are obtained through the substitution of the absorbance value of the enzyme protein α-amylase in the regression equation of the protein standard curve. As a result, the protein levels of the enzyme α-amylase showed a value of 16.478 $\mu g.\mu L^{-1}$, which can be seen in Table 5.

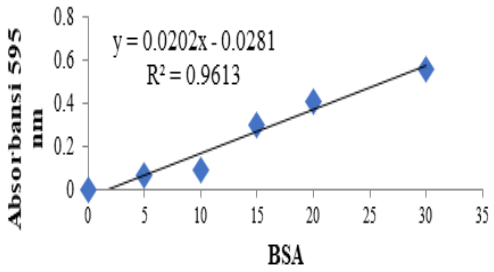

Figure 3. Protein Standard Curve

The enzyme α-amylase extracted from the brown stem leafhopper showed the ability to hydrolyze the substrate well to the highest concentration on the test. Enzymes show increased activity with the addition of the concentration of the amylase substrate until it reaches the highest activity value at the substrate concentration of 160 µL. The number of enzymes added to each substrate concentration influence treatment is 20 µL or equivalent to 330 µg, referring to the dissolved protein content.

Table 5. Dissolved protein levels of brown planthoppers

Sample Protein	Absorbance Value 595 nm	Protein up to ($\mu g.\mu L^{-1}$)
Brown planthopper	0.329	16.478

The pH conditions and optimum temperature obtained previously were used as standard in this test. Thus, the optimal enzyme performance to even the highest substrate concentration. The activity of the enzyme α-amylase increased under conditions of high substrate concentration [24]. Research on the enzyme α of the *Mythimna* separate species showed an increase in enzyme activity as the concentration of the amylase substrate increased. The increase or decrease in enzyme activity at various concentrations depends on the active site of the available enzymes. The decrease in enzyme activity can be caused by the active site of the enzyme is full. This research shows that under optimum conditions, brown planthoppers can hydrolyze the substrate at several concentrations by utilizing the enzyme α on its own [25].

Lineweaver-Burk analysis showed maximal velocity (Vmax) of the enzyme α-amylase brown planthopper was recorded at 43.47 $U.mg^{-1}$ protein and Km 1%. The Km value has an inverse relationship with the substrate concentration needed to unify the active site of the enzyme α-amylase. So that the lower the value of Km then indicates a strong bond [26]. The value of Km enzyme α-amylase use of this amylase substrate is not much different from the results obtained by Zibaee *et al.* [27]. The enzyme α-amylase of the species Andrallus spinidens has Vmax 7.14 $U.mg^{-1}$ protein and Km 1.04% in the starch substrate.

CONCLUSION

Supernatant extracted from brown planthoppers produces a clear zone on the medium to which iodine is added. It indicates the activity of enzyme α-amylase in the hydrolysis of starch.

A pH value of 6 provides the most optimum conditions for the activity of enzymes α-amylase extracted from brown planthoppers. An increase in pH value of more than 6 leads to a gradual decrease in enzyme activity.

The enzyme α extracted from brown planthoppers can work optimally in the temperature range of 30°C - 45°C and decreases in activity when the temperature reaches 50°C. The enzyme α-amylase from brown planthoppers showed the ability to hydrolyze amylase substrates to a concentration of 0.8 $\mu g.\mu L^{-1}$. Enzyme activity tends to increase with the addition of substrate concentrations.

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Isolation and Screening of Lactic Acid Bacteria from Sumbawa Buffalo Milk (*Bubalus bubalis*) as Potential Starter Cultures

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Abstract

The Sumbawa buffalo (*Bubalus bubalis*) is one of the ruminant livestock in Indonesia that not only contributes to fulfilling meat requirements but also milk. Besides containing nutrients that are very beneficial for human health, buffalo milk is also a potential source of lactic acid bacteria (LAB) with technological and functional properties. Lactic acid bacteria have been utilized as starter cultures in various fermented products. This study aimed to isolate LAB from Sumbawa buffalo milk and to identify the potential isolate as a starter culture. The screening of LAB as a starter culture was based on some technological properties, including proteolytic activity, lipolytic activity, exopolysaccharide (EPS) production, antibacterial activity, antibiotic sensitivity, hemolytic activity, and acidification activity. Data were analyzed statistically using one-way ANOVA and Tukey's post-hoc test at a 5% significance level. A total of 21 isolates were isolated from fresh buffalo milk, with a LAB total was $4.7x10^5$ CFU.mL^{-1}. All the isolates were characterized as Gram-positive with cocci-shaped. The SA8 isolate was selected as the most potential candidate as a starter culture because it has fulfilled the criteria such as the highest proteolytic activity, the lowest lipolytic activity, producing EPS, potential antagonistic activity against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhi, and sensitivity to cefazolin, intermediate to erythromycin and cinoxacin, non-pathogen, as well as the most rapid acidification activity. The SA8 isolate was identified as *Enterococcus lactis* with a similarity level of 99.99% towards strain BT159. This indigenous LAB was a potential starter culture of Sumbawa fermented buffalo milk to increase the diversification of products derived from buffalo milk.

Keywords: *Enterococcus lactis*, lactic acid bacteria, technological properties, starter culture, Sumbawa buffalo milk.

INTRODUCTION

Milk is a commodity produced by livestock that is important for fulfilling nutritional needs to improve public health. One of the main livestock supply areas for several regions in Indonesia is West Nusa Tenggara Province. This province is ideal for livestock farming due to the availability of grazing land that provides feed for the needs of ruminant livestock, for example, buffalos, cattle, and horses [1]. Based on the Central Bureau of Statistics data in 2019, the three provinces with the most buffalo farming sectors are East Nusa Tenggara, West Nusa Tenggara, and North Sumatra. In West Nusa Tenggara, the Sumbawa buffalo has different morphological and genetic characteristics than mud buffalo or other local buffalo clumps. Sumbawa buffalo has distributed in the Sumbawa Island, West Nusa Tenggara Province. The buffalo milk produced by farmers in Sumbawa averages 1-3 liters per cows per day. This milk production is quite high compared to the average buffalo milk production in general [2].

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Buffalo milk has some benefits for human health. It contains bioprotective, immunoglobulins, lactoferrins, lysozymes, lactoperoxidase, and bifidogenic [3]. Buffalo milk has prominent nutritional content, such as fat, protein, lactose, and minerals (calcium, iron, magnesium, and phosphorus). It has cholesterol levels almost twice the content of conjugated linoleic acid. Conjugated linoleic acid is one of the other forms of essential fats that human needs [4]. Monounsaturated fatty acids contained in buffalo milk can provide antioxidant activity [5], improve bone health and reduce the risk of osteoporosis [6], and maintain blood pressure levels [7]. Buffalo milk also has potential economic value through fermentation for producing fermented milk products manufactured either traditionally or industrially [8]. In addition, the fermentation process can reduce milk spoilage due to the role of lactic acid bacteria (LAB) in inhibiting the growth of undesirable microbes.

Lactic acid bacteria are characterized by a Gram-positive, catalase-negative, with a rod or cocci-shaped [9]. During fermentation, glucose can be converted by LAB into lactic acid [10], and some LAB probiotics are beneficial to maintain intestinal microflora balance [11]. The use of indigenous LAB, native microflora that inhabit

certain habitats, as a starter culture for producing fermented milk products can increase the fermented product quality in terms of aroma, taste, and texture [12] and can inhibit the growth of pathogenic bacteria [13]. Starter cultures are defined as microbes used to start, direct, and accelerate the fermentation process [14]. The milk fermentation, with the addition of starter cultures, can also increase nutritional values through increasing nutritional bioavailability and the production of bioactive substances with functional properties [15].

Indigenous bacteria are believed to be more effective when used as a starter culture in fermentation. Some criteria of starter cultures in producing fermented products, especially yogurt, are high proteolytic activity, low lipolytic activity, producing exopolysaccharide (EPS), high antibacterial activity, high sensitivity to antibiotics, non-pathogen, and high acidification activity. The LAB exploration of Sumbawa buffalo milk and its use as a starter culture has not been widely reported yet. Therefore, this study aimed to isolate LAB from Sumbawa buffalo milk and to identify the potential isolate as the starter culture candidates.

MATERIAL AND METHOD

Sampling Collection of Sumbawa Buffalo Milk

The determination of the sampling location was carried out by purposive sampling by taking into account the high level of the buffalo population. Buffalo milk samples were taken from two different locations, namely Srangen Hamlet (Sample S) in Bugis Village and Tanakakan Hamlet (Sample T) in Menala Village, each of which is in Taliwang District, West Sumbawa Regency, West Nusa Tenggara. The buffalo milk samples obtained directly from farmers were put into sterile bottles, and then stored in a cool box (± 4°C) during transportation from the sampling site to the laboratory.

Nutritional Content Analysis

The nutritional content (proximate) analysis was carried out following the AOAC procedure [16]. The parameters measured include water content, ash content, fat content, and protein content. The proximate content provides information about the nutritional values of the Sumbawa buffalo milk.

Isolation of LAB

The LAB isolation used a selective medium of MRS and M17 agar containing 1% $CaCO_3$ [9]. The serial dilution of the sample was carried out in 9 mL of sterile saline water (NaCl 0.85%). A total of 100 µL of the samples was inoculated in the media using a spread plate technique. The cultures were incubated in aerobic conditions at 37°C for 48 h. The colonies with a clear zone were selected. The total colony of LAB was calculated based on the total plate count (TPC) method. Furthermore, isolates were characterized using Gram staining and catalase tests to confirm the LAB characteristics. The purified LAB isolates were maintained in MRS agar slant for stock cultures.

Proteolytic Activity Test

The proteolytic activity was conducted using a 1% skim milk agar medium, which was enriched with 0.05% NaCl, 0.1% yeast extract, 0.2% tripton, 0.01% $CaCl_2$, and 1.5% Bacto agar [17]. The 24-h LAB cultures were equalized using a spectrophotometer with a wavelength of 600 nm, and then 50 µL of the cultures were dripped onto a blank disk (Oxoid, UK). The disks were then placed on the surface of skim milk agar and incubated at 37°C for 24 h. A negative control used was media without cultures. Proteolytic activity was observed, indicated by the presence of clear zones around the blank disks, and it is measured in millimeters and calculated using Formula 1. The isolates with high proteolytic activity were selected for the subsequent assay.

$$\mathrm{DCZ} = \mathrm{DT}\text{-}\mathrm{DC} \quad\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Description :

DCZ : Diameter of the clear zone (mm)
DT : Diameter of total (mm)
DC : Diameter of colony (mm)

Lipolytic Activity Test

The lipolytic activity test was conducted by inoculating 50 µL of the selected LAB cultures onto a blank disk (Oxoid, UK) and placed on the Sierra agar media containing 1 % peptone, 0.5% NaCl, 0.01% $CaCl_2.2H_2O$, 1.5% Bacto agar, 1% Tween 80, and 0.01% methyl red. Subsequently, the cultures were incubated at 37°C for 96 h [18]. A negative control used was media without cultures. The lipolytic activity was observed by the appearance of clear zones around the blank disks, and it is measured in millimeters and calculated using Formula 1. The isolates with the absence or low lipolytic activity were selected for the subsequent assay.

Exopolysaccharide Production Test

Exopolysaccharide (EPS) production test was carried out by culturing the LAB isolates in MRS agar supplemented with 5% sucrose and incubated at 37°C for 24-48 h [19]. The positive results of EPS production were indicated

qualitatively by forming thick slime or mucoid colonies. The isolates showing this character were then selected for the next assay.

Antibacterial Activity Test

The antibacterial activity test was performed using the disk-diffusion agar [17]. The indicator bacteria used were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* Typhi. These indicator bacteria were selected to represent the enteric pathogens and are usually found as contaminant bacteria in milk. The selected LAB cultures with a density of 10^7 CFU.mL^{-1} were centrifuged at 10,000 x g at 4°C for 10 mins. The cell-free supernatant (CFS) was taken, and the pH was adjusted using NaOH 4 M to pH 7. The CFS was filter-sterilized using a 0.22 µm milipore membrane. The filter-sterilized CFS (50 µL) was dripped onto a blank disk (Oxoid, UK). The disks were then put onto nutrient agar containing 100 µL of indicator bacteria with a cell density of 10^6 CFU.mL^{-1} and then incubated at 37°C for 24 h. The inhibition zones around the disks were in millimeters and calculated using Formula 1. A negative control used was media without cultures, while streptomycin 10 µg was used as a positive control.

Antibiotic Sensitivity Test

The antibiotic sensitivity test was conducted using the disk-diffusion agar technique referring to James [20]. The antibiotics used were Kanamycin 30 µg, Erythromycin 15 µg, Cinoxacin 100 µg, and Cefazolin 30 µg. After the selected LAB cultures achieved 10^8 CFU.mL^{-1}, 50 µL of the LAB cultures were spread onto the MRS agar using a sterile cotton swab, and the antibiotic disks were placed on the surface of the agar media and incubated at 37°C for 24 h [20]. The disk dripped with MRS broth media was used as a negative control. The inhibition zones around the disks were in millimeters and calculated using Formula 2. The sensitivity to antibiotics was categorized as shown in Table 1 [20].

$$\text{DCZ} = \text{DT-DC} \ldots\ldots\ldots\ldots \text{(2)}$$

Description :

DCZ : Diameter of the clear zone (mm)
DT : Diameter of total (mm)
DC : Diameter of disk (mm)

Table 1. Criteria for sensitivity to antibiotics

Antibiotics	Diameter of inhibition zone (mm)		
	Resistant (≤)	Intermediates	Sensitive (≥)
Kanamycin (30 µg)	13	14-17	18
Erythromycin (15 µg)	13	14-22	23
Cinoxacin (100 µg)	15	16-20	21
Cefazolin (30 µg)	14	15-22	23

Hemolytic Test

The hemolytic test was performed on blood agar containing 5% sheep blood. The 24-h LAB cultures were streaked on the blood agar and incubated at 37°C for 24 h [21]. The observed of clear zones indicated hemolytic activity. The isolates with no hemolytic activity were selected for the next assay.

Acidification Activity Test

The acidification activity test was carried out using skim milk media supplemented with 0.3% yeast extract and 0.2% glucose [22]. The 3 mL of selected LAB cultures were inoculated into 27 mL of skim milk and then incubated at 30°C for 24 h. The skim milk without inoculum was used as a control. The sampling was conducted at 0, 2, 4, 6, and 24 h of incubation to evaluate pH change (ΔpH) and calculated using Formula 3. The cultures were considered to have rapid, moderate, or slow acidification rate when ΔpH=0.4 and were achieved after 3, 3-5, or >5 h, respectively.

$$\Delta\text{pH} = \text{initial pH} - \text{final pH} \ldots\ldots\ldots \text{(3)}$$

Molecular Identification

The selected isolate was identified using a molecular technique based on 16s rDNA sequences. The total DNA was extracted using the Zymo-Spin™ Lysis Kit following the manufacturer's instructions. The PCR program was run at 95°C for 5 min for initial denaturation, followed by 35 cycles of 95°C for 30 seconds of denaturation, 52°C for 45 seconds of annealing, 72°C for 90 seconds of extension, and the final extension at 72°C for 5 min. The extracted DNA was amplified using 16S rDNA universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT CTG ACT ACT T-3'). The amplicon was confirmed using gel agarose electrophoresis (1.5%) and then sequenced at 1st BASE, Malaysia. The sequences were analyzed for constructing a phylogenetic tree using MEGA 6 software with the Neighbor-Joining algorithm and the Tamura-Nei model with a bootstrap of 1000 [17].

Data Analysis

Data of proteolytic activity, lipolytic activity, antibacterial activity, antibiotic sensitivity, and acidification were analyzed by ANOVA with IBM SPSS v.25 software with a 95% confidence level. The data were considered significant when $p \leq 0.05$. In addition, the Tukey test was carried out to determine the difference among isolates. Each test was carried out in triplicates.

RESULT AND DISCUSSION

The nutritional contents of buffalo milk play an important role in determining the quality of the milk as well as supporting the growth of indigenous microbes. The proximate analysis of Sumbawa buffalo milk samples (in the average of two samples) revealed that protein content of 5.52%, a fat content of 11.13%, the water content of 77.48%, ash of 0.86%, carbohydrates of 5.01%, and pH of 6.82 (Table 2). The highest protein content in sample S was 6.03%, and the highest fat content in sample T was 12.82%. The buffalo milk samples from North Lintau Buo District, Tanah Datar Regency, West Sumatra, has protein content of 5.23–6.49%, fat content of 7.84–12.44%, the water content of 76.27–80.61%, and pH 5.6–6.0 [23]. It is not much different from the proximate content in Sumbawa buffalo milk, but the pH of Sumbawa buffalo milk was closer to a neutral pH.

Table 2. Proximate content in Sumbawa buffalo milk samples

Parameters	Proximate Content		
	Buffalo Milk Sample S	Buffalo Milk Sample T	Average
Protein (%)	6.03	5.01	5.52
Fat (%)	9.44	12.82	11.13
Water (%)	78.63	76.32	77.48
Ash (%)	0.88	0.84	0.86
Carbohydrate (%)	5.02	5.01	5.01
pH	6.83	6.81	6.82

In general, buffalo milk has protein content of 3.6%, fat content of 7.4%, and lactose content of 5.5% [24]. Therefore, the protein and fat content of the Sumbawa buffalo milk samples were higher than of buffalo from other regions. The Sumbawa buffalo milk has the potential to be used as raw material for developing milk products with a high nutrition. The buffalo found on Sumbawa Island is a swamp or mud buffalo. Swamp buffaloes produce less milk than River buffaloes but have more nutritional qualities, including protein, lean, dry weight, and higher fat than River buffalo [25]. However, further research is still warranted to collect more diverse samples to ensure this result.

Lactic acid bacteria are commonly found as indigenous bacteria in fresh milk, including buffalo milk. It is supported by the result of this study that the total LAB was $4.7x10^5$ CFU.mL^{-1}. This cell density was lower than that of fresh buffalo milk from North Lintau Buo District, Tanah Datar Regency, West Sumatra, which the total LAB reported by $2x10^7$–$1.2x10^8$ CFU.mL^{-1} [23]. It is probably due to the pH difference. The pH of fresh buffalo milk from Lintau Buo Utara District, Tanah Datar Regency, West Sumatra, is lower (5.6–6.0) than the pH in Sumbawa buffalo milk (6.82).

Based on phenotypic characteristics, a total of 21 LAB isolates were obtained. The entire LAB isolates showed Gram positive and catalase negative. The proteolytic activity test was determined for the initial screening stage of LAB as a starter culture. Based on the proteolytic activity, 17 isolates were categorized as high proteolytic activity, including SA8 (5.97 mm), SA1 (5.70 mm), TA6 (5.68 mm), SA9 (5.63 mm), TA12 (5.57 mm), SA7 (5.33 mm), TA5 (5.27 mm), TA8 (5.08 mm), TA11 (4.90 mm), SA2 (4.73 mm), SA4 (4.67 mm), TA9 (4.53 mm), TA1 (4.47 mm), SA3 (4.33 mm), TA2 (4.27 mm), SA6 (4.23 mm), TA4 (4.17 mm) (Fig. 1). Of these isolates, ten isolates exhibiting proteolytic activity of more than 4.70 mm were selected for the next screening stage. In this proteolytic activity, the protein (casein) in milk is hydrolyzed and used as a nutrient. Proteolytic activity plays an important role during the fermentation process. The decomposition rate of polypeptides into peptides and amino acids determines the acceleration of the fermentation process.

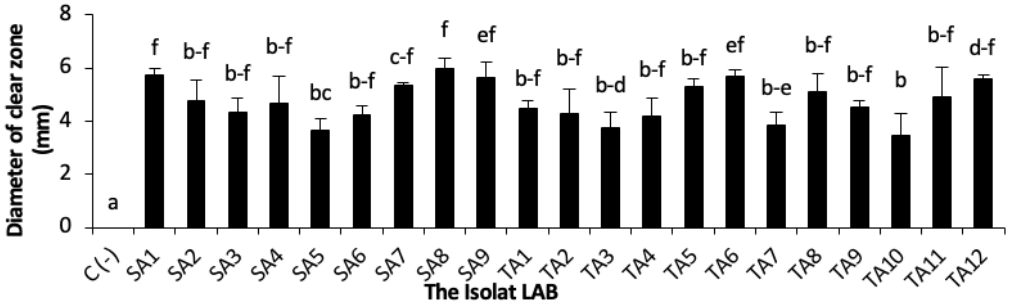

Figure 1. Proteolytic activity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** C (-) = negative control. SA = sample form Srangin Village, 1-9 = isolate number, TA = sample from Tanakakan Village, 1-12 = isolate number.

The LAB proteolytic system plays a key role in the fermentation process, allowing bacteria to grow and adapt inside the milk, thus ensuring a successful fermentation process [27]. Besides supporting bacterial growth, the proteolytic activity of LAB is essential for contributing to the development of organoleptic properties of fermented milk products, especially flavor quality [28]. In fermentation products, the proteolytic bacteria can increase the number of peptides and free amino acids, which affects its flavor. From the perspective of the fermentation industry, the proteolytic properties and the ability of lactic acid production from sugar are the main criteria for determining the starter culture [29]. The selection of starter cultures based on enzyme production and proteolytic activity is essential, in addition to other characteristics such as acidification properties and resistance to high NaCl concentrations [30].

The lipolytic activity was performed to obtain LAB with low activity in utilizing lipids as a carbon source to avoid the rancid flavor. Of 10 selected LAB isolates, seven LAB isolates (TA6, TA11, SA8, SA9, TA5, SA2, and TA12) were selected with low lipolytic activity (Fig. 2).

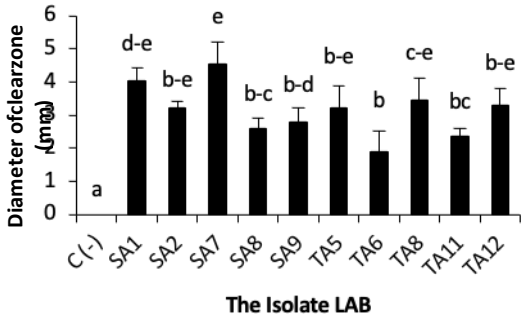

Figure 2. Lipolytic activity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** C (-) = negative control. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

Lipase from LAB contributed to the development of flavors in food [31]. In the case of fermented milk products, except cheese, the rancid odor is categorized as an undesirable flavor. The impact of lipolytic activity can result in the appearance of rancid odors caused by short fat chain reactions such as butyrate [32]. Therefore, high lipolytic activity is desirable for starter culture in cheese production. However, this study was designed to explore the starter culture for fermenting buffalo milk to produce non-cheese products, such as yoghurt and other beverage fermented products. Therefore, the LAB isolates with low lipolytic activity were selected. The seven LAB were confirmed qualitatively in producing exopolysaccharides as indicated by the appearance of ropy or mucoid colonies. The colonies still resembled sticky threads when pulled. However, it is necessary to conduct quantitative analysis to ensure the production of EPS and its concentrations.

Exopolysaccharide-producing bacteria show a ropy or mucoid (slimy) colony [19]. In addition, some LAB produces extracellular capsules or polysaccharides with technological properties and desired biological activity. The polysaccharides produced by LAB are called EPS and can be used to modify rheological properties (viscosity) and play a role in emulsification and flocculation [33]. EPS are produced extracellularly by microbes, and it is connected to the cell in the form of a capsule or mucus of the outside the bacterial cell wall or cell surface. The production of EPS can be easily detected in response to sucrose contained in the media.

However, detection of EPS production cannot be merely dependent on qualitative tests. The LAB isolated from Indonesian traditional foods obtained 108 isolates, and only 60% of isolates produced EPS [34]. Furthermore, from six LAB isolates from Sumbawa fermented mare's milk products, there was only one isolate (BC7 isolate) capable of producing EPS, as indicated by the texture of the ropy colony. The other isolates were classified as soft-type colonies. However, the soft-type colony which did not form sticky threads produced a high EPS concentration which was detected quantitatively [17].

The antibacterial activity test showed the three LAB isolates were able to inhibit the indicator bacteria (*B. cereus*, *E. coli*, and *S.* Typhi), namely SA8, SA9, and TA6 (Fig. 3). Interestingly, seven LAB isolates were able to inhibit the growth of *S.* Typhi, but none can inhibit *S. aureus*. The SA8 isolates exhibited a potential antibacterial activity as it showed the highest inhibitory activity against *E. coli* (1.80 mm) and *S.* Typhi (2.60 mm) but moderately against *B. cereus* (2.27 mm).

Lactic acid bacteria used as starter cultures can extend the products due to the presence of natural antibacterial compounds in the form of organic acids as primary metabolite products, such as lactic, acetic, and formic acids. It leads to a decrease in pH, consequently inhibiting the growth of foodborne pathogens [35]. However, other antibacterial compounds are also produced, such as ethanol, H_2O_2, diacetyl,

reuterin, and bacteriocins. All such antibacterial compounds can resist the growth of pathogenic bacteria in food and control most undesirable organisms [36]. The antibacterial activity shown in this study was the action of non-acid compounds as the neutralized CFS was applied. Therefore, it is suggested to conduct more studies to confirm whether the bacteriocin is responsible for this inhibitory activity.

Some metabolites, such as antibacterial peptides, can play a role in LAB's performance and metabolism, affecting the safety of fermented products. LAB that are often used as starter cultures are *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp., or *Pediococcus* spp. showed a low level of virulence factor, including antibiotic resistance and biogenic amine-related genes, and can be considered safe [30]. Bacteriocins as bactericidal peptides has modes of action, such as pore formation, DNA cell degradation, and inhibition of peptidoglycan synthesis. Bacteriocins can be absorbed by teichoic acid and lipoteichoic acid. These acid compounds are found on the surface of Gram-positive bacteria. The activity of bacteriocins is categorized as a narrow spectrum against bacteria that are still closely related to their producing bacteria [37].

Staphylococcus aureus was the only indicator bacteria that cannot be inhibited. Adha [38] reported that bacteriocin produced by *Lactobacillus plantarum* isolated from curd was not able to inhibit *S. aureus. Staphylococcus aureus* can form layers in the extracellular matrices (biofilm), which might increase tolerance to antibiotics, disinfectants, and resistance to other immunocompetent cells [38].

Based on the antibacterial activity test, three isolates were the potential antibacterial producer (SA8, SA9, and TA6) because they can inhibit three indicator bacteria, and the SA8 isolate showed the most potential antibacterial producer. The selected isolates showed a sensitivity to the antibiotic cefazolin and resistance to kanamycin (Fig. 4). Although the TA6 isolate showed the presence of a clear zone (5.43 mm), based on James's interpretation [20], the zone formed is still categorized as resistant to kanamycin. The SA2 and SA9 isolates were sensitive to erythromycin and cefazolin. But they were resistant to kanamycin and cinoxacin. The TA5 isolate were sensitive to the erythromycin and cefazolin but intermediates to cinoxacin.

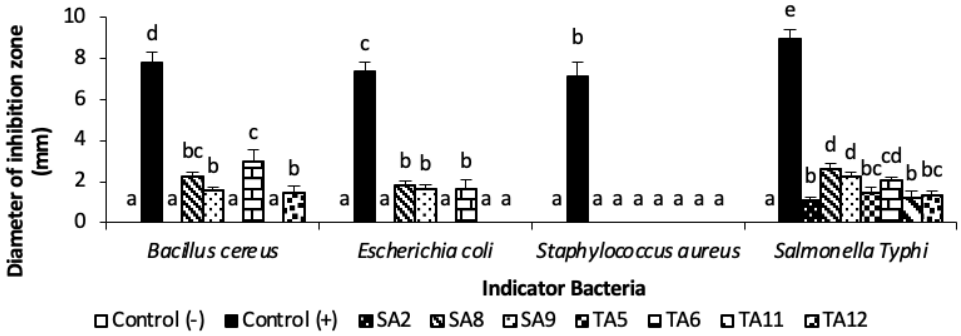

Figure 3. Antibacterial Activity by LAB isolates isolated from Sumbawa Buffalo milk. The notation on the graph shows the real difference of Tukey at p<0.05. **Description:** C (-) = negative control, C (+) = positive control. SA = sample form Srangin Village, TA = sample from Tanakakan Village

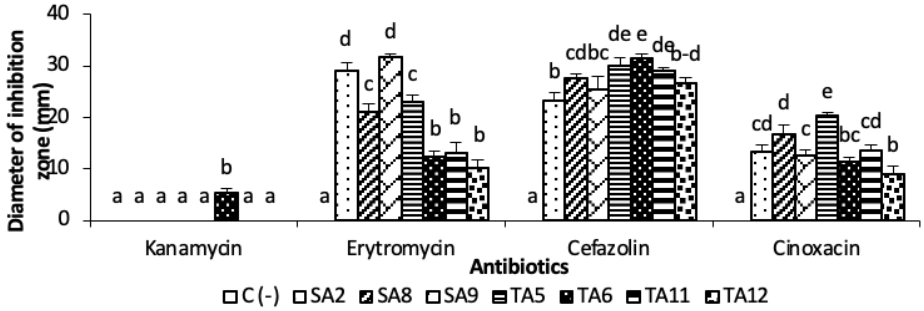

Figure 4. Antibiotic sensitivity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates (p<0.05). **Description:** C (-) = negative control. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

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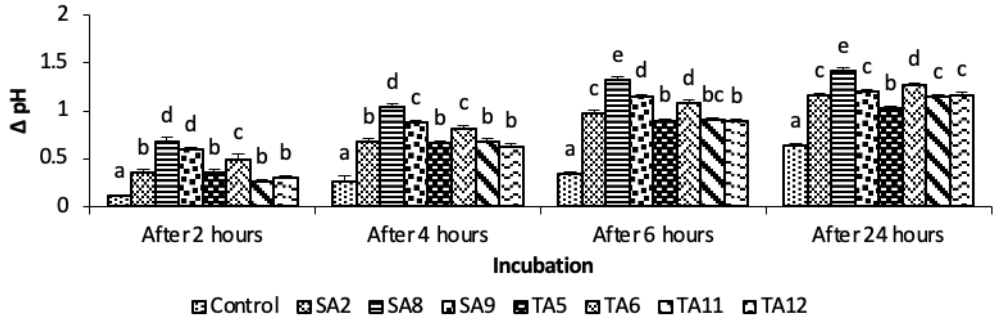

Figure 5. Acidification activity of LAB isolates isolated from Sumbawa Buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** Control = without inoculum. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

The resistance to kanamycin has also been reported before. Four out of six LAB isolates from Sumbawa fermented mare's milk products were resistant to kanamycin [17]. Microbial resistance to antibiotics is caused by the excessive use of antibiotics and causes selective pressure on the proliferation of microorganisms [39].

To confirm the pathogenicity potency, the seven LAB isolates, which have been selected from a range of starter culture criteria before, showed that these LAB isolates did not exhibit lysis activity towards red blood cells contained in the blood agar media. These isolates were categorized as gamma haemolysis. It can be concluded that the selected LAB isolates were safe for use in food production.

The acidification test was the final screening for the starter culture used in this study. The isolates of SA8, SA9, and TA6 were categorized as having very rapid acidification rates with a pH change above 0.4 achieved after 2 h of incubation (Fig. 5). Meanwhile, the other isolates (SA2, TA5, TA6, TA11, and TA12) had moderate acidification activity with a pH change of 0.4 achieved after 4 h of incubation. The isolates of SA8 and SA9 were the fastest in lowering the pH of milk after incubating for 2 h, with pH changes of 0.68 and 0.59, respectively. Meanwhile, the TA11 isolate was the lowest acidification activity, with a pH change being 0.26. The SA8 isolate was the most rapid in lowering the pH of milk at every sampling.

Acidification activity as a key mechanism of fermentation showed a significant influence on the product's sensory profile and the LAB's strain stability. The initial stage of metabolism in fermentation using mixed microorganisms occurs in a very rapid acidification process. It is due to an exchange of several metabolites, indicating a strong acid production capacity. As fermentation progresses, an increase in acid concentration might inhibit the growth of LAB, thereby affecting acid production [40]. Acidification and rapid growth of the LAB starter culture during food fermentation minimize the risk of spoilage and failure of the fermented products. In addition, starter culture plays a major role in the change of taste. Recent studies on different microbial species have shown that high growth rates were influenced by the degree of expression of metabolic enzymes and stress proteins. In starter cultures, such exchanges will affect the formation of sensory profiles and bacterial survival [41]. Based on all screening steps conducted, the SA8 isolate was selected as a potential candidate for a starter culture. Furthermore, the phylogenetic analysis successfully identified this isolate as *Enterococcus lactis* with a similarity level of 99.99% toward *E. lactis* BT159 (Fig. 6).

Genus *Enterococcus* was rarely found in buffalo milk originating from Indonesia. *Enterococcus faecalis* was identified in the milk of Belang Toraja buffalo [21]. On the contrary, Genus *Lactobacillus* was dominantly reported. *Lactobacillus plantarum*, *L. brevis*, *L. pentosus*, and *L. Lactis* were found in buffalo milk from North Sumatra [9]. *Lactobacillus brevis*, *L. paracasei*, *L. pentosus*, *L. plantarum*, and *L. Lactis* were detected in buffalo milk from Pampangan, South Sumatra [42]. Moreover, *L. fermentum* strain NRIC 0129 was reported in buffalo milk from LintauBuo Utara District, Tanah Datar Regency [23]. *Enterococcus* is the main culture found in cheese products, acting as a natural starter during fermentation. It plays an important role in cheese ripening by giving the cheese its distinctive taste and taste [43]. *Enterococcus* is a hardy species and can adapt well to harsh environmental conditions [44].

Enterococcus lactis strains were susceptible to

ampicillin, gentamicin, penicillin, vancomycin, clindamycin, sulfamethoxazole, and chloramphenicol. But it was resistant to erythromycin and tetracycline [45]. Some strains of *E. lactis* were considered potential probiotic strains because they lack a specific virulence and antibiotic resistance gene [46]. *Enterococcus lactis* produces enterocins A, B, and P and exhibits antagonistic activity against pathogenic bacteria and fungi [47].

The SA8 isolate was genetically closely related to *Enterococcus lactis* strain BT159. The strain BT159 was first isolated from Bitto cheese made from cow's and 10% goat's milk. According to Morandi *et al.* [43], this strain was characterized as Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, catalase-negative, coccus-shaped, and arranged in pairs or short chains. The characteristics of the colonies on MRS agar were whitish, smooth, and circular, with a thorough border texture. This strain was able to hydrolyze gelatin and tributyrin, as well as produce gas from glucose. All *Enterococcus lactis* strains can grow on NaCl with concentrations of 2, 4, and 6.5% and withstand temperatures of 10 and 45 °C. They also can grow in litmus milk and cause acidification of cheese at 24-hour incubation [43].

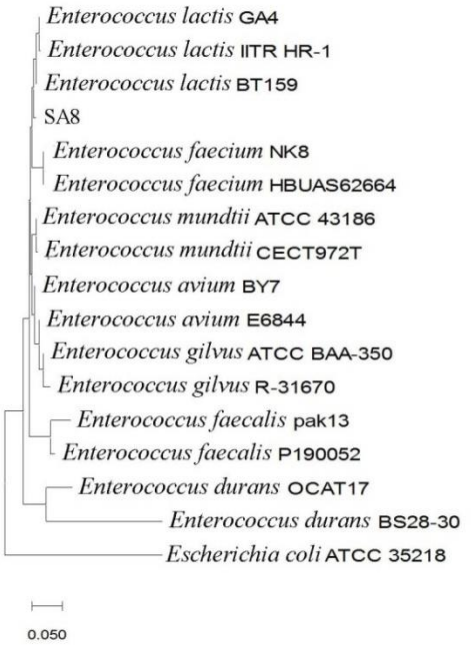

Figure 6. Phylogenetic tree showing the relationship between SA8 isolates and reference bacterial strains based on analysis of 16S rDNA sequences.

CONCLUSION

A total of 21 LAB isolates were obtained from Sumbawa buffalo milk with a total LAB of $4.7x10^5$ $CFU.mL^{-1}$. All isolates were Gram-positive, catalase-negative, and cocci-shaped. The SA8 isolate was selected as the most potential candidate for a starter culture. This isolate has met the starter culture criteria used such as the best ability in proteolytic activity, the lowest lipolytic activity, producing EPS, the best inhibitory activity against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhi, and sensitivity to cefazolin, intermediate to erythromycin and cinoxacin, no hemolytic activity, as well as the fastest acidification rate. The isolate SA8 was identified as *Enterococcus lactis* with a similarity level of 99.99%.

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Curcuma mangga Ethanol Extract Improves Sperm Quality of Mice Exposed to Monosodium Glutamate

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Abstract

The current study aimed to determine variations effect in the dose of *Curcuma mangga* ethanol extract on male mice exposed to monosodium glutamate (MSG). The 24 adult male mice with the body weight of 25-30 g and 3-4 months old were used, then divided into six groups (n= four per group). The groups are K- (untreated mice), K+ (induced MSG) for 45 days, P1 (MSG + *C. mangga* extract 25 mg.g^{-1} BW), P2 (MSG + *C. mangga e*xtract 50 mg.g^{-1} BW); P3 (MSG + *C. mangga* extract 75 mg.g^{-1} BW); and P5 (MSG + *C. mangga* extract 100 mg.g^{-1} BW) for 30 days. Animals were dissected on the 46th day. The epididymis was isolated, and sperm quality was evaluated. The results showed that25-75 mg.g^{-1} BW *C. mangga* ethanolic extract could increase sperm viability by 24-29%, while at a dose of 25-100 mg.g^{-1} BW the sperm motility decreased by 58-92%. The administration of 100 mg.g^{-1} BW *C. mangga* could decrease it by 58% and concentration at a dose of 100 mg.g^{-1} BW increased sperm concentration by 96%. The use of MSG can increase sperm abnormalities compared to controls. This study showed that *C. mangga* effectively improved the sperm quality of mice exposed to MSG.

Keywords: *Curcuma manga*, Epididymis, Monosodium Glutamate, Sperm quality.

INTRODUCTION

Monosodium glutamate (MSG) is a sodium salt of L-glutamic acid from glutamate purification or a combination of several amino acids with a small amount of peptide produced from the hydrolyzed vegetable protein (HVP) process. Glutamic acid is an essential amino acid because the human body can also produce glutamic acid [1]. Glutamic acid is indispensable in human metabolism and is also one of the main essential components in high-protein food products from animals such as meat, milk, fish, and cheese. It is also found in plants such as tomatoes and mushrooms [2,3,4]. Glutamate will diffuse throughout the body through the bloodstream and bind to NMDA receptors (N-Methyl-D-Aspartate), Metabotropic Glutamate (Glu), and Kainate (Ka). Increased glutamate concentration can lead to increased activation of glutamate receptors.

The increase in the production of Reactive Oxygen Species in the Tricarboxylic Acid (TCA) cycle in the mitochondria was due to an increase in intracellular ca^{2+} ions [5]. The imbalance between antioxidants and reactive oxygen species (ROS) causes cell damage due to oxidative stress. Increased free radicals and lipid peroxidation production at the testicular tissue level cause changes in spermatozoa morphology accompanied by an increase in cholesterol in the testes, causing degeneration of gonadal cells. It will affect the motility of spermatozoa [3].

The spermatozoa characteristics are used to observe the effect of MSG, such as the decreased amount of motility, viability, and abnormalities of sperm. Administration of MSG also causes excitotoxic effects, such as disturbances in the Hypothalamus-Pituitary Axis. The hypothalamus is a homeostatic regulatory center. It regulates the secretion of hormones, including gonadal hormones. Excessive levels of MSG cause damage to the arcuate and ventromedial nuclei in the hypothalamus. It can cause a decrease in the secretion of (Gonadotropin Releasing Hormone) GnRH, which affects the anterior pituitary in secreting gonadotropin hormones, such as (Luteinizing Hormone) LH and (Follicle Stimulating Hormone) FSH [6].

Curcumin is the primary pigment of ginger used as a spice, food coloring, cosmetic and medical agent. The potential preventive or therapeutic properties of curcumin correlate with its antioxidant, anti-inflammatory, antitumor, antiviral, and antiallergenic properties [7,8]. Antioxidants are essential in protecting against lipid peroxidation, which has a protective effect by preventing oxidative damage. Antioxidants are essential in many biological processes that can reduce sperm cell abnormalities and motility caused by chemicals [9]. One of the most widely cultivated medicinal plants comes from the Zingiberaceae family, known as *temu-mangga*

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(Indonesian). *Curcuma mangga* is one of this family's plants that contains antioxidants [10]. The antioxidants in *C. mangga* are curcumin, polyphenols, flavonoids, and P-hydroxycinnamic acid compounds [11]. The presence of flavonoid compounds in *C. mangga* is closely related to its antioxidant activity. It happens because phenol groups can inhibit the formation of lipid peroxidation in the early stages and act as an antidote to reactive oxygen free radicals and hydroxyl radicals by donating hydrogen. Atoms become peroxyl radicals which form flavonoid radicals to form flavonoid radicals. React reactively and neutrally to oxygen [7].

Jalip *et al.* [12] investigated the antioxidant activity of *C. manga* methano extract using the DPPH radical scavenging method and showed an IC_{50} value of 90.42 $g.mL^{-1}$. This research found that the ethanol extract of *C. mangga* has higher antioxidant activity, thus becoming the object of this research. In addition, ethanol is also widely used as a solvent for extracting polar compounds such as phenolics and flavonoids because of the principle of like dissolving like so that polar compounds will be more soluble in more polar solvents. The antioxidant content in *C. mangga* can decompose free radicals produced by MSG, which are stable and not reactive in the body. Based on this description, this study aims to determine the effect of the ethanol extract of *C. mangga* on the quality of spermatozoa in MSG-induced male muscle.

MATERIAL AND METHOD

Experimental animals were randomly divided into six groups (n= four per group, Table 1). MSG and *C. mangga* extract was administered daily by oral gavages. The animals were housed in conventional plastic cages measuring 22 x 31 x 9.5 cm (1 animal per cage) with wood shavings and standard laboratory conditions at room temperature with a light/dark cycle every 12 hours.

Table 1. Experimental animals groups

Group	4 $mg.g^{-1}$ BW MSG intake (days)	*C. mangga* Dose ($mg.g^{-1}$ BW)	*C. mangga* intake (days)
K-	-	-	-
K+	45	-	-
P1	45	25	30
P2	45	50	30
P3	45	75	30
P4	45	100	30

Notes: K- = Negative control, K+ = positive control, P = treatments, BW = body weight.

Standard food (HI-PRO – VIT 511) and water (Le mineral PT Mayora Indah Tbk (MYOR)) are available ad libitum. Experimental animals were dissected on day 46th by dislocation, then the epididymis was isolated, and the spermatozoa were evaluated. It following the principles of Animal Care (No: 023-KEP-UB-2022).

Spermatozoa Collection and Analysis

The right and left cauda epididymis were taken and placed in a watch glass containing 1.5 ml of NaCl solution at 37°C [13]. Viability, motility, abnormalities, and sperm concentration were observed using a light microscope with 100 and 400x magnification.

Sperm Motility

Semen is added to the object-glass as much as 10 L. Sperm movement was observed using a light microscope with a magnification of 100x for five fields of view [14]. Assessed and recorded the results of the percentage of sperm motility (Table 2) and analyzed according to the criteria [15].

Table 2. Criteria of sperm motility categories [16]

Score	Category	Progressive Motile
1	Very Poor	0-20%
2	Poor	20-40%
3	Good	40-60%
4	Very Good	60-80%
5	Excellent	80-100%

Sperm Viability and Abnormality

A total of 10 µL of liquid cement was added to the object-glass and dyed with 1% eosin/5% negrosin. Sperm viability and abnormalities percentage were examined using a light microscope with 400x magnification in 3 fields of view (or up to 200 cells). The formula is as follows [17]:

$$viability\ (\%) = \frac{live\ sperm\ count}{total\ sperm\ count}\ x\ 100\%$$

$$Abnormality\ (\%) = \frac{Normal\ sperm\ count}{total\ sperm\ count}\ x\ 100\%$$

Sperm Concentration

The sperm was diluted 50x by 20 µL liquid semen that added to 980 µL fixative solution (1:1) sodium bicarbonate (NaHCO3) and 10% formalin. The liquid semen was taken and placed in a Neubauer Haemocytometer. Spermatozoa were observed and counted with a microscope with a magnification of 400x, and the number of sperm was counted in five small chambers per sample using the formulation [16].

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$$SC = n \times k \times FP \times 10^4$$

Description:
n = number of spermatozoa counted
10^4 = hemocytometer chamber volume
FP = dilution factor
k = number of small squares counted

Statistical Analysis

Control and experimental group data are presented as mean and Standard Deviation (SD). Significant differences between treatment groups were analyzed using one-way ANOVA with SPSS 26.0 for the windows. If the results of the one-way ANOVA analysis are significantly different ($P<0.05$), then it is continued with the analysis of the Least Significant Difference (BNT) test.

RESULT AND DISCUSSION

The quality of semen was analyzed with the following results Table 3. The quality include sperms' motility (SM), viability (SV), abnormality (SA), and concentration (SC).

Table 3. The quality of spermatozoa

Group	Semen Quality ± SD			
	SM (%)	SV (%)	SA (%)	SC (10^6.mL^{-1})
K-	60 ± 9.1	81 ± 3.2	46 ± 15.1	11.2 ± 4.1
K+	30 ± 12.4	61 ± 4.5	56 ± 12.3	5.6 ± 0.7
P1	47 ± 15.7	78 ± 2.5	51 ± 17	9.7 ± 2.8
P2	50 ± 9.4	79 ± 4.2	61 ± 20.2	9.9 ± 3.6
P3	57 ± 9.1	76 ± 6.3	44 ± 13.9	12.2 ± 3.5
P4	49 ± 6.9	66 ± 8.9	23 ± 6.1	1.1 ± 2.9

Description: SD = Standard Deviation. The same letter in the treatment variable shows a non- real difference based on Duncan's 5% test.
K- = Control, K+ = Treatment with MSG
P1 Treatment = MSG + *C. mangga* 25 mg.g^{-1} BW
P2 Treatment = MSG + *C. mangga* 50 mg.g^{-1} BW
P3 Treatment = MSG + *C. mangga* 75 mg.g^{-1} BW
P4 Treatment = MSG + *C. mangga* 100 mg.g^{-1} BW

Sperm Motility

The results showed that MSG treatment could decrease sperm motility (24%) compared to the control. Administration of *C. mangga* extract at a dose of 25-100 mg.g^{-1} BW increased the sperm motility of mice induced by MSG, specifically in *C. mangga* at a dose of 75 mg.g^{-1} BW, with the highest value of 91.5% (Table 3).

Giving MSG at a dose of 4 mg.g^{-1} BW for 45 days can reduce sperm motility. Spermatozoa motility is divided into four categories, fast-moving spermatozoa, slow-moving spermatozoa, spermatozoa move in place and stay still. In the results of the study, MSG causes a decrease in the number of fast-moving spermatozoa and also an increase in the number of stationary spermatozoa.

MSG exposure can stimulate lipid peroxidation in the testes, which indicates oxidative stress due to ROS formation and decreased antioxidants in the body. The increase in ROS production will be followed by PARP^{-1} activation, where PARP^{-1} catalyzes the hydrolysis of NAD$^+$ to nicotinamide PAR. It happens because of the reduced supply of NAD$^{+,}$ so the production of ATP as an energy source for sperm cells decreases and causes cell death [18]. Reduced ATP synthesis by mitochondria can decrease sperm motility [19].

The administration of *C. mangga* extract at a dose of 25-100 mg.g^{-1} BW increased sperm motility due to Curcumin compounds acting as antioxidants. Curcumin acts as a scavenger against free radicals [20]. Curcumin can reduce oxidative stress and increase motility, viability, and plasma membrane integrity. Mammalian spermatozoa are rich in unsaturated fatty acids in the plasma membrane, so they are susceptible to ROS attack [21]. The decrease in free radicals can improve mitochondrial function, increasing ATP production [22]. The availability of sufficient ATP is also a condition of good membrane fluidity to increase sperm movement and capacitation, increasing sperm motility [23]. It is because curcumin inhibits the formation of superoxide and hydroxyl anion radicals by preventing the oxidation of Fe^{2+} and FE^{3-} through the Fenton reaction. However, the increased concentration of ROS molecules due to curcumin supplementation may be another reason for increased sperm motility [20,24,25].

Sperm Viability

The low percentage of viability occurs due to the administration of high doses of MSG. Because MSG contains sodium, which, if consumed in excessive amounts, will cause an increase in the amount of sodium in the body, excessive consumption of MSG can also form ROS. Increased levels of ROS that exceed the body's antioxidant limit can produce oxidative stress that causes damage to cell walls, cell nuclei, and capsules in sperm [26-28]. Increased ROS caused by excessive doses of MSG can cause oxidative stress that can damage DNA in the nucleus of spermatozoa. Causes of other degenerative changes, such as increased cell death through apoptosis and cell necrosis [29].

The body needs antioxidants to neutralize free radicals and prevent damage caused by free radicals. Antioxidants and free radicals complement the electron deficiency of free

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radicals and the formation of free radicals, causing oxidative stress [29]. Besides a high antioxidant content in *C. mangga*, other bioactive compounds found are flavonoids, polyphenolic curcumin, and p-hydroximic acid [30]. The presence of flavonoid compounds in *C. mangga* is related to its antioxidant activity due to the presence of a phenyl group that can inhibit the formation of lipid peroxidation at the initiation stage. It also acts as a scavenger of reactive oxygen free radicals or hydroxyl radicals by donating hydrogen atoms to peroxyl radicals, which form flavonoid radicals so that they react with oxygen, so that it becomes neutral [31]. The curcumin in *C. mangga* also a compounds which act as good antioxidants. This is supported by previous studies that curcumin acts as a free radical scavenger [20]. Curcumin also reduces oxidative stress and improves the plasma membrane's motility, viability, and integrity [32].

Sperm Abnormality

MSG exposure can increase sperm abnormalities by 20.6% when compared to controls. In this case, the administration of *C. mangga* ethanol extract was able to significantly reduce spermatozoa abnormalities at a dose of 100 $mg.g^{-1}$ BW by 58% (Table 3). After being treated for 45 days, the morphological abnormalities of spermatozoa, headless spermatozoa, double-headed spermatozoa, curved tail, and micro-heads are in Figure 1.

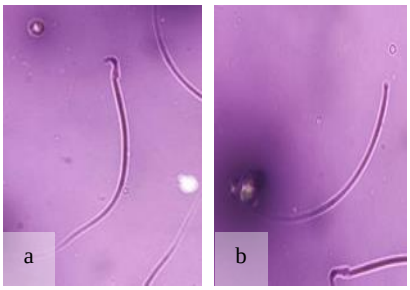

Figure 1. Spermatozoa Morphology (magnification 400x)
(a) Normal spermatozoa,
(b) Headless spermatozoa

This study found primary and secondary abnormalities in spermatozoa. The primary abnormalities were small heads, amorphous heads, and spiral tails, while the secondary abnormalities were headless and tailless spermatozoa. It is because ROS affects the plasma membrane of spermatozoa which contains large amounts of phospholipids and unsaturated fatty acids, so unsaturated fatty acids are susceptible to ROS, especially hydroxyl radicals, which are the most reactive derivatives. After all, hydroxyl radicals cause a chain reaction called lipid peroxidation. It resulted in the breaking of fatty acid chains into compounds. These compounds are toxic to spermatozoa cells, including 9-hydroxy-none malondialdehyde (MDA) and ethane (C_2H_6) [22].

Peroxyl radicals are reactive enough to attack surrounding fatty acids to form lipid hydroperoxides and new carbon-centered radicals called hydroxyl radicals. The accumulation of lipid hydroperoxides in the membrane disrupts cell function. It is the primary agent of changes in spermatozoa morphology from regular to abnormal [33]. Curcumin inhibits lipid peroxidation by absorbing free radicals and increasing the activity of endogenous antioxidant enzymes, CAT and SOD [34]. A significant relationship exists between increased free radicals and sperm malformations [35]. Based on this, the antioxidants present in the extract could protect the cell structure of spermatozoa from damage caused by free radicals. Thus, the plasma membrane could be maintained, and the abnormal spermatozoa produced decreased compared to the group that was only exposed to MSG [36].

Spermatozoa Concentration

The results showed that MSG treatment could cause a decrease in sperm concentration when compared to the control. The reduction was 50%. The highest sperm concentration was found in the treatment with *Curcuma mangga* extract at a dose of 25 $mg.g^{-1}$ BW by 72% compared to the treatment with monosodium glutamate (Table 3).

Based on these results, MSG can reduce the concentration of spermatozoa. Decreased ROS and lipid peroxidation as a result of MSG, proven by the previous research, which states that MSG can increase the formation of MDA as a result of the reaction between OH radicals and unsaturated fatty acids, which are usually present in the liver, kidneys, brain and spermatozoa [37]. Therefore, the increase in MDA due to MSG can be used to indicate a high increase in ROS in the body. Damage to cell membranes and DNA, both mitochondria and cell nuclei, caused by lipid peroxidation, causes spermatozoa to undergo apoptosis [38]. As a result of this apoptosis, the concentration and percentage of normal spermatozoa morphology decreased [39].

From the study results, *C. mangga* extract was able to increase the concentration of spermatozoa, which was quite significant compared to the treatment that was only given

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MSG. It follows the statement that the bioactive compound curcumin acts as an antioxidant and increases sexual libido [40]. The curcumin compound in Curcuma is protective and reduces oxidative stress. The administration of Curcuma extracts from the tests gives a preventive effect characterized by an increase in spermatogonia, primary spermatocytes, and spermatids on microscopic images [41]. In addition, a study conducted [42] stated that there was an increase in sperm concentration, SOD levels, and testosterone levels in the group given curcumin.

CONCLUSION

This study concluded that *C. mangga* extracts at doses of 75 and 100 $mg.g^{-1}$ BW was more effective in improving sperm quality. The quality included spermatozoa motility by 57% and 49%, viability by 76% and 66%, abnormalities by 44% and 23%, and concentration of 12.2 x 10^6 mL^{-1} and 1.1 x 10^6 mL^{-1}, respectively.

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Textile Industry Waste Pollution in the Konto River: A Comparison of Public Perceptions and Water Quality Data

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Abstract

Textile industry production activities in one of the Badas areas can have implications for the occurrence of dye liquid waste pollution in the Konto river flow. This implication leads to the public perception that there has been river pollution from textile dyeing liquid waste. In this case, public perceptions need to be analyzed as a form of river environmental monitoring activities. Therefore, public perceptions of pollution were compared with actual water quality data, especially related to dyes in river water, to link public perceptions with environmental resource management efforts. This study aims to analyze public perceptions of textile dye waste pollution and compare the analysis results with the river water quality analysis. Analysis of river water quality (dye content, BOD, COD) was conducted at five sampling points in Badas, Kuwik, Balungjeruk, and Wonorejo villages. Public perception was measured descriptively through interviews with respondents referring to the Slovin method. The analysis of public perceptions shows that the Public considers that there has been pollution of river water, mainly due to textile industry waste, along the Konto River. The results of the water quality analysis showed that dye concentrations were found at four sampling points except for Wonorejo Village. This result shows that the correlation between public perception and data on dye contamination only occurs in Badas, Kuwik, and Balungjeruk villages. In addition, the BOD/COD ratio indicates that pollution has occurred at all observation points in this study.

Keywords: Aquatic Ecosystem, Dyes, Pollution, Public Perception, Waste.

INTRODUCTION

The need for clothing has encouraged the development of the textile and textile product (TPT) industry in Indonesia. The development of TPT in Indonesia is driven by the increasing value of the investment. BPS (Statistic Center) noted that in 2021 there was an increase in the investment value from US$ 238.89 million to US$ 279.79 million, with total exports of apparel products amounting to 5,856,500 tons [1,2]. The development of textile production certainly encourages the growth of textile and textile product production. One of the main activities of TPT is fabric processing, which is divided into weaving and fabric dyeing activities.

One of the primary components needed in fabric coloring activities and can be a source of pollutants is the dye. In the textile industry, about 200,000 tons of dye are released into the environment each year as a result of the dyeing and finishing process [3]. In general, the textile industry uses more synthetic dyes. This is because synthetic dyes are stable, have many color choices, are easy to obtain and practical [4]. The use of synthetic dyes encourages the creation of waste that is harmful to the environment.

Dyestuff waste can cause continuous material damage to environmental components [5]. This impact comes from textile industry dye waste that pollutes the environment, especially in the aquatic environment. Dyestuff waste in waters will be difficult to degrade because bacteria unable to degrade dye waste. Thus, these polluted waters cannot be used as life support by humans [6]. Plants that have habitats around waters will experience a decrease in chlorophyll content so that the plant's metabolic system will be disrupted [7].

Consumption of water that has been contaminated by synthetic dyes will result in cell mutagenicity in living things, especially in humans, resulting in the emergence of cancer cells [8]. Side effects of dyes in humans also cause several diseases, such as kidney malfunction, reproductive system, liver, brain, and central nervous system [9].

The impact of continued contamination on humans occurs when the dye is in direct contact and over a long period. A large amount of direct human contact occurs when humans use water contaminated with liquid dye waste for their daily needs. Of course, the industry wants to get as much profit as possible but sometimes forgets

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about the environmental health aspects that must be met along with industrial activities It can trigger upheaval between the industry and the public with several complaints and even conflicts. Society certainly has a point of view that is connoted through perceptions that arise and develop in discrediting existing pollution [10]. People want the polluted environment to recover. However, the existing contamination has made the environment change in terms of environmental health.

Environmental health, especially the aquatic environment contaminated with textile waste, can be viewed from the content of textile waste in the waters. Decree No.P.16/MENLHK/SETJEN/ KUM.1/4/2019 of the Minister of Environment concerning the Second Amendment to the Regulation of the Minister of the Environment Number 5 of 2014 concerning Wastewater Quality Standards has strictly regulated the content of waste that can be disposed of in waters. Among these several parameters, Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) are crucial in determining water quality. BOD is the amount of dissolved organic matter in the waters, while COD is the chemical oxygen demand used to describe the amount of dissolved organic matter in the solution.

BOD COD ratio can be used to determine the level of pollution in the aquatic environment [11]. From this indicator, there are no specific controls governing the concentration of dye waste allowed in the waters. It makes the perception of the industry and the public can be different. The industry has a strong assumption that the contamination of dye liquid waste is still in accordance with the existing quality standards. However, people do not understand it, and the perception that arises is a direct observation of the characteristics of the waste and the impact it has on the environment.

These different perspectives need to be clarified through scientific studies to obtain facts between the perceptions of the public and the industry regarding the state of the surrounding aquatic environment. Scientific studies that can be carried out are measuring and analyzing the content of dye waste in polluted waters, measuring several environmental parameters, and making observations about assumptions and perceptions that arise in the public. In this article, the results of the analysis carried out on the communities around the river flow affected by textile waste disposal, as well as direct analysis of the river water around the textile industry. This study aims to analyze public perceptions of textile dye waste pollution and compare the results with the analysis of river water quality.

MATERIAL AND METHODS

Research Design

The method used in this study is interviews with the public regarding perceptions of indications of river water pollution and laboratory tests to determine the truth of indication of pollution by textile industry waste. The study was conducted in January – April 2022. Interviews were conducted in Badas, Kuwik, Balungjeruk, and Wonorejo villages, with the total number of respondents measured by the Slovin formula [12]. The level of water pollution caused by textile industrial waste are carried out with several tests. It included testing the content of dyes in pure textile waste (before being discharged into waters), testing the levels of dyes in waters and testing environmental parameters that focus on the BOD and COD tests. The test was carried out at the Research Laboratory of the Department of Chemistry, State University of Malang.

Public Perception Data Collection

Interviews were conducted on public perceptions of textile industry waste pollution. The selection of the number of respondents was based on several considerations. The respondent's residence must be close to the river flow through which the waste passes, and the respondent must be at least 17 years of age and over so that their opinion can be justified. The determination of the number of respondents being interviewed is determined by the Slovin formula [12]. The results of calculations using the Slovin method show that the number of respondents needed to meet the public perception data is 100 respondents.

$$n = \frac{N}{1 + N(e)^2} \qquad n = \frac{16{,}658}{1 + 16{,}658(0.1)^2}$$

$$n = 99{,}403 \approx 100$$

Description:

n = Number of samples (number of respondents)
N = Total Population (16,658)
e = Error Tolerance Limit (0.1)

Water Sampling

Samplings of deep river water measure the concentration of dyes discharged in the waters and measurement of water quality parameters COD and BOD using a random pumping test [13]. The sampling point taken is shown in Figure 1.

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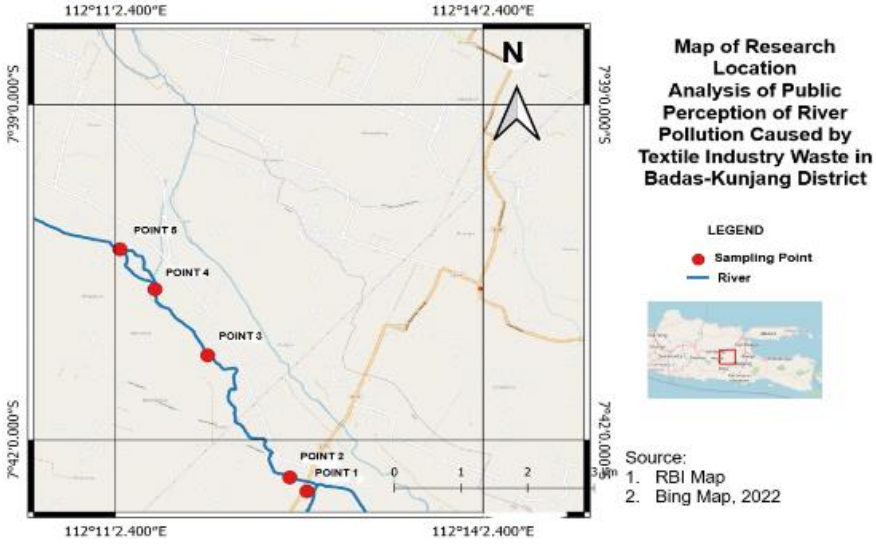

Figure 1. Sampling Points of Konto River

Measurement of Dyestuff Concentration in Pure Waste

Information on the type of dye used is known directly from the textile industry concerned. The dyes used are Yellow RGB, Yellow HR, Red 3BS, Red RGB, Everzol Black GSP, Reactive Black WNN, Remazol Navy RGB, and Turq-Blue. Pure dyes are also obtained directly from the textile industry. The related textile industry states that it emits 600-700 m^3 of liquid dye waste daily.

Measurement of various concentrations of dyes in the waste begins by making various mother liquors with a concentration of 100 ppm pure dye, which is then converted into five concentrations of solutions with the same concentration range. Then each absorbance was measured at the maximum wavelength possessed by the dye. The absorbance value obtained is plotted on a calibration curve with the ordinate axis of absorbance and the abscissa axis being the concentration of waste with the general equation $\mathbf{y = a + bx.}$

Measurement of Dye Concentration in Konto River Water Samples

River water samples were measured at each of the characteristic wavelengths of the dye using UV-Vis Spectronics. The absorbance value is plotted on the line equation $\mathbf{y = a + bx}$ between the abscissa axis ($\mathbf{x}$) is the concentration, and the ordinate axis is ($\mathbf{y}$) the absorbance value.

COD Test

The COD test was carried out by taking 10 mL of river water, which was suspected to be contaminated with textile waste, and was put into a 250 mL reflux vessel. The solution was added 0.2 g of $HgSO_4$ and 25 mL of $K_2Cr_2O_7$ 0.25 N. Then 20 mL of concentrated H_2SO_4 was added. The mixture was refluxed for 2 hours and then cooled to room temperature. After cooling the solution, ± 50 mL of distilled water and three drops of ferroin indicator were added. The resulting reflux solution was titrated with 0.25N $Fe(NH_4)_2SO^4$ until the color changed from blue-green to reddish-brown. COD results can be calculated by the equation:

$$COD\ Level = \frac{1000}{V_{sample}} |A - B| \times N \times 8$$

Description:

A = Volume of $Fe(NH_4)_2SO_4$ solution used in blank titration

B = Volume of $Fe(NH_4)_2SO_4$ solution used in sample titration

N = Normality of $Fe(NH_4)_2SO_4$ solution

BOD Test

BOD measurement is carried out by pouring the diluted sample into 1 300 mL Winkler bottle and 1 bottle of Winkler 150 ml. The solution in a 300 mL Winkler bottle was put into an incubator at 20 oC for 5 days which was then used to measure BOD_5. The sample for measuring BOD0 was added 1 mL of 50% $MnSO_4$. The mixture that has been formed is then added with 1 mL of NaOH and 1 mL of KI. The mixture was then added ± 4 mL of 4N H_2SO_4 and titrated with 0.1 N $Na_2S_2O_3$ until a light yellow color appeared. After that, 5 drops of starch indicator were added until the solution turned blue and the titration was continued until the blue color disappeared. BOD results can be calculated by the equation below along with the calculation of 5-day BOD (BOD5).

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$$DO\ Level = \frac{1000\ x\ V_1\ x\ N_{thio}}{(V_2 - 2)} \times 8$$

Description:
DO = Dissolved Oxygen
V_1 = Volume of Na_2SO_3 used for titration
N_{thio} = Concentration of $Na_2S_2O_3$ solution
V_2 = Volume of water sample tested

$$BOD_5 = DO_0 - DO_5$$

Description:
BOD_5 = Biochemical Oxygen Demand ($mg.L^{-1}$)
DO_0 = Dissolved oxygen value test on the 0 day ($mg.L^{-1}$)
DO_5 = Dissolved oxygen test value on 5^{th} day ($mg.L^{-1}$)

RESULTS AND DISCUSSION

Analysis of Public Perception

Public perceptions are divided into three general categories, which are: (1) public perceptions of the physical characteristics of waste, (2) impacts on agriculture, and (3) environmental health. Physical waste characteristics are divided into two indicators, consisting of the level of odor and color change. The impact of waste on agriculture can be seen in terms of quantity and quality of agricultural products. Meanwhile, the impact of textile industry waste pollution can be categorized into the impact on public health and the effect on the number of aquatic flora and fauna.

Physical characteristics of waste

The level of odor is related to the comfort of the Public around the watershed affected by textile industry waste, where this odor level will significantly correlate with air pollution. The results of public recognition in the four areas affected by the waste flow on the level of odor are shown in Table 1.

Table 1. Public Perception of the Odor Level of Waste along the River Flow

Village	Odor Level		
	Very Stink	Stink	No Stink
Badas	2	9	14
Kuwik	3	7	15
Balungjeruk	0	4	21
Wonorejo	0	2	23
Total	**5**	**22**	**73**
Percentage	**5%**	**22%**	**73%**

In general, the public perception of the level of odor states that the characteristics of the waste flowing along the river flow do not cause a strong odor. This result is indicated by the statement of 73% of the respondents interviewed. Meanwhile, 22% of respondents stated that the smell of waste felt by the public was stink. This statement is mostly made by people in the Badas area, where this area is the initial area that waste passes through. Of these areas, the Wonorejo area contributed the least to the level of this pungent odor. It is because the distance between the initial center of the waste and the Wonorejo area is already ±2 km apart. From the results presented in Table 1, it can also be seen that the farther the distance from the disposal center to the flow area, the lower the odor level.

The second physical indicator observed is the change in the color of river water caused by contamination of textile industry waste. This indicator is easy to observe because the color of river water before and after contamination from dye waste is clearly distinguished. Public perception of the change in the color of the flowing waste is shown in Table 2.

Table 2. Public Perception of Textile Waste Color Level

Village	Color Change Rate		
	Significantly changed	Temporarily Changed	No changes
Badas	1	23	1
Kuwik	1	24	0
Balungjeruk	0	25	0
Wonorejo	1	20	4
Total	**3**	**92**	**5**
Percentage	**3%**	**92%**	**5%**

Textile waste contamination can be observed from the aspect of the color changing of river water, which previously appeared colorless to several striking colors such as blue, green and other colors. A total of 92 respondents stated that the color changes that occur in river water only change temporarily. From the large number of respondents, it seems that the respondents from the Balungjeruk Public are compact in saying that the color changes that occur are only temporary. It is because the people of Balungjeruk still seem to use river water in their daily activities such as washing and bathing. When the waste flows, people have a tendency to use river water until it appears that the flowing waste has run out or the river water has returned to normal. Documentation of indications of pollution that people complained about is shown in Figure 2.

Figure 2. Indications of Pollution Complained by the Public (**Source:** Personal Documentation)

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People thought there was no change at all in river water because the waste flow, is only 5% or five respondents, with 4 out of 5 respondents stating this is from the Wonorejo Village Public. It is possible because the waste that flows in the river has been distributed and used by the Public in previous villages such as Badas, Kuwik, and Balungjeruk villages so that the concentration of color is relatively reduced. The facts are supported by direct observations from waste sampling at the first point of waste disposal. The color obtained from the observations at the waste source is shown in Figure 3.

Figure 3. Observations at Waste Sources

Public assumptions regarding changes in water color also state that river water has changed significantly. The magnitude of this assumption is relatively small compared to the total assumptions obtained from respondents, which is only 3%. The significant change in question is a permanent change in the color of river water.

Impacts on agriculture

The villagers of Badas, Kuwik, Balungjeruk, and Wonorejo generally make a living as rice farmers [14]. Public work indirectly demands the use of river water as material for agricultural irrigation. Along the watershed, there are many rice fields and fields, so the use of river water is the primary thing for the Public. Land use along the area around the Konto River can be seen in Figure 5.

The river water that is contaminated by textile waste is still used to meet agricultural irrigation needs. The public does not seem to have deeper knowledge about the use of water contaminated by the textile waste in processing rice fields. Textile waste can certainly affect the quality and quantity of existing agricultural products [15].

The accumulation of textile waste in water used in irrigating rice fields will directly pollute plants and soil. Soil, as a primary component for plants, will be disturbed in its composition due to the presence of pollutants. Djuwansah [16] suggested that textile industrial waste contains NaHPO_4.2H_2O. This content can cause soil salinity to increase so that plant growth rates will decrease due to decreased osmotic pressure. The problem of decreased quality of food products has also been observed by several related farmers. Several opinions regarding the effect of textile waste contamination on agricultural products can be seen in Table 3.

Table 3. Public Perception of Agricultural Product Quality

Village	Agricultural Product Quality		
	Not Worth Selling	Quality decrease	No effect
Badas	0	2	23
Kuwik	0	8	17
Balungjeruk	0	2	23
Wonorejo	0	2	23
Total	**0**	**14**	**86**
Percentage	**0%**	**14%**	**86%**

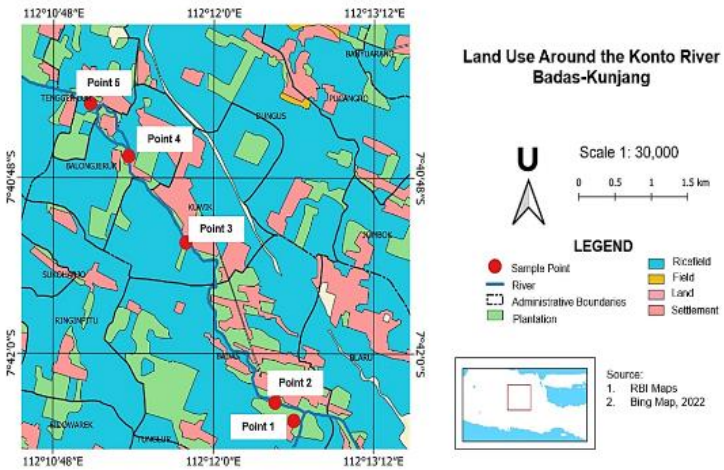

Figure 5. Land Use around the Konto River

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Public opinion polls regarding the impact of waste contamination on the quantity of agricultural products indicated a decrease in the quality of agricultural products. However, the general public also believed that agricultural products were not affected by contamination. According to the Public, textile waste contamination does not cause agricultural products to become unfit for sale so that agricultural products can still be consumed as usual. Nevertheless, they admit that there is a decrease in the quality of agricultural products, which is indicated by the weight of the harvest that is no longer what it used to be (reduced).

A total of 14% of respondents stated an indication of a decrease in the quality of the harvest compared to before the textile waste contamination. Eight respondents from Kuwik dominate the public's assumption that there is a decline in the quality of agricultural products. It is possible because the land use around the watershed that passes through Kuwik Village is mostly used as agricultural land [14].

In general, the public states that the contamination of textile waste flowing along the river does not affect the quality of the harvest. As many as 86% of the total respondents assumed that there was no effect on the quality of the harvest. This assumption was made on the basis that during the research process, the agricultural yields fluctuated. The public also mentioned that, for now, some farmers tend to drain their farms with water from bore wells. Therefore, the exact impact on the quality of agricultural products due to the contamination of textile products cannot be ascertained directly by farmers.

The impact on the quality of agricultural products is also supported by data on the quantity of agricultural products. The data are obtained as long as there is the contamination of waters by textile waste. Plants that are in the spotlight due to textile waste contamination are rice plants. The effect of textile waste contamination on the quantity of agricultural products can be seen from the perception of people who are livelihoods as rice farmers. Some public perceptions of the quantity of agricultural products are presented in Table 4.

The results of interviews with the surrounding public revealed that the contamination of textile waste on rice plants did not cause a significant impact on rice yields in quantity. The existing impacts do not cause crop failure so that rice growth is still in a relatively good realm.

Table 4. Public Perception of Agricultural Product Quantity

Village	Agricultural Produce Quantity		
	Crop Failure	Yield Declining	No Effect
Badas	0	2	23
Kuwik	0	5	20
Balungjeruk	0	1	24
Wonorejo	0	2	23
Total	**0**	**10**	**90**
Percentage	**0%**	**10%**	**90%**

A number of respondents stated that the existing contamination did not seem to have any impact on the quantity of agricultural produce. It can be seen that 90% of the respondents stated that the quantity of existing agricultural products was still relatively the same in the conditions before and after the textile waste contamination. However, there were several respondents who stated that there was an impact on the quantity of yields, namely a decrease in the quantity of yields that was quite felt by farmers. This opinion is supported by the presence of 10% of the total respondents expressed this.

Environmental health

The public also expressed their perception of the primary aspect apart from the impact of agriculture, namely in the field of environmental health. The environmental health highlighted is divided into two important aspects, namely public health and the impact on the Public population. Interviews to explore people's assumptions about environmental health are directly related to public health itself.

Some people still use polluted river water for bathing and washing purposes. Such water is still widely used by the people of Balungjeruk Village. This habit is of course understandable because of the breakdown of the Ketandan Dam, which makes several rivers right in front of residential areas. The use of contaminated water is also still widely used by several communities in Badas, Kuwik and Wonorejo villages.

Several respondents stated that the public was actually afraid to do bathing and washing activities, but the existence of a deep-rooted habit made the public continue to carry out these activities. If the contamination is obvious, the public will wait until the striking water color changes fade, then bathing and washing activities will be carried out again. The existing pattern of water use is still likely to have a direct impact on public health. Public perceptions of the direct perceived impact of the use of contaminated river water can be seen in Table 5.

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Table 5. Public Perception of Public Health

Village	Public Health		
	Cause Chronic Disease	Cause Minor Disease	Does not cause disease
Badas	0	13	12
Kuwik	0	14	11
Balungjeruk	0	15	10
Wonorejo	0	16	9
Total	**0**	**58**	**42**
Percentage	**0%**	**58%**	**42%**

All respondents answered in unison that the use of contaminated water does not cause chronic disease directly. Chronic diseases in question are cancer, severe digestive disorders, and the emergence of various deadly diseases. There is a strong assumption expressed that the use of river water has caused some minor ailments such as itching. However, the assumption is opposed by the assumption that the use of river water does not cause any disease. The difference in assumptions, which is only 16% of the total assumptions, has made contradictory assumptions. It makes a possible impact on public health that cannot be measured by the results of a poll of respondents alone.

The health impacts due to the contamination of liquid dye waste are not only in humans, but also in aquatic flora and fauna that are in direct contact with the waters. Aquatic flora that is commonly monitored by residents includes water spinach, water hyacinth, and bamboo. In addition, some people also plant elephant grass around the watershed (DAS). Meanwhile, the aquatic fauna that is most easily observed by residents is in the form of various types of fish, such as *wader* fish (Cyprinidae), *keting* fish (*Mystus nigriceps*), *sili* fish (*Macrognathus aculeautus*) and shrimp.

Since the liquid dye waste was discharged into the waters, 26% of respondents said that there was a decline in the population of aquatic fauna. Some of these were seen floating on the water's surface when the waste flowed into the waters. This observation is not permanent at all times, but temporarily adjusts the arrival time of waste in each area. Some fish contaminated with the waste looked like they were drunk but did not die.

This phenomenon has attracted the attention of residents because some residents still use several types of fish as a source of food. Public assumptions due to indications of the influence of aquatic flora and fauna populations can be seen in Table 6.

Table 6. Public Perception of Aquatic Flora and Fauna Population

Village	Aquatic Flora Fauna Population		
	Significantly Decreased	Decreased Slightly	No Effect
Badas	0	5	20
Kuwik	0	11	14
Balungjeruk	0	5	20
Wonorejo	1	5	19
Total	**1**	**26**	**73**
Percentage	**1%**	**26%**	**73%**

The existence of this problem makes people afraid to use fish as usual. This assumption mostly comes from the respondents of the Kuwik Village Public. It is because some communities are in direct contact with the river in their daily activities, so observations about the decline in the population of aquatic flora and fauna can be taken into consideration. However, 14 respondents from Kuwik Village stated that there was no effect on the aquatic flora and fauna due to textile waste contamination.

In general, 73% of the total respondents from various villages stated that there was no effect caused by textile waste contamination on the population of aquatic flora and fauna. The assumption held by respondents who think so is because respondents thought that contamination does not affect the population because fish are still able to survive. Fish can still regenerate despite exposure to waste contamination, but to know for sure the impact of textile waste contamination on aquatic flora and fauna, further studies and research need to be carried out.

Analysis of Dyestuff Content in Pure Waste

Analysis of the dye content in pure waste was carried out with the aim of knowing the concentration of each pure dye contained in the waste. The waste sample is waste that has not been disposed of in the waters. The concentration of pure dye in the waste has been identified by measurement using a UV-Vis Spectrophotometer. The identification of the concentration of dyes contained in the textile waste can be seen in Table 7.

Table 7. Concentration of Dyes in Waste

Dye	Wavelength (nm)	Ref	Waste Concentration (ppm)
Yellow RGB	410	[17]	56.00
Yellow HR	416	[18]	90.26
Red 3BS	512	[19]	22.80
Red RGB	520	[20]	28.54
Everzol Black GSP	596	[21]	34.97
Reactive Black WNN	596	[22]	39.61
Remazol Navy RGB	620	[23]	44.27
Turq-Blue	662	[24]	11.60

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In Table 7, it can be seen that textile dye waste has eight dye contents with different concentrations. The highest concentration of dye produced is Yellow HR. The dye belongs to the azo type with a character that is difficult to decompose naturally. It is because the dye have a nitrogen group with double bonds (R-N=N-R) [25]. This group of dyes are generally carcinogenic and harmful to the environment. It is because the dyes are resistant to degradation by light, microorganisms and processing carried out by related industries [26].

Analysis of Dyestuff Content in the Konto River

Analysis of dye concentration in the Konto River refers to the initial dye that has been identified in the pure dye waste. The concentration of dyes in the Konto River is shown in Table 8. Waste at the first and second sampling points comes only from textile industry waste, while waste at the third, fourth and fifth sampling points can come from textile industry waste and household waste. It is supported by the fact that the first and second sampling points are not located near people's homes, while the third to fifth sampling points of the Konto river water sampling are already in a densely populated area.

The concentration of dyes in the waste appears to have decreased compared to the concentration of dyes in pure waste, before being discharged into the waters. It is made possible by the dilution of river water so that the concentration of waste decreases. This decrease is also influenced by the Konto river water discharge. Sampling was taken during the rainy season so that the river's water discharge was also large. Wonorejo Village does not appear to have experienced any contamination at all. It is due to the distance and branching of the river.

At the first point of sampling, river water showed differences in the concentration of dyes compared with the results of measurements at the second point. Even though the distance between the first and second points is not too far, only ± 100 m, the intensity of the waste that is flowed by the factory three times a day with a total discharge of waste water issued is ± 700 m^3. The large volume of wastewater that flows into the Konto River does not seem to change the characteristics of the Konto River permanently.

Table 8. Concentration of Dyes in the Konto River Flow

Dye	Waste Concentration (ppm)				
	Point 1 (Waste disposal starting point)	Point 2 (Badas Village)	Point 3 (Kuwik Village)	Point 4 (Balungjeruk Village)	Point 5 (Wonorejo Village)
Yellow RGB	22.42	12.99	10.67	10.67	0
Yellow HR	68.13	53.21	46.16	45.34	0
Red 3BS	5.30	0.13	0	0	0
Red RGB	12.68	8.40	0	0	0
Everzol Black GSP	2.08	0.95	0	0	0
Reactive Black WNN	5.37	2.22	0	0	0
Remazol Navy RGB	3.70	1.63	0	0	0
Turq-Blue	0.15	0.35	0	0	0

COD Analysis

COD is a parameter used to measure the ability of aquatic microorganisms to decompose organic substances by an oxidation process so that it can result in a decrease in dissolved oxygen in the waters [27]. The permissible level of COD in waters due to the presence of textile industry waste is 150 $mg.L^{-1}$. COD parameter measurements were also carried out at several points in each village which was fed by the Konto River. The results of measuring COD parameters at each sampling point are shown in Table 9.

One of the factors that can affect COD in water is the presence of inorganic molecules that interact with dichromate [28]. Dichromate in water is an oxidizing agent that causes pollutants in the form of carbon dioxide in the waters [29]. Carbon dioxide in the waters is needed in the decomposition carried out by bacteria. In a state of excess carbon dioxide in the waters, it inhibits the binding of oxygen by aquatic organisms so that the metabolism will be disrupted. The disturbance of the metabolic process in question is in the process of photosynthesis by aquatic organisms.

The measurement results shown in Table 9 show that the COD levels have exceeded the predetermined limit, namely at sampling points 2 and 4. The observations showed that the COD levels at points 2 and 4 were caused by several factors. The sample at point 2 was taken right at the Konto dam, so a lot of dye waste may settle

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on the riverbed so that the COD level of the waters is high. Point 4 (Balungjeruk Village) shows COD levels exceed the limit as in the COD level measurement at point 2. It is possible because of the use of Konto River water by the people of Balungjeruk Village for washing clothes and bathing.

The use of Konto River water by the people of Balungjeruk Village is driven by several factors, such as knowledge, attitudes, and actions of the Public in using river water in daily activities [30]. Knowledge, attitudes, and actions can be formed because of the convenience of the Public in utilizing river water for bathing and washing purposes. Surfactants from bathing and washing activities can reduce water quality [31]. Surfactants in waters contain alkyl benzene compounds, which are non-biodegradable and can increase COD levels in waters [32].

Table 9. COD Parameter Measurement Results

Sampling Point	COD level (mg.L^{-1})	Description
Sampling Point 1	40	Do not exceed the quality standard
Sampling Point 2	320	Exceeding the quality standard
Sampling Point 3	40	Do not exceed the quality standard
Sampling Point 4	220	Exceeding the quality standard
Sampling Point 5	100	Do not exceed the quality standard

Table 10. BOD Parameter Measurement Results

Sampling Point	BOD Level (mg.L^{-1})	Description
Sampling Point 1	5.7240	Do not exceed the quality standard
Sampling Point 2	3.0574	Do not exceed the quality standard
Sampling Point 3	2.6363	Do not exceed the quality standard
Sampling Point 4	5.0925	Do not exceed the quality standard
Sampling Point 5	5.8644	Do not exceed the quality standard

Table 11. BOD/COD Ratio Value of Konto River

Sampling Point	BOD/COD Ratio Value	Description
Sampling Point 1	0.1400	Non-Biodegradable
Sampling Point 2	0.0095	Non-Biodegradable
Sampling Point 3	0.0650	Non-Biodegradable
Sampling Point 4	0.0230	Non-Biodegradable
Sampling Point 5	0.0580	Non-Biodegradable

BOD Analysis

BOD is the amount of dissolved oxygen needed by aquatic organisms in decomposing organic matter [33]. BOD analysis is needed to determine the amount of oxygen needed by aquatic organisms in stabilizing organic matter in waters. The BOD content that is allowed in waters due to the presence of textile industry waste is 60 mg.L^{-1}. Measurement of BOD in the Konto River was carried out by taking samples of the water from the Konto River that flows in the villages of Badas, Kuwik, Balungjeruk, and Wonorejo. The results of BOD measurements on the Konto River are shown in Table 10.

The results of the BOD measurement shown in Table 10 are that none of the BOD levels in all analyzed river water samples exceeded the predetermined quality standard. The value of BOD in waters is due to textile waste influenced by the concentration of dyes and the presence of surfactants in the production process [34]. Dyes and surfactants in waters cause the BOD value to increase due to dye and surfactant waste which can form dissolved compounds, colloids, and suspension compounds. The formation of colloids due to textile industry waste substances will result in the need for oxygen in oxidizing organic matter to increase.

BOD levels are related to dissolved oxygen in the water. If the BOD level is high, it will result in a decrease in dissolved oxygen, and it can reduce water quality, especially in the life of aquatic organisms [35]. Dissolved oxygen plays a role in degrading pollutants by aquatic organisms. It is because dissolved oxygen (DO) can help the oxidation and reduction of pollutants. Low DO conditions in the waters cause the waters to be anaerobic. This situation will trigger the decomposition of organic substances into carbon dioxide gas and methane gas, while nitrogen compounds in the water will turn into ammonia. This situation can be exacerbated as follow. If

DO sulfur in the waters is low, it will turn into sulfide acid, which makes the waters smell badIt is because sulfide acid is a source of unpleasant odors, a smell like rotten eggs. The results of the BOD measurement in the waters shown in Table 10 show that DO in the river in each village is still good. It indicates no shortage of dissolved oxygen needed by aquatic organisms.

BOD/COD Ratio Analysis

The biodegradability of waste in waters can be seen from the large ratio of BOD/COD. It is related to the ability of the waters to decompose pollutants [36]. The value of the BOD/COD ratio can be classified into three groups, namely biodegradable, slow biodegradable, and non-biodegradable [37]. Analysis of the BOD/COD ratio can be used to measure the quality of the indications of pollution in the Konto River. The value of the BOD/COD ratio at several sampling points of the Konto River is shown in Table 11.

The results of the calculation of the BOD/COD ratio in the Konto River at five sampling points indicate that the textile industry dye waste is non-biodegradable. It shows that the Konto River has been polluted by dye waste. The status of the largest pollution is at sampling point 2. It is possible because the sampling point is a dam, so it is possible for dye waste to form in the waters. This deposit may continue to grow along with the textile industry's dyestuff waste disposal activities. Over time the quality at sampling point 2 will deteriorate.

Analysis of the Correlation of Public Perceptions with Water Quality Data

The results of interviews with public perceptions indicate that there was a temporary change in the color of the river, which caused some minor illnesses River pollution, according to the public, does not result in a decrease in the quality and quantity of agricultural products. Indications of existing pollution also do not cause a decrease in aquatic flora and fauna. The impact on the agricultural sector and the population of aquatic flora and fauna cannot be observed by the public in detail. The public issued a complaint about the textile industry's dye waste, especially on changes in river color. This indicator of color change is the basis for the strongest assumption by the public, which states that there is river water pollution.

Public perception of the indications of water pollution in the Konto River was studied scientifically by measuring the concentration of dye in the Konto River, testing the levels of COD and BOD as indicators to prove the presence of water pollution that occurred. The measurement of the dye concentration showed that the presence of the dye was found at several sampling points. At the first sampling point (the point of direct waste disposal), the largest concentration of dye was Yellow HR. The concentration of yellow HR at the first point is 68.13 ppm. The concentration of this dye in pure waste was found to be 90.26 ppm. This dye was still dominantly found at several sampling points from the second to the fourth, namely 53.21 ppm, 46.16 ppm, and 45.34ppm, respectively. At sampling point five in Wonorejo Village, there was no concentration of dye waste found, so there was no dye pollution in this area. It can happen because there is a decrease in the concentration of pollution along with the flow of river water.

The results of the measurement of dyes in Wonorejo Village can be an indication that there is a difference between public perception and data on dye concentration. However, from COD measurements, the results of COD in Wonorejo Village have exceeded the quality standard limit. Based on these facts, the high COD results may not be caused by the concentration of textile industry dyes. The results of the analysis of the water quality of the Konto River in the villages of Badas, Kuwik, Balungjeruk, and Wonorejo stated that the pollution in the Konto River was non-biodegradable. It is indicated by the high value of the COD/BOD ratio.

This fact corresponds with the results of interviews with Public perceptions with data on dye concentrations and water quality tests only in Badas, Kuwik, and Balungjeruk villages. Wonorejo Village also experienced water pollution, but the water pollution that occurred was not due to dye contamination in the waters, as people said. This result is supported by the absence of dye concentration in Wonorejo Village, but the COD/BOD ratio indicates the presence of pollutants in Wonorejo village.

Sources of pollution based on public perception may differ from actual conditions. It may be due to the influence of the time of observations made during the rainy season. Research shows that river water quality analysis must be carried out comprehensively. Not only based on public perception but also water quality data. Public perception can be used as an indicator in measuring water quality. Further research needs to be done by taking samples

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during the dry season to determine the level of river pollution that occurs throughout the year.

CONCLUSION

The public perception of the indications of water pollution in the Konto River by textile waste is proven to correspond. It shows a corresponding between dye concentration measurement and water quality in Badas, Kuwik, and Balungjeruk villages. However, it was notcorrespond with Wonorejo Village because the dye concentration was not found at the measurement point in the Wonorejo Village flow. On the other hand, the measurement of water quality in Wonorejo Village shows that there is pollution based on the analysis of the BOD/COD ratio, but it is not because of the dye liquid waste but the presence of other pollutant sources.

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Anti-inflammatory Activity of *Elephantopus scaber* and *Sauropus androgynus* Combination in Pregnant Mice Infected with *Escherichia coli*

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Abstract

The current study aimed to investigate the effectiveness of *Elephantopus scaber* and *Sauropus androgynus* as anti-inflammatory agents in pregnant mice infected with *Escherichia coli*. This study used seven treatments group (*n*=3): K- (healthy pregnant mice), K+ (pregnant mice infected with *E. coli*), P1 (pregnant mice infected with *E. coli* + *E. scaber* 100%), P2 (pregnant mice infected with *E. coli* + *E. scaber* 75% and *S. androgynus* 25%), P3 (pregnant mice infected with *E. coli* + *E. scaber* 50% and *S. androgynus* 50%), P4 (pregnant mice infected with *E. coli* + *E. scaber* 25% and *S. androgynus* 75%), P5 (pregnant mice infected with *E. coli* + *S. androgynus* 100%). Flow cytometry analysis was used to analyze cell populations expressing $CD4^+TNF\alpha^+$, $CD4^+IFN\gamma^+$ and regulatory T cells ($CD4^+CD25^+CD62L^+$). All treatment groups significantly ($p<0.05$) decreased TNFα and IFNγ levels, while the P2 group was more effective in increasing regulatory T cells at the 1st and 2nd trimesters of the pregnancy. This study showed that *E. scaber* and *S. androgynus* combination alleviated inflammation by reducing inflammatory cytokines (TNFα and IFNγ) and increasing T-regulatory cells. Therefore, *E. scaber* and *S. androgynus* combination could suppress the inflammation during pregnancy and infection.

Keywords: *Elephantopus scaber,* immune system, infection, inflammation, *Sauropus androgynous.*

INTRODUCTION

The immune system protects the body from antigens like bacteria and viruses. Immunological pregnancy detection is essential for maintaining pregnancy and inadequate detection of fetal antigens leads to abortion [1]. These changes make pregnant women more susceptible to infectious agents. Pregnant women are considered a special group because of their specific susceptibility to several infections due to the unique immunological condition caused by pregnancy. Thus, pregnancy presents some challenges when deciding how to deal with, prevent, and treat infectious diseases [2].

The maternal immune system has many changes throughout the pregnancy period. These conditions protect the mother and fetus from pathogens while avoiding a detrimental immune response to the allogeneic fetus. Although there is little evidence that the maternal immune system is totally suppressed during pregnancy, the increased risk of certain infections indicates significant qualitative immunological changes [3]. Pregnancies are complex and unique circumstances. Therefore consideration should be given to understanding how specific endocrine, physiological and immunological factors increase the risk of infection. Specifically, urinary tract infections during pregnancy may occur more frequently, or pneumonia may become more severe, mainly due to decreased functional residual capacity of the lungs due to changes in the circulatory system and increased abdominal pressure) [4,5].

Several diseases may cause by bacterial infection [6]. Bacteria is one of the agents that can infect humans [6]. *Escherichia coli* is a pathogenic bacterium that can infect humans and cause various diseases [7]. During pregnancy, urinary tract infections are a widespread occurrence. The most common pathogen isolated from the urinary tract is *E. coli*. *Escherichia coli* has high resistance to Ampicillin, so it should not be used for *E. coli* infection therapy. Furthermore, some antibiotics harm the mother and fetus, like Pyelonephritis. Pyelonephritis can cause morbidity and be life-threatening to both the mother and fetus [8]. Placental infections by *E. coli* can produce inflammatory cytokines such as Tumor Necrosis Factor (TNF)-α, Interferon (IFN)-γ, Interleukin (IL)-12, and high levels of IL-6 activate the maternal immune system, leading to placenstal damage and miscarriage or premature birth [9]. Therefore alternative therapy is needed for *E. coli* infection in pregnant women.

Elephantopus scaber is a small herb native to the Neotropical Realms, Europe, Asia, Africa, and Australia. Sesquiterpene lactone compounds from *E. scaber* such as deoxyelephantopin,

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isodeoxyelephantopin, scabertopin, and isocabertin have emerged as potent anticancer compounds. Other biological activities, including antibacterial, hepatoprotective, antioxidant, anti-diabetic, and anti-inflammatory, were also reported [10]. Al Fahad *et al.* in 2012 showed that petroleum ether, chloroform, and methanol extracts from above-ground parts of *E. scaber* tested for antibacterial activity against *Staphylococcus aureus, Salmonella paratyphi A, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shigella sonnei, Escherichia coli,* and *Salmonella typhimurium* have a significant activity to inhibit the growth of bacteria. However, methanol extract proved most effective against the organisms tested [11].

Sauropus androgynus, also known as *Katuk* in Indonesia, have some pharmacology activities [12]. Anju *et al.* recently showed that *S. androgynus* leaves are used as antitussives, tonics, and sedatives to the lungs and relieve internal heat [13]. The dark green leaves of *S. androgynus* contain valuable hematopoietic elements, cell rejuvenation, and the circulatory promoter chlorophyll. *S. androgynus* leaves were previously reported to have significant alkaloids in fresh leaves [14]. Selvi and Bhaskar confirmed that the leaves of *S. androgynus* may be beneficial in treating inflammation, pain, and fever [15]. These activities may be partly due to phytochemicals such as flavonoids, alkaloids, steroids, and terpenes.

Herbal medicine has been chosen as an alternative treatment for decreasing antibiotic utilization during pregnancy. The combination of *S. androgynus* and *E. scaber* is presumed to stimulate the immune system synergistically through anti-inflammatory effects. This study investigates the effectiveness of *E. scaber* and *S. androgynus* formulations as anti-inflammatory agents in pregnant mice with *E. coli* infection.

MATERIAL AND METHOD

Herbs Material

The powder leaves of *E. scaber* and *S. androgynus* were purchased from Balai Materia Medica Batu in Malang, Indonesia. Taxonomists have identified the leaves at Balai Materia Medica Batu in Malang. The leaf powder was put into a closed bottle and added 70% ethanol. Furthermore, the bottles were stored in a dark place overnight. The mixture was filtered and replaced with new ethanol and soaked again until the original color of ethanol was seen, indicating that the compound had been completely extracted. The extraction results were evaporated at a temperature of 50°C in a water bath using a vacuum pump evaporator (Brushless DC Motor). Each evaporation result in paste form was weighed according to the treatment dose and dissolved in aquadest for each day. The starting dose of *E. scaber* and *S. androgynus* was 200 and 150 mg.kg^{-1} body weight, respectively [16,17].

Animal preparation

This study used twenty-one female mice (*Mus musculus*) strain BALB/c as an experimental animal. The mice used are 4-5 weeks old with active health conditions, do not lose hair, and do not have deformed limbs. Mice were acclimatized for seven days since they arrived in Animal Laboratory, Biology Department, Brawijaya University Malang, Indonesia, from Gajahmada University, Yogyakarta, Indonesia. After acclimation, mice were mated with male mice, and a vaginal plug in female mice indicated day 1 of mice pregnancy.

Escherichia coli injection

Escherichia coli (EPEC) isolates were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. *E. coli* were injected intraperitoneally at five days of mice's gestation. The number of *E. coli* was 10^7 CFU.mL^{-1} in 0.5 mL per mouse. *E. coli* were detected using the Gram Staining and Catalase Test. *E. coli* infection was confirmed by collecting mice's tail vein blood after 24 h of infection.

Treatment of *E. scaber* and *S. androgynus* extract

The pregnant mice were randomly divided into seven experimental groups (n=3). All treatment groups are presented in Table 1. The treatment group consisted of control and extract treatment. The combination of *E. scaber* and *S. androgynus* leaf extract was administered orally from day 1 until day 16 of pregnancy.

Table 1. Experimental groups (*n*=3)

Group	*E. coli**	ES extract	SA extract
K-	-	-	-
K+	+	-	-
P1	+	200	-
P2	+	150	37.5
P3	+	100	75
P4	+	50	112.5
P5	+	-	150

Note: *Infected of 10^7 CFU.mL^{-1} *E. coli*, ES= *Elephantopus scaber* (mg.kg^{-1} BW); SA= *Sauropus androgynus* (mg.kg^{-1} BW).

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Cell isolation

Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocyte cells were isolated from the spleen. Spleen squeezed with the tip of the syringe, crushed clockwise, and suspended with PBS. Furthermore, the obtained cells were filtered. The crushed spleen was centrifuged at a speed of 3200 rpm at a temperature of 20°C for 5 min. The supernatant was discarded, and the pellet was resuspended with 1 ml of PBS. A total of 100 µL homogenate was transferred to a new microcentrifuge tube, and 500 µL of PBS was added. Then it was centrifuged at 3200 rpm at 20°C for 5 min. The obtained pellet was stained with the specific antibody.

Flow cytometry analysis

Flow cytometry was used to analyze the cell populations expressing $CD4^+TNF\alpha^+$, $CD8^+IFN\gamma^+$, and $CD4^+CD25^+CD62L^+$. Firstly, the obtained pellet was stained with extracellular antibodies (FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD25, and PE-conjugated rat anti-mouse CD62L) provided by Biolegend, San Diego, CA, and then incubated for 20 min at 4°C. Subsequently, the cells were added with 50 µl fixative solution (cytofix/cytoperm) and incubated for 20 min at 4°C. The residual of the fixative solution was removed by washing solution (washperm) and then centrifuged at 2500 rpm at 10°C for 5 min. The supernatant was discarded and pellets were stained with intracellular antibodies (PE-conjugated rat anti-mouse TNFα, and PE/Cy5-conjugated rat anti-mouse IFNγ) provided by Biolegend, San Diego, CA, and then incubated for 20 min at 4°C. The sample was then transferred to a flow cytometry cuvette and pipetted. Flow cytometry analysis was carried out using a flow cytometer BD FACS Calibur™ and a computer that was installed with the BD Quest Pro™ software.

Data analysis

The data were analyzed using BD CellQuest PRO™ software and then tabulated. One-way ANOVA was used for statistical analysis with a $p<0.05$. Statistical analysis was performed using SPSS version 16.0 for Windows.

RESULT AND DISCUSSION

TNFα is a proinflammatory cytokine secreted by $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages. This study determined the relative number of TNFα produced by CD4+ T cells. The relative number of $CD4^+TNF\alpha^+$ in the infected pregnant mice (K^+) significantly increased compared to the healthy pregnant mice (K-) (Figure 1).

The results also demonstrated that P1-P4 groups significantly ($p<0.05$) reduced the relative number of $TNF\alpha^+$ compared to the K^+ group. The lowest decrease was found in the P1 and P2 groups. However, the P1 and P2 were not significantly different in reducing TNFα levels produced by CD4 T cells. Combining *E. scaber* and *S. androgynus* is expected to relieve inflammatory conditions by decreasing TNFα levels. *Sauropus androgynus* has been known to contain high flavonoid content. Gresso [18] showed that flavonoids could reduce LPS-induced TNFα levels through phosphorylation of p38 MAP kinase.

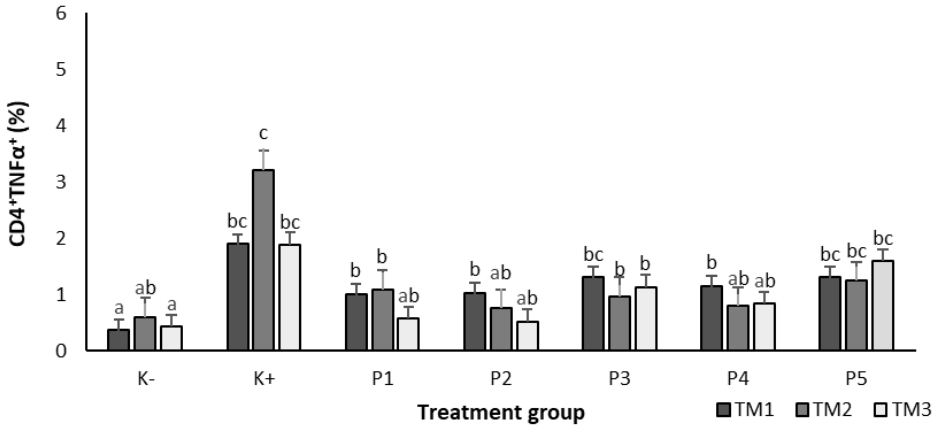

Figure 1. The relative number of TNF-α produced by $CD4^+$ T cells in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed with flow cytometry. Data were presented as mean ± SD from 3 mice in each treatment group with $p < 0.05$.

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The increase in the TNFα was also accompanied by the rise in other proinflammatory cytokines, such as IFNγ. IFNγ is a proinflammatory cytokine that plays a vital role in inflammation. Therefore, reducing the relative number of IFNγ is expected to reduce inflammation. Figure 2 showed that all treatment groups (P1-P5) significantly (p<0.05) suppressed the production of IFNγ in CD4 T cells compared to the K+ group. Vitamins A and E in *E. scaber* contribute to inhibiting proinflammatory cytokines by Th2 cells [16].

The results showed that peritoneal injection of *E. coli* caused a significant decrease in the relative number of regulatory T cells in the K^+ group compared to the K- control group (Fig. 3). The combination of *E. scaber* and *S. androgynus* significantly increased the relative number of regulator T cells (p<0.05) in all treatment groups compared to the K+ group at all trimesters. The highest increase in the relative number of regulatory T cells was found in the P2 group (*E. scaber* 75% and *S. androgynus* 25%. *Sauropus androgynus* contains a lot of tannins, saponins, flavonoids, and alkaloids, which can trigger MAPK activity to stimulate Treg production [15].

The function of the immune system is to protect the host from pathogens. This function depends on the ability of the innate immune system to regulate cell migration to monitor, recognize and respond to invading microorganisms. Mor *et al.* showed that 70% of decidua leukocytes are NK cells, 20-25% are macrophages, and 1.7% are dendritic cells [2]. Although B cells are absent in the adaptive immune system, T lymphocytes comprise about 3-10% of decidual immune cells [19].

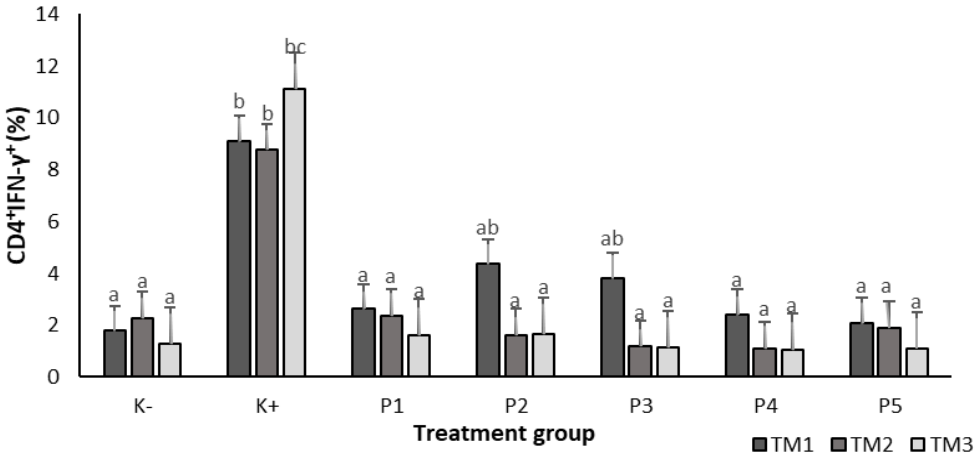

Figure 2. The relative number of IFN-γ^+ produced by $CD4^+$ cells in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed with flow cytometry. The data presented as mean ± SD from 3 mice in each treatment group with $p < 0.05$.

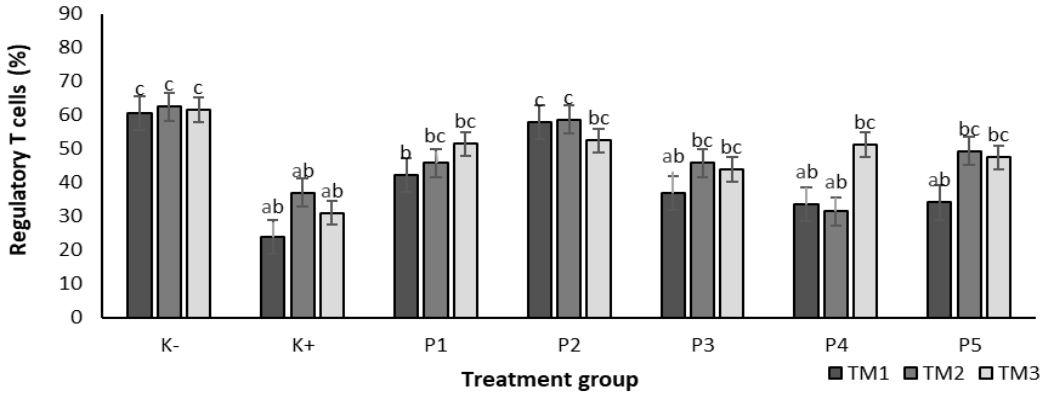

Figure 3. The relative number of regulatory T cells ($CD4^+CD25^+CD62L^+$) in all treatment groups. Mice were dissected in 1st, 2nd, and 3rd trimesters of pregnancy. Lymphocytes were isolated from the spleen and then analyzed by flow cytometry. The mean ± SD value from 3 mice in each treatment group ($p < 0.05$).

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In early pregnancy, NK cells, dendritic, and macrophages infiltrate the decidua and accumulate around the invading trophoblast cells [20]. Deletions of macrophages, NK cells, or dendritic cells (DCs) have adverse effects [21]. Hanna *et al.* studies have shown that in the absence of NK cells, trophoblast cells cannot reach the endometrial vascular distribution, leading to abortion. These studies suggest that NK cells are important for uterine endometrial infiltration [22]. Similarly, DC depletion prevented blastocyst implantation and decidua formation. DC is required for decidua formation and may affect the angiogenic response by inhibiting vascular maturation [23].

This study proves that the combination of *E. scaber* and *S. androgynus* has anti-inflammatory activity. *E. scaber* contains many saponin and flavonoid compounds. Flavonoids and saponins have an essential role in suppressing inflammation. *Sauropus androgynus* extract had activity in reducing inflammation. Activation of cyclooxygenase can increase prostaglandins, especially PGE2, and this production can inhibit inflammation, pain, and fever [24]. Ginwala *et al.* showed that flavonoids have an anti-inflammatory role through several different mechanisms as inhibitors of regulatory enzymes and transcription factors that have essential functions in mediating inflammation [25]. Flavonoids also have antioxidant potential that can reduce free radicals and their formation. So, flavonoids have an important influence on immune mechanisms in the inflammatory process [25].

Flavonoids can inhibit protein kinase as an anti-inflammatory mechanism. Protein kinases are proteins involved in signal transduction during cell activation in inflammation. Certain flavonoids can target several kinases through several mechanisms [26]. The decreased T-reg cells in sick mice K^+ was associated with increased cytokines, including IL-6, TNF-α, and IFN-γ. Usually, the presence of TGF-β will induce naive T cells to become Treg cells, but the presence of IL-6 will change this function. IL-6 and TGF-β together cause the differentiation of naive T cells into Th17 cells and the formation of inactivated Treg cells so that the number of Treg cells decreases. It is related to Dienz and Rincon, who described IL-6 as a proinflammatory cytokine that could inhibit the differentiation of naive T cells into TGF- induced Tregs [27].

Another research also reported that a combination of *S. androgynus* and *E. scaber* possessed antibacterial activity during pregnant and infection. Christina *et al.* [28] stated that a ratio of 75:25 exhibits significant protection in the renal and hepatic of infected pregnant mice. An equal ratio of both extracts also decreased the inflammation mediated by granulocyte (Gr-1). The combination of *S. androgynus* and *E. scaber* also have the potential effect as a hormonal balancer in infected pregnant mice. Djati *et al.* [29] also reported that *S. androgynus* and *E. scaber,* at a ratio of 75:25, also altered the hormonal changes and erythropoiesis in infected pregnant mice. Therefore, *E. scaber* and *S. androgynus* combination could prevent damage from bacterial infection during pregnancy.

CONCLUSION

This study showed that *E. scaber* and *S. androgynus* combination alleviated inflammation by reducing inflammatory cytokines (TNFα and IFNγ) and increasing T-regulatory cells. Therefore, *E. scaber* and *S. androgynus* combination could suppress the inflammation during pregnancy and infection.

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Aquatic Environmental Analysis of the Hematological Profile of *Barbonymus altus* in the Brantas River, Jombang, East Java

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Abstract

The Brantas River is the longest in East Java. One of the areas included in the flow of the Brantas River is the Jombang Regency. The Jombang Brantas River is used by the community to meet their daily needs in agriculture, plantations, industry, and fishing grounds. The input of waste generated from several community activities will result in the survival of fish in the waters. Fish as a biomarker agent is quite significant in estimating the level of heavy metal pollution. Red Bader Fish (*Barbonymus altus*) is often found and lives around the Jombang Brantas River. This study aims to determine the condition of the aquatic environment by observing the health of the fish through hematological observations of the Red Bader Fish (*B. altus*). Sampling was carried out at three stations with three replications every two weeks, which was carried out in February – March 2022 in the Jombang Brantas River. Data analysis used the Canonical Correspondence Analysis (CCA) method as an analytical method to determine the correlation between the dependent variable (hematological profile) and the independent variable (water quality conditions). In this study, the results showed that the water quality at Station 1 and Station 2 was classified as normal, while at Station 3, it was classified as polluted. Data analysis showed that erythrocytes, hemoglobin, and hematocrit had a relationship with temperature, pH, DO, BOD, ammonia, TSS, and TDS in low concentrations. Meanwhile, leukocytes and micronuclei showed a relationship with high concentrations of ammonia, BOD, TDS, and TSS.

Keywords: Brantas River, Bader fish (*Barbonymus altus*), CCA, hematology, water quality.

INTRODUCTION

The Brantas River is one of the longest rivers in East Java, with a length of ±320 km and an area of ±12,000 km^2 [1]. The Brantas River is often used by the surrounding community to meet water needs in agriculture, households, and industry, as well as a place to find fish for consumption and trade [2]. Community activities in meeting their needs will produce waste that causes a decrease in river water quality.

Jombang is one of the districts in the central part of East Java. Jombang has a very strategic position because it is at the crossroads of the north and south of the Island of Java [3]. This condition is accompanied by an increasingly rapid industrial development so that it cannot be separated from the problem of environmental pollution due to the waste generated. Waste is a by-product of the production process that is not used in the form of liquid, solid, gas, and so on. This condition will result in natural and environmental degradation that affects the quality of life of the community [4]. Water pollution produced in the form of heavy metals can accumulate in the body quickly and is easily absorbed by aquatic organisms, one of which is fish that live in the waters.

Fish is a biomarker that is quite significant in estimating the level of heavy metal pollution and can describe changes in water characteristics [5]. The fish that is often found in the Jombang Brantas River is the Red Bader fish (*Barbonymus altus*). Red Bader Fish is included in the Cyprinidae species, which is generally used in toxicity tests so that it can be used as a biomarker agent in determining river pollution that gets input from the pollutant load from community activities [6]. Heavy metals enter the body's tissues through the respiratory tract, digestion, and penetration through the skin. The entry of heavy metals into the body will be absorbed by the blood and distributed to all body tissue [7].

Blood is one of the parameters used to see fish abnormalities that occur due to disease or environmental conditions [8]. Blood has an important function in an organism's life. Blood plays a role in circulating food substances from digestion, carrying oxygen, and carrying enzymes and hormones to all parts of the body [9]. Therefore, information is needed to determine the health status of fish based on the hematological profile used as an indicator of the aquatic environment in the Jombang Brantas River.

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MATERIAL AND METHOD

Sampling Area

This research was conducted in the Jombang Brantas River, East Java. Sampling was carried out using the purposive sampling method [10]. The location of the research was carried out at three stations suspected of receiving different waste inputs, including Station 1, adjacent to agricultural land and boat crossings; Station 2, adjacent to land use in agriculture; and Station 3, is adjacent to the waste disposal from the animal feed industry. The research locations of the three stations can be seen in (Fig. 1). Water and fish blood samples were taken three times every two weeks from February – March 2022. Fish blood sampling was taken as many as three fish for each time at each station.

Water Quality Measurement

Water quality measurements were carried out in situ and *Ex Situ*. The water samples were measured *in situ*, such as temperature using a water thermometer (SNI) [11], pH using a pH meter (SNI) [12], and Dissolved Oxygen (DO) using the Winkler method [13]. Meanwhile, water samples were measured ex-situ by inserting the sample that had been taken in a bottle container and then put into a coolbox for testing at the Freshwater Fisheries Laboratory, Brawijaya University. The parameters are Biological Oxygen Demand (BOD) using the Winkler method, which had been incubated for five days [14], ammonia (NH_3) using a spectrophotometer, Total Suspended Solid (TSS), and Total Dissolved Oxygen (TDS) [15].

Fish blood sampling and observation of hematological profile

A sampling of fish was carried out using fishing nets by taking three fish from each location. The Red Bader Fish (*Barbonymus altus*) used in this study can be seen in (Fig. 2). Blood was drawn by wetting the syringe and Eppendorf tube with a 3.8 % Na-citrate anticoagulant, then puncturing the linea lateralis at a slope of 45°C and slowly taking the blood with a 1 mL syringe, then putting it into the Eppendorf tube [16]. After that, it was put into a cool box that has been given ice gel for observations at the Freshwater Fisheries Laboratory, Universitas Brawijaya.

Observation of the hematological profile was carried out by counting the number of erythrocytes and leukocytes under a microscope using a hemocytometer [17]. Hemoglobin using a Hb meter and hematocrit using a hematocrit scale [18]. Then micronuclei by counting cell damage using a microscope [19].

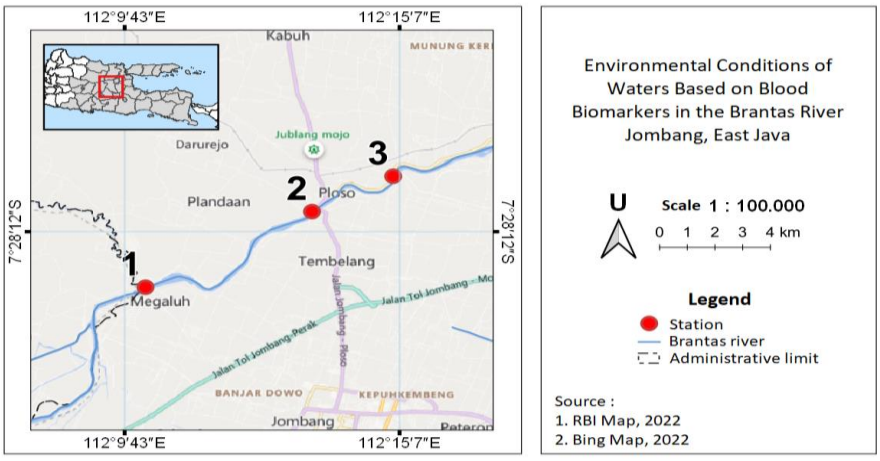

Figure 1. Map of sampling location

Figure 2. Red Bader fish (*Barbonymus altus*) sample (body length ±20 cm)

Data Analysis

This study connects water quality and hematological profiles as variables in the analytical test using the Canonical Correspondence Analysis (CCA) method [20]. This analysis is a multivariate statistical model that identifies and quantifies the relationship between two variables. This analysis focuses on the correlation between a linear combination of one set of variables with a linear combination of another set of variables. The independent variables in this study were all water quality parameters, while the dependent variable was the observation of the hematological profile of the red Bader fish (*B. altus*).

RESULT AND DISCUSSION

Water Quality Measurement

Water quality measurements are carried out with parameters of temperature, pH, Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), ammonia (NH_3), Total Suspended Solid (TSS), and Total Dissolved Oxygen (TDS) (Fig. 3). In this study, the average temperature parameter at the three research locations was 28-30.5°C. At each station, the values are 28-29.5°C (Station 1), 28-30°C (Station 2), and 28-30.5°C (Station 3). The highest temperature increase was in the third sampling, with an average of 30°C due to the hot weather. This value is still considered optimal in waters where the temperature range is 28-32°C, while at a temperature of 18-25°C, it will cause a decrease in fish appetite and death if the temperature is too cold [21]. An increase in temperature that is too high will result in fish stress, which can affect fish hematology [22].

The pH parameters of the waters at the three stations were obtained in the range of 7.12-7.95. The permissible pH value in the waters according to the Class II quality standard is 6-9 [23]. The pH value of the three stations is still considered optimal in the waters. Water conditions that have a very acidic or alkaline pH will endanger the survival of organisms because it causes metabolic and respiratory disorders [24]. Other effects of pH on fish physiology include stunted fish growth, sensitivity to bacteria and parasites, and water toxicity to fish [25].

Parameters of DO were obtained in the range of 7.11-10.77 $mg.L^{-1}$ at the three stations. With values at each station of 7.11-9.01 $mg.L^{-1}$ (Station 1), 7.9-10.77 $mg.L^{-1}$ (Station 2), 8.11-8.76 $mg.L^{-1}$ (Station 3). The value of DO allowed in the waters according to the Class II quality standard is 4 $mg.L^{-1}$ [23]. The main source of oxygen in waters can come from the process of diffusion of free air and the results of photosynthesis of organisms that live in the waters. Increasing the depth will result in a decrease in DO levels due to the reduced photosynthesis process and oxygen levels that are too much used for respiration and oxidation of organic and inorganic materials [26]. When dissolved oxygen has a low concentration, the fish will respond in various forms, including blood flow can be increased by opening the lamellae and then a secondary response to increase the area of effective breathing. Furthermore, the concentration of red blood cells can be increased by carrying oxygen from the blood per unit volume [27].

The BOD parameter is the amount of dissolved oxygen needed by microorganisms to decompose organic matter under aerobic conditions. BOD levels in waters are influenced by temperature, an abundance of plankton, the presence of microbes, and the content of organic matter in the waters [28]. In this study, the measurement of BOD parameters in the waters ranged from 9.15-28.25 $mg.L^{-1}$. Measurement of BOD obtained the highest value at the time of the second sampling at Station 3 of 28.25 $mg.L^{-1}$ and the lowest value at the time of the third sampling at Station 1 of 9.15 $mg.L^{-1}$. BOD values in high waters can reduce the availability of dissolved oxygen in the water because it is used in the oxidation process of organic matter that can be decomposed by microorganisms [29]. The value of BOD of river water quality standard with Class II category is 3 $mg.L^{-1}$ [23].

Ammonia (NH_3) is one of the inorganic nitrogen that can be dissolved in water. Ammonia in water generally comes from urine and feces, oxidation of organic matter, industrial waste and community activities [30]. Ammonia values at the three stations ranged from 0.056-0.45 $mg.L^{-1}$. The increase in ammonia value occurred at Station 3 with a range of 0.266-0.45 $mg.L^{-1}$. The value of Ammonia (NH_3) in river waters according to the Class II quality standard is 0.2 $mg.L^{-1}$ So, Station 3 obtained a value that exceeds the quality standard, which is suspected to be sampling close to the industrial waste disposal site.The high concentration of ammonia in the waters can come from the animal metabolism and the decomposition of organic matter by bacteria. Relatively high levels of ammonia indicate contamination of organic matter from industrial, agricultural, and domestic waste [31].

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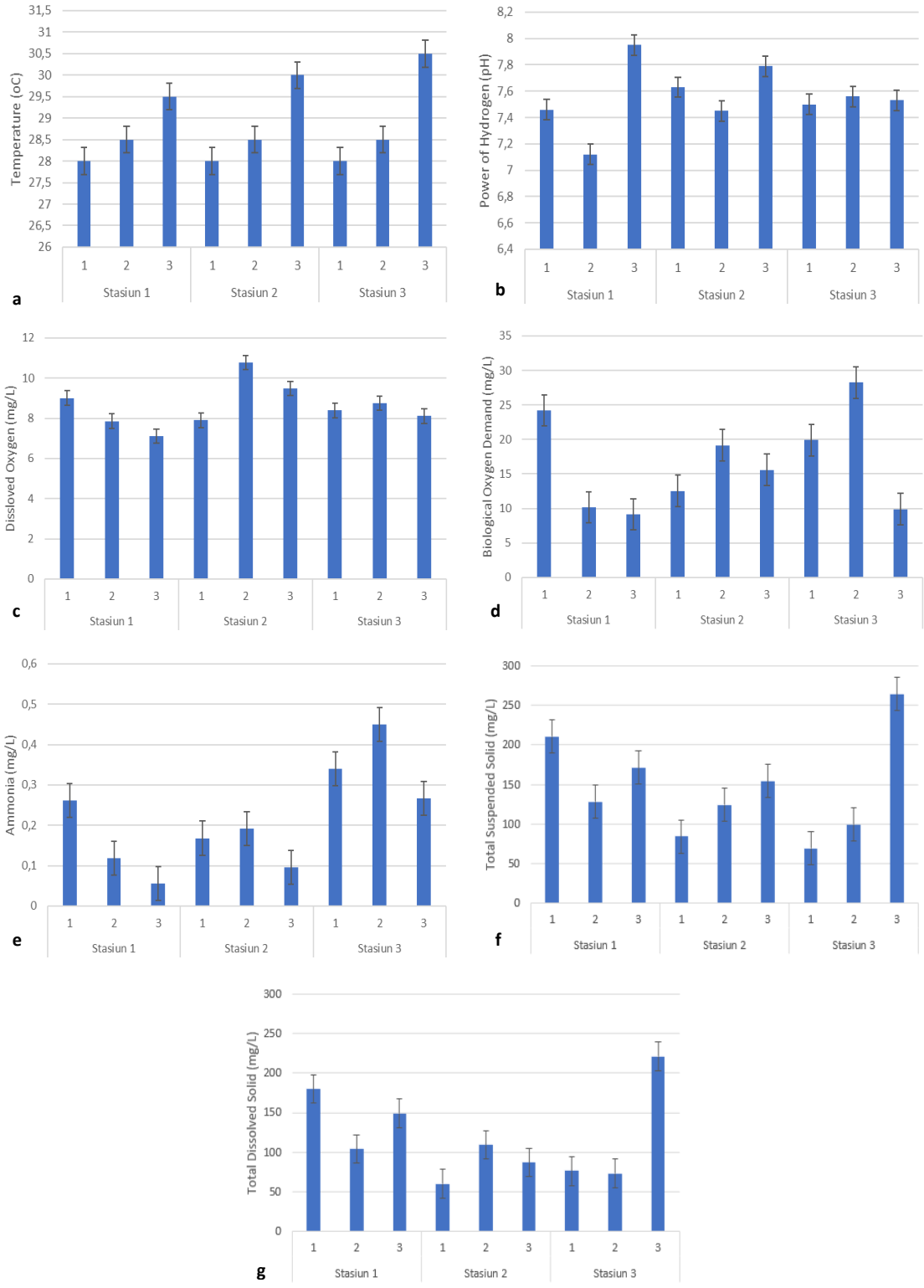

Figure 3. Water quality measurement result. a) Temperature, b) pH, c) *Dissolved Oxygen* (DO), d) *Biological Oxygen Demand* (BOD), e) *ammonia* (NH_3), f) *Total Suspended Solid* (TSS), and g) *Total Dissolved Solid* (TDS).

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The excessive concentration of Total Suspended Solid (TSS) is due to the low quality of the waters due to the presence of heavy metals that can bind to suspended sediments in the waters. It will result in a high value of TSS in the waters, thereby increasing the value of turbidity, which has an impact on inhibiting the life of aquatic biota. Penetration of light into water bodies will be reduced, thereby inhibiting the photosynthesis process thereby reducing water productivity [32]. TSS consists of mud and fine sand, and micro-organisms caused by soil erosion and soil erosion carried into water bodies [33]. In this study, the TSS values of the three stations ranged from 69-264 mg.L^{-1}. The highest TSS value indicates Station 3, which is near the disposal of industrial waste, which is 154-264 mg.L^{-1}. Allowable TSS value in waters, according to class II, is 50 mg.L^{-1} [23]. This condition illustrates that the TSS value still meets the water quality standards.

The value of the concentration of Total Dissolved Solids (TDS) in waters is a natural component of surface water whose main constituents can be organic salts, organic materials, and various other dissolved materials found in waters. The TDS value in the waters is based on the Class II quality standard of 1000 mg.L^{-1} [23]. The TDS values at the three research stations showed that the average was 60-221 mg.L^{-1} and the highest value was obtained at station 3 of 87-211 mg.L^{-1}. So it can be concluded that the TDS value is still in the optimal range in the waters. However, for station 3, the highest score was obtained due to the location adjacent to industrial waste disposal. Changes in TDS concentration in waters can be caused by industrial waste, increased rainfall, or seawater intrusion [34]. The high value of TDS can be caused by weathering of rocks and water runoff that carries soil erosion. The process of rock weathering is related to strong currents so that it can erode rocks and sediments at the bottom of the water [35].

Observation of Hematological Profile of Red Bader Fish (*Barbonymus altus*)

Hematological observations were carried out by looking at several blood profiles of the Red Bader Fish (*B. altus*), such as erythrocytes, leukocytes, hemoglobin, hematocrit, and micronuclei. Hematology is often used to detect physiological changes caused by environmental stress conditions and fish health status [22]. Erythrocytes play an important role in the transportation of oxygen throughout the body and are the largest component of blood cells and contain Hemoglobin [36]. Erythrocyte counts at the three stations obtained an average of 870.000-2.560.000 cells.mm^{-3} (Fig. 4). At each station, the erythrocyte values were 1.180.000-2.270.000 cells.mm^{-3} (Station 1), 1.670.000-2.560.000 cells.mm^{-3} (Station 2), 870.000-1.190.000 cells.mm^{-3} (Station 3). The number of erythrocyte levels in fish ranges from 1.050.000-3.000.000 cells.mm^{-3}. So the research shows that the lowest value occurs at Station 3, which can be presumed because of the input of industrial waste, while at Stations 1 and 2, the erythrocyte values are still in the normal range.

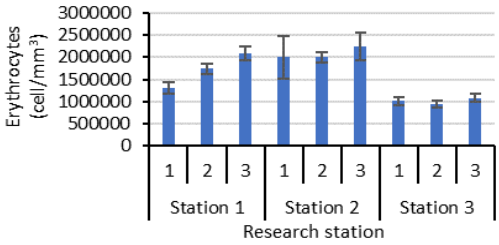

Figure 2. Erythrocyte in Bader fish hematological profile

Leukocytes are components of blood cells that act as the defense system of the fish body. The number of leukocytes in fish ranges from 20.000-150.000 cells.mm^{-3}. An increase in the number of leukocytes is called leukocytosis. The number of leukocytes is influenced by several factors, including fish species, age, nutrition, and stress [8]. The leukocyte count at the three stations yielded an average of 71.400-190.800 cells.mm^{-3} (Fig. 5). At each station, the leukocyte values were 77.400-158.400 cells.mm^{-3} (Station 1), 71.600 – 117.400 cells.mm^{-3} (Station 2), 130.000-190.800 cells.mm^{-3} (Station 3). The highest increase in the number of leukocytes occurred at Station 3, while the lowest value was at stations 1 and 2. The increase in the number of leukocytes at Station 3 may be due to industrial waste disposal. An increase in the number of leukocytes is an increase in antibody production to help fish survive and fight toxins as a form of immunological reaction [37].

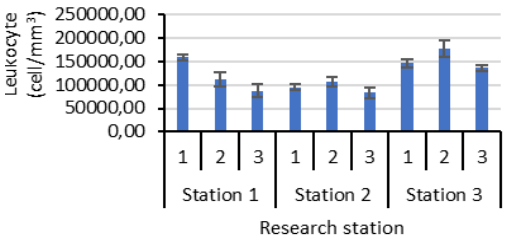

Figure 3. Leukocyte in Bader fish hematological profile

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Hemoglobin (Hb) in the fish body functions to bind oxygen, so the presence of heavy metals will interfere with Hb synthesis which causes the ability to bind oxygen to be small. The lack of oxygen bound in the fish's body will affect the metabolic process [38]. The amount of hemoglobin in fish is influenced by the type of species, gender, age, physical condition, season, air pressure, and living habits [39]. Measurement of hemoglobin at the three stations obtained an average result of 3.4-8 g% (Fig. 6). At each station, the hemoglobin value was 4.8-8.6 g% (Station 1), 6.2-7.6 g% (Station 2), and 3.6-4.8 g% (Station 3). The optimal amount of hemoglobin in fish in fish is 6 - 11 g% [40]. So at station 3, the value is below the normal threshold for the amount of hemoglobin, while Stations 1 and 2 are relatively normal. Anemia can occur due to increased destruction of erythrocytes or reduced release of erythrocytes in the blood circulation. The impact of anemia is the inhibition of fish growth due to the low number of erythrocytes which results in reduced food supply to cells, tissues, and organs so that the metabolic process of fish will be hampered [41].

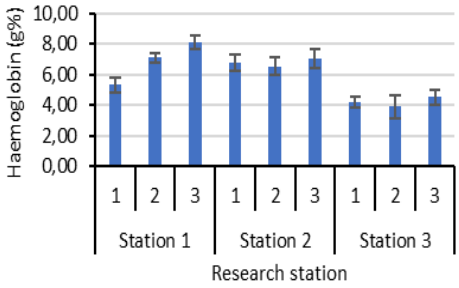

Figure 4. Hemoglobin in Bader fish hematological profile

Hematocrit is influenced by season, diet, and hormonal factors. Fish hematocrit levels are generally relatively constant between 10 - 30% [42]. Hematocrit calculations at each station obtained hematocrit values of 18 - 28% (Station 1), 21 - 30% (Station 2), and 10 - 21% (Station 3). The results of the hematocrit calculation are shown in Figure 7. The lowest hematocrit value comes from Station 3, which is thought to be due to the location close to the waste disposal site. The content of a relatively low hematocrit value describes a low erythrocyte volume which causes fish to experience anemia. On the other hand, high erythrocytes indicate homeostatic efforts in the fish body (pathogenic infection) in the body to produce more blood cells to replace infected erythrocytes, which will cause stress in fish [43].

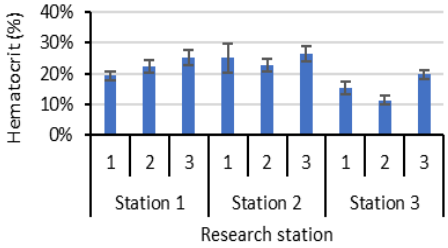

Figure 5. Hematocrit in Bader fish hematological profile

Micronuclei are extra-nuclear bodies containing chromosomal fragments and whole chromosomes that do not fuse with the nucleus after cell division [44]. Micronuclei calculations at the three stations obtained an average result of 18-64 cells.1000^{-1} (Fig. 8). At each station, the micronuclei values obtained were 17-34 cells.1000^{-1} (Station 1), 19-30 cells.1000^{-1} (Station 2), 31-64 cells.1000^{-1} (Station 3). Micronuclei observations obtained the highest results at station 3 with a range of 31-64 cells.1000^{-1}. The high levels of micronuclei at this station are caused by the location of the extraction, which is close to the industrial waste disposal site.

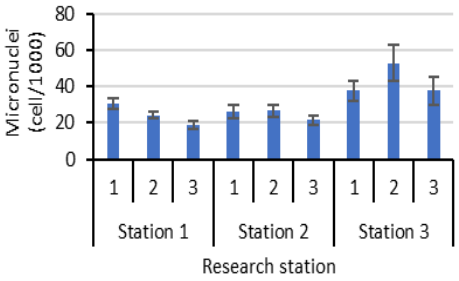

Figure 6. Micronuclei in Bader fish hematological profile

Relationship of Water Quality with Hematological Profile of Red Bader Fish (*B.altus*)

This research was conducted by analyzing data using the CCA (Canonical Correlation Analysis) method. Data analysis using the Canonical Correspondence Analysis (CCA) method is a multivariate analysis that can explain the relationship between biological communities and environmental parameters in the form of ordinances [45]. This method related to the dependent variables including erythrocytes, leukocytes, hemoglobin, hematocrit, and micronuclei with independent variables including temperature, pH, Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), ammonia (NH3), Total Suspended Solid (TSS), and Total Dissolved Solids (TDS) (Fig. 9). The results of the data analysis showed three conclusions. First, erythrocytes, hematocrit, and hemoglobin correlated with temperature, pH, DO, BOD,

ammonia, TSS, and TDS with moderate concentrations. Second, leukocytes and micronuclei have a strong association with ammonia, BOD, TSS, and TDS. Third, leukocytes and micronuclei showed a correlation with temperature and pH parameters with low concentrations. The number of erythrocytes affected by overall air quality is due to not being able to display all air quality parameters closely. Hematological parameters as a toxicity index are used in monitoring the aquatic environment [37]. Blood characteristics used in unifying fish physiological and pathological changes are effective and sensitive. Blood is an essential tool in diagnosing health with different levels of fish blood response and different stress factors [46].

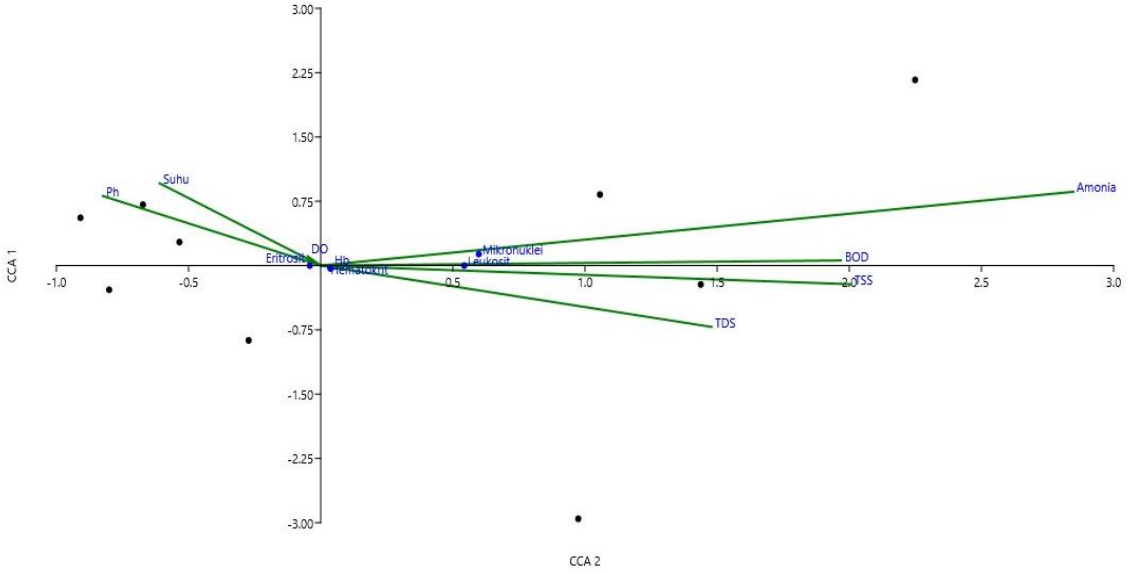

Figure 9. Result Canonical Corelation Analysis (CCA)

CONCLUSION

Based on the results of the analysis of the water quality parameters of the Jombang Brantas River, it was found that Station 1 and Station 2 were classified in normal conditions, while Station 3 was classified in relatively abnormal/polluted conditions. This condition is related to the hematology of Red Bader fish (*Barbonymus altus*), such as the values of erythrocytes, hemoglobin, and hematocrit, which are too low at Station 3, while Stations 1 and 2 have optimal values. The increase in the value of leukocytes and micronuclei was quite significant at Station 3, which was close to the location of industrial waste disposal. The results of data analysis show that the value of water quality affects the number of erythrocytes, hemoglobin, and hematocrit, which are moderate/low. Meanwhile, the values of ammonia and BOD at high concentrations were strongly correlated with leukocytes and micronuclei.

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Exploring YidR Gene from *Klebsiella pneumoniae* To Design a Multi-epitope Vaccine Using Immunoinformatics Approach

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Abstract

Klebsiella pneumoniae is the main leading cause of hospital-acquired infection, leading to severe infections such as pneumonia and urinary tract infection. There is an urge to develop an efficient *Klebsiella* vaccine, and epitope-based vaccines may offer a practical choice. Therefore, this study aims to design an epitope-based vaccine construct. YidR gene is expressed in all strains of *Klebsiella* with 97.6% of sequence homology, which makes it a potential vaccine candidate. *In silico* approach was used to make a multi-epitope vaccine construct consisting of immunogenic epitopes against the YidR gene of *Klebsiella*. Acquired multi-epitopes vaccine was analyzed for antigenicity, allergenicity, toxicity, and various physicochemical parameters. Molecular docking simulation was performed using TLR2 and TLR4 to asses binding affinity with the designed vaccine construct. The results showed that the predicted multi-epitope-based vaccine candidate is non-allergen, non-toxic, and has an efficient binding affinity towards TLR2 and TLR4 that could effectively induce immune responses.

Keywords: *In Silico*, *Klebsiella pneumoniae*, vaccine design, YidR gene.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic, gram-negative bacterium and a common etiology in severe infections, such as pneumonia and vascular infection, which increase the healthcare-associated infection burden [1]. It has also been demonstrated that it can infect intracellularly through bladder epithelial cells in urinary tract infections [2].

Due to mutation as a form of evolution, *K. pneumoniae* developed an ability to acquire new genetic material. Because of that, two strains of *K. pneumoniae* are classified as classic and hypervirulent. The difference between these two is that the hypervirulent one has a higher virulence than the classic one, enabling it to infect a healthy individual, cause many sites of infection, and spread metastatically [3].

The hypervirulent strain of *K. pneumonia* explicitly has a virulence plasmid that differentiates it from the classic strain [4]. This virulence plasmid contains an antimicrobial resistance gene that is acquired in some way:

1. Plasmid acquisition that encodes antimicrobial resistance gene [5];
2. Gene insertion to the plasmid [6];
3. The classic strain acquires virulence plasmid from the hypervirulent strain [7].

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The antimicrobial resistance of *K. pneumonia* is increasing steadily, thus making the therapy given more difficult than usual. Antibiotic therapy may not be effective because there is an increase in infection prevalence of drug-resistant strains or unwanted reactions from high-dosing antibiotic usage [8].

A vaccine is one measure to control infection by introducing epitopes of an antigen to the immune system. Epitopes are a small part of an antigen that can induce immune response through cell-mediated or antibody-mediated immunity. The whole system is required to acquire immunity [9].

Despite the alternative way to control infection, until now, no *Klebsiella* vaccine has been available, partly due to complicated production processes and high production costs [8]. Following those difficulties, *Klebsiella* is well-known for its strain's heterogenicity, which complicates the matter more [8].

YidR gene is found in all isolates acquired from bovine and human samples (308/308, 100%) [10]. This gene shared high homology (97.6%) across different isolates. This prevalence and similarity made this gene a potential antigen candidate for *K. pneumoniae* [10]. YidR gene is predicted to encode ATP/GTP-binding protein which appears to facilitate hyper-adherence phenotype for biofilm production in *Salmonella enterica* [8].

Using *In Silico* approach, researchers can develop a vaccine design with a rational and effective construct while still being reasonable in time need and production cost. This approach has several advantages, such as minimizing the unwanted immune responses, like allergenicity and toxic properties it may carry. With a more specific vaccine design, the vaccine can be designed with desired immunity response and is cost-effective [11,12]. In this study, we analyzed the potential of the YidR gene from *K. pneumonia* as an epitope-based vaccine through epitope prediction, physiochemical parameters analysis, and molecular docking.

MATERIAL AND METHOD

Protein Selection and Sequence Retrieval for Epitope Prediction

The previous iteration of the YidR gene from Rodrigues *et al.* [8] was used in this study. The obtained sequence was then submitted to NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi;accessed in April 2022) to acquire the FASTA sequence of YidR. The sequence with the highest query cover and percent identity is then chosen to be used in this study [13].

HLA Population Selection

Due to the vast amount of HLA in human populations, this study limits the population target to Indonesia. Using Allele Frequency Net Database (allelefrequencies.net; accessed April 2022) [14], the HLA allele with more than 20% of the population was selected. The chosen HLAs are listed in the Table 1 below.

Table 1. HLA populations in Indonesia chosen for this study

Allele	Population	% of Individuals with the allele
A*11:01	Indonesian Java Western	30.1
A*24:02	Indonesian Java Western	25.8
A*24:07	Indonesian Java Western	39.4
A*33:03	Indonesian Java Western	29.7
B*15:02	Indonesian Java Western	22.9
B*25:13	Indonesian Java Western	21.6
DRB1*07:01	Indonesian Java Western	25.4
DRB1*12:02	Indonesian Java Western	59.7
DRB1*15:02	Indonesian Java Western	41.1

Cytotoxic T-Cell Epitope Prediction

Cytotoxic T-Cell recognizes epitopes presented by HLA Class I on the surface of the antigen-presenting cells. Epitopes for Cytotoxic T-cell is predicted using NetMHCpan EL 4.1. (https://www.iedb.org/; accessed April 2022) as the method. Seven HLAs from the previous step were selected, which are HLA-A*11:01, HLA-A*24:02, HLA-A*24:07, HLA-B*15:02, HLA-B*15:13, HLA-B*15:21, and HLA-A*33:03 as our prediction target. The results are considered a good/suitable binder if the result is under 1% of the percentile rank. Later on, the predicted epitopes were evaluated [15] for (all accessed in April 2022):

1. Immunogenicity with IEDB https://www.iedb.org/
2. Antigenicity with VaxiJen; http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html
3. Toxicity with ToxinPred https://webs.iiitd.edu.in/raghava/toxinpred/design.php
4. Allergenicity with AllerTOP https://www.ddg-pharmfac.net/AllerTOP/method.html.

Helper T-Cell Epitope Prediction

Helper T-cell recognizes epitopes presented by HLA Class II on the surface of the antigen-presenting cells. Epitopes for T cell helper are predicted using the consensus method (iedb.org). Three HLAs from the previous step, DRB1_07:01, DRB1_12:02, and DRB1_15:02, were tested for the prediction target. The results are considered a strong binder if the result is under 2% of the adjusted rank. Later on, the predicted epitopes were evaluated further [15] (all accessed in April 2022):

1. Antigenicity by VaxiJen: http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html
2. Toxicity by ToxinPred: https://webs.iiitd.edu.in/raghava/toxinpred/design.php
3. Allergenicity by AllerTOP: https://www.ddg-pharmfac.net/AllerTOP/method.html)
4. IFN induction by IFNepitope: https://webs.iiitd.edu.in/raghava/ifnepitope/predict.php
5. IL4 induction by IL4Pred: https://webs.iiitd.edu.in/raghava/il4pred/predict.php
6. IL10 induction by IL10Pred: https://webs.iiitd.edu.in/raghava/il10pred/predict3.php

B Cell Epitope Prediction

B cell has important role in producing immunity memory. Epitopes for B cell is predicted using BEPIPRED 2.0 method in IEDB (https://www.iedb.org/; accessed April 2022). The prediction epitopes were determined afterwards [15] (all accessed in April 2022):

1. Antigenicity with VaxiJen; http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html
2. Toxicity with ToxinPred https://webs.iiitd.edu.in/raghava/toxinpred/design.php
3. Allergenicity with AllerTOP https://www.ddg-pharmfac.net/AllerTOP/method.html.

Selection of Adjuvant and Protein Linker for Vaccine Construction

The adjuvant is a component used in a vaccine construct to enhance vaccine efficacy [16]. Cholera toxin subunit B was utilized in this study as an adjuvant because it increased immune response in mucosal tissues or systemic [15]. To connect all of the epitopes, we used linkers as described by Shey *et al*. [17], which are EAAAK linker to connect adjuvant with the whole sequence, AAY linker to connect epitopes from T cell cytotoxic, and GPGPG linker to connect epitopes from T cell helper and B cell. Structure for multi-epitope construct was generated using trRosetta in April 2022 with this link https://yanglab.nankai.edu.cn/trRosetta/ [18]. This vaccine's modeling was evaluated using Ramachandran's geometrical analysis (https://molprobity.biochem.duke.edu/index.php ; accessed April 2022) [19].

Evaluation of Physicochemical Properties of Vaccine Construct

All epitopes obtained from the prediction and vaccine construct were evaluated for physicochemical parameters. It includes molecular weight, instability index, aliphatic index, GRAVY, theoretical isoelectric point, and estimated half-life using ExPasy Protparam tools (http://web.expasy.org/protparam; accessed April 2022) [16].

Molecular Docking of Vaccine with TLR2 and TLR4

Toll-Like Receptor (TLR) is a transmembrane protein that functions in recognizing part of infecting pathogen to activate the immune response. In *K. pneumoniae* infection, TLR2 and TLR4 expression is elevated as a response [16]. Therefore, this study used TLR2 and TLR4 as receptors in molecular docking.

TLR2 and TLR4 protein structure was acquired using Protein Data Bank (www.rcsb.org) with a PDB number of 2Z7X for TLR2 and 4G8A for TLR4. All of the protein was purified from another ligand and other unusable molecules using PyMOL software. Molecular docking was performed using the HDOCK server (https://hdock.phys.hust.edu.cn/; accessed May 2022) [20] with reference to Pizzuto *et al.* [20] for TLR2 binding site and the previously acquired conformation for TLR4.

RESULT AND DISCUSSION

A total of 85 epitopes for T cell cytotoxic, 11 epitopes for T cell helper, and 14 epitopes for B cell were found (Supplementary 1–3). The acquired epitopes are then evaluated for a vaccine candidate's required characteristics (antigenicity, allergenicity, and toxicity). It was done to ensure that no untargeted response is triggered and to minimize the adverse effect of the vaccine (Table 2, Table 3, and Table 4) [21].

Table 2. Result of the evaluated epitopes used in this study

Epitope	Peptide	Antigenicity	Toxicity	Allergenicity
B-Cell	TFAPRHHQLTN	Antigen	Non-Toxin	Non-Allergen
B-Cell	HVLHQRDPALDLR	Antigen	Non-Toxin	Non-Allergen
B-Cell	PSGASFTGE	Antigen	Non-Toxin	Non-Allergen
Cytotoxic T-Cell	VTVHPTQER (HLA-A*11:01)	Antigen	Non-Toxin	Non-Allergen
Cytotoxic T-Cell	WQYDFHHRR (HLA-A*33:03)	Antigen	Non-Toxin	Non-Allergen
Helper T-Cell	GTVETIYRATQGAHV (HLA-DRB1*07:01)	Antigen	Non-Toxin	Non-Allergen
Helper T-Cell	VETIYRATQGAHVGV (HLA-DRB1*07:01)	Antigen	Non-Toxin	Non-Allergen

Table 3. Result of the immunogenicity evaluation of Cytotoxic T-Cell epitopes used in this study

Epitope	Peptide	Immunogenicity
Cytotoxic T-Cell	VTVHPTQER	0.29415
Cytotoxic T-Cell	WQYDFHHRR	0.28446

Table 4. Result of the cytokines induction evaluation of Helper T-Cell epitopes used in this study

Epitope	Peptide	IFNepitope	IL4	IL10
Helper T-Cell	GTVETIYRATQGAHV	Negative	Inducer	Inducer
Helper T-Cell	VETIYRATQGAHVGV	Positive	Inducer	Inducer

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Two cytotoxic T-cell epitopes were chosen based on the highest immunogenicity score (Table 3). These two epitopes are presented by HLA-A*33:03, covering 29.7% of the Indonesian population (Table 1). Two helper T-cell epitopes were chosen despite one having a negative IFNgamma score, and both are positive for inducing IL-4 and IL-10 (Table 4). Both T-helper epitopes are presented by HLA-DRB1*07:01, which is one of the major HLA Class II alleles, covering 25.4% of the Indonesian population (Table 1). Despite their ability to induce IL-4, which associated with the allergic reaction, the epitopes are still chosen since they are promising/suitable antigens, non-toxin, and non-allergen (Table 2).

Then, a construct was designed based on Shey *et al.* [17] with Cholera Toxin subunit B (CTB) as an adjuvant (Fig. 1). Adjuvant is required to raise the efficacy of the vaccine [16]. At the same time, CTB is selected because of its properties to increase immunogenicity and augment immune response in mucosal tissues and systemic [15]. According to Shey *et al.* [17], the vaccine construct can be connected with these linkers:

1. Adjuvant is located in the N-terminal of the sequence, and connected with the rest using EAAAK linker.
2. Epitope sequences from T cell cytotoxic are connected using AAY linker.
3. Epitope sequences from T cell helper and B cell are connected using GPGPG linker.

The trRosetta server was employed for the 3D modeling of the vaccine construct (https://yanglab.nankai.edu.cn/trRosetta/). A Template Modelling (TM) score of 0.576 was obtained for the model predicted, which was higher than 0.5, indicating that the model predicted has high confidence in topology prediction [18].

Next, the model was evaluated with the help of Ramachandran geometry analysis. This tool verified the model using indirect stereochemical properties and the geometrical complex of an amino acid according to electrostatic rules [19]. It showed that the vaccine construct has 98.82% in 'Favored rotamers' and 94.42% in 'Ramachandran favored. The number is over 90%, implying that the amino acid position in the 3D model is in stable and correct position [19]. There were also 3 Ramachandran outliers in Asp59, Ile140, and Gln163 (Table 6, Fig. 2).

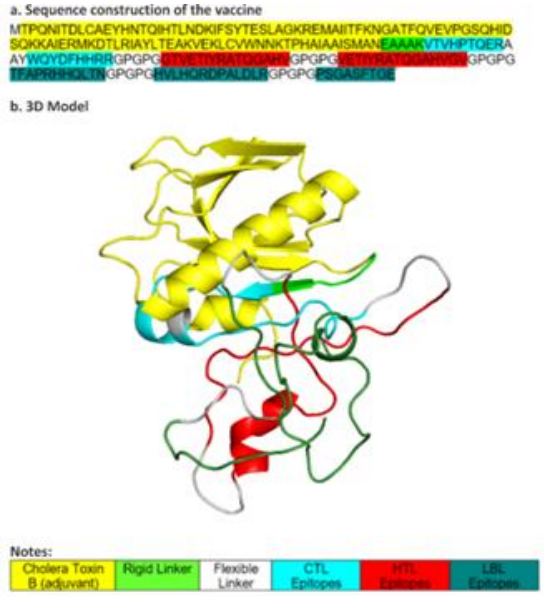

Figure 1. Vaccine Construction;
a) Sequences construction of the vaccine;
b) 3D model acquired from trRosetta server

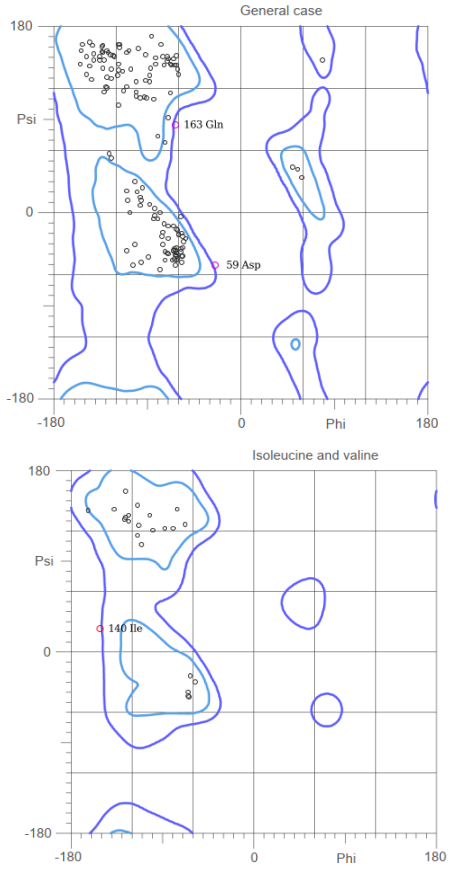

Figure 2. Ramachandran plot analysis from the vaccine model

The physiochemical parameters of this vaccine were also observed using ExPasy Protparam tools. The result is presented below (Table 5, Supplementary 4).

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Table 5. Physicochemical properties of the vaccine

Length	Molecular Weight	Theoretical pI	Instability Index
225	24532.47	8.54	22.48
Aliphatic Index	**GRAVY**	**Estimated Half Life**	
63.82	-0.620	30 hours (mammalian reticulocytes, in vitro)	

TLR2 and TLR4 are transmembrane proteins that recognize part of pathogens to activate the immune system. In *K. pneumoniae* infection, TLR2 and TLR4 expression is elevated in epithelial tissue [16]. Within the myeloid differentiation primary response gene (MyD88) signaling pathway, TLR2, and TLR4 are prominent for many cytokines' excretion, including TNF-α and Interleukin-1, Interleukin-6 and Interleukin-8 which are essential as a chemoattractant for neutrophils, also NF-κB which have roles in inflammation [22].

Molecular docking was performed as a reference to determine the binding affinity of the vaccine to TLR2 and TLR4. TLR2 and TLR4 structure data was accessed from Protein Data Bank (www.rcsb.org) with PDB numbers 2Z7X and 4G8A. The obtained protein structure was then processed using PyMOL software to obtain pure TLR2 and TLR4 protein.

According to Pizzuto *et al.* [20], TLR2 has a binding site listed specifically in the main chain, which are L324, F325, F349, L350, S329, and the side chain, which is N294. For TLR4, we used a specific binding site from the Protein Data Bank crystal structure, which is already bound to a complex of TLR4 and bacterial lipopolysaccharide.

The HDOCK server was employed for the molecular docking of our vaccine construct. HDOCK is an integrated place for homology search, template-based modeling, structure prediction, macromolecular docking, and other information with an accurate and fast docking result [23]. Surprisingly our vaccine constructs binding affinity scores of -244,72 and -266,51 as docked with TLR2 and TLR4 subsequently (Table 6, Fig. 3, Fig. 4).

Table 6. Binding affinity score from molecular docking results

Receptor	TM-Score	R-Outlier	R-Favored (%)	Z-Score	Binding Affinity Score
TLR2	0.576	3	94.42%	-0.42	-244.72
TLR4	0.576	3	94.42%	-0.42	-266.51

Notes
R-Outlier = Ramachandran Outlier
R-Favored = Ramachandran Favored

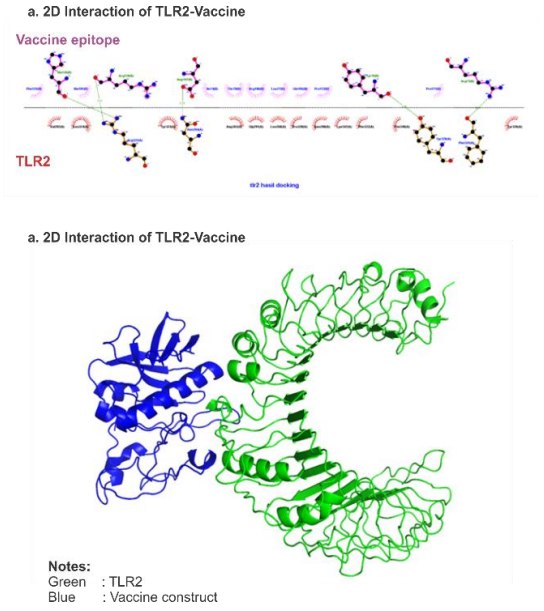

Figure 3. Molecular docking results of TLR2 and vaccine construct

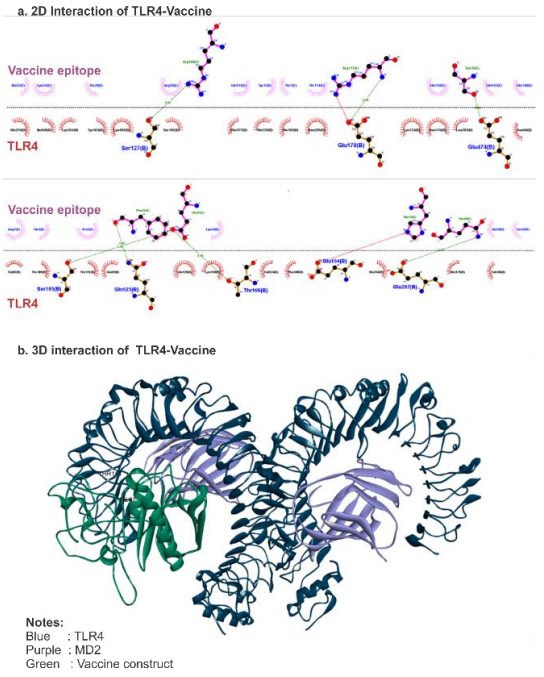

Figure 4. Molecular docking results of TLR4 and vaccine construct

Binding affinity is scored from conformational results from docking, which has the lowest energy. It represents the similarity of the result with the crystallography structure [24]. Negative results mean hydrogen bonding and van der walls are the most prominent interactions between protein and ligand complex stabilization, suggesting the binding process is spontaneous [25]. With a binding affinity score of -244,72 and -266,51 for TLR2 and TLR4, respectively, the

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vaccine construct is able to bind effectively to TLR2 and TLR4.

Despite that, this study is limited and needs further study before this vaccine construct is clinically approved. This study uses using *in silico* approach, thus making this study only a prediction-based analysis using simulations and predictions from computational algorithms and data banks from past studies. This study is yet unable to represent actual protein properties when expressed in laboratory conditions and needs further study for clinical approval.

CONCLUSION

The present study shows an *in-silico* approach to designing vaccines against *Klebsiella pneumoniae*. Multi-epitope-based vaccine constructs based on epitopes for T cells and B cells using the YidR gene demonstrated that the vaccine constructs could bind to TLR2 and TLR4 effectively. This study could be beneficial in delivering information on the epitope-based construct's potential as a protective approach for immunization against bacterial infection.

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SUPPLEMENTARY

Supplementary 1. Candidate Epitope MHC I

ALLELE	#	START	END	LENGTH	PEPTIDE	CORE	ICORE	SCORE	PERC. RANK	Immunogenicity	Antigenicity	Toxicity	Allergenicity
HLA-A*11:01	1	67	75	9	VTVHPTQER	VTVHPTQER	VTVHPTQER	0.554665	0.25	0.03243	Antigen	Non-Toxin	Non-Allergen
HLA-A*11:01	1	379	387	9	AIVFSPDGK	AIVFSPDGK	AIVFSPDGK	0.467985	0.33	-0.00182	Antigen	Non-Toxin	Non-Allergen
HLA-A*11:01	1	68	76	9	TVHPTQERY	TVHPTQERY	TVHPTQERY	0.237656	0.77	0.04284	Antigen	Non-Toxin	Non-Allergen
HLA-A*24:07	1	5	13	9	TFAPRHHQL	TFAPRHHQL	TFAPRHHQL	0.718941	0.04	0.04201	Antigen	Non-Toxin	Non-Allergen
HLA-A*33:03	1	67	75	9	VTVHPTQER	VTVHPTQER	VTVHPTQER	0.669339	0.13	0.29415	Antigen	Non-Toxin	Non-Allergen
HLA-A*33:03	1	89	97	9	WQYDFHHRR	WQYDFHHRR	WQYDFHHRR	0.571215	0.21	0.28446	Antigen	Non-Toxin	Non-Allergen
HLA-A*33:03	1	143	151	9	YNDHVLHQR	YNDHVLHQR	YNDHVLHQR	0.437298	0.39	0.13668	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	110	118	9	DAMDITPPY	DAMDITPPY	DAMDITPPY	0.899684	0.02	0.11562	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	68	76	9	TVHPTQERY	TVHPTQERY	TVHPTQERY	0.870672	0.03	0.04284	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	130	138	9	HVYSPNGQF	HVYSPNGQF	HVYSPNGQF	0.810082	0.05	-0.22364	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	149	157	9	HQRDPALDL	HQRDPALDL	HQRDPALDL	0.332884	0.49	0.06875	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	358	366	9	HAGTGDITF	HAGTGDITF	HAGTGDITF	0.261319	0.67	0.23894	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:02	1	266	274	9	GVSQRRLTF	GVSQRRLTF	GVSQRRLTF	0.210936	0.88	-0.05782	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	110	118	9	DAMDITPPY	DAMDITPPY	DAMDITPPY	0.371252	0.13	0.11562	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	70	78	9	HPTQERYVF	HPTQERYVF	HPTQERYVF	0.25244	0.3	0.06326	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	337	345	9	FNWHPSGEW	FNWHPSGEW	FNWHPSGEW	0.221636	0.35	0.02502	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	358	366	9	HAGTGDITF	HAGTGDITF	HAGTGDITF	0.152492	0.6	0.23894	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	266	274	9	GVSQRRLTF	GVSQRRLTF	GVSQRRLTF	0.116657	0.81	-0.05782	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	5	13	9	TFAPRHHQL	TFAPRHHQL	TFAPRHHQL	0.112322	0.85	0.04201	Antigen	Non-Toxin	Non-Allergen
HLA-B*15:13	1	149	157	9	HQRDPALDL	HQRDPALDL	HQRDPALDL	0.105504	0.91	0.06875	Antigen	Non-Toxin	Non-Allergen

Notes: Epitope Candidate

Supplementary 2. Th cell (MHC II) properties

Peptide	adjusted rank	Antigenicity	Toxicity	Allergenicity	IFNepitope	IL4	IL10
ETIYRATQGAHVGVV	1.7	Probable Antigen	Non-Toxin	Allergen	Negative	Inducer	Inducer
TIYRATQGAHVGVVT	1.7	Probable Antigen	Non-Toxin	Allergen	Positive	Inducer	Inducer
GTVETIYRATQGAHV	1.8	Probable Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Inducer
VETIYRATQGAHVGV	1.8	Probable Antigen	Non-Toxin	Non-Allergen	Positive	Inducer	Inducer
FVSFTYNDHVLHERD	1.9	Probable Non-Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Non-Inducer
GQFVSFTYNDHVLHE	1.9	Probable Non-Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Non-Inducer
NGQFVSFTYNDHVLH	1.9	Probable Non-Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Non-Inducer
PNGQFVSFTYNDHVL	1.9	Probable Non-Antigen	Non-Toxin	Allergen	Negative	Inducer	Non-Inducer
QFVSFTYNDHVLHER	1.9	Probable Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Non-Inducer
SPNGQFVSFTYNDHV	1.9	Probable Non-Antigen	Non-Toxin	Allergen	Negative	Inducer	Non-Inducer
YSPNGQFVSFTYNDH	1.9	Probable Non-Antigen	Non-Toxin	Allergen	Negative	Inducer	Non-Inducer

Peptide	adjusted rank	Antigenicity	Toxicity	Allergenicity	IFNepitope	IL4	IL10
GTVETIYRATQGAHV	1.8	Probable Antigen	Non-Toxin	Non-Allergen	Negative	Inducer	Inducer
VETIYRATQGAHVGV	1.8	Probable Antigen	Non-Toxin	Non-Allergen	Positive	Inducer	Inducer

Notes:

Epitope Candidate

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Supplementary 3. B cell properties

Start	End	Peptide	Length	Antigenicity	Toxicity	Allergenicity
5	15	TFAPRHHQLTN	11	Antigen	Non-Toxin	Non-Allergen
32	40	PSGASFTGE	9	Antigen	Non-Toxin	Non-Allergen
84	92	RPDAQWQYD	9	Antigen	Non-Toxin	Allergen
114	126	ITPPYTPGALRGG	13	Antigen	Non-Toxin	Allergen
146	158	HVLHQRDPALDLR	13	Antigen	Non-Toxin	Non-Allergen
167	180	GPVTPQGQHPREYG	14	Non-Antigen	Non-Toxin	Non-Allergen
191	214	TTPAPAPGSDEINRAYEEGWVGNH	24	Non-Antigen	Non-Toxin	Non-Allergen
220	229	GDTLAENGDK	10	Antigen	Non-Toxin	Allergen
238	254	LPQDEAGWKQPGGAPLA	17	Non-Antigen	Non-Toxin	Non-Allergen
274	289	FTHHRRYPGLVNVPRH	16	Non-Antigen	Non-Toxin	Allergen
320	333	GGEPRQLTHHASGI	14	Antigen	Non-Toxin	Allergen
367	377	LTDTHAHAPSA	11	Antigen	Non-Toxin	Allergen

Start	End	Peptide	Length	Antigenicity	Toxicity	Allergenicity
5	15	TFAPRHHQLTN	11	Antigen	Non-Toxin	Non-Allergen
32	40	PSGASFTGE	9	Antigen	Non-Toxin	Non-Allergen
146	158	HVLHQRDPALDLR	13	Antigen	Non-Toxin	Non-Allergen

Notes:

Epitope Candidate

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Supplementary 4. Physico-chemical properties

Epitope	Peptide	Length	ID*	MW	II**	AI***	GRAVY	Theoretical pI	The estimated half-life
Cytotoxic T-Cell	VTVHPTQER	9	KP	1066.18	9.28	64.44	-1,033	6.72	100 hours (mammalian reticulocytes, in vitro)
Cytotoxic T-Cell	WQYDFHHRR	9	KP, PR	1344.46	82.09	0	-2,422	8.76	2.8 hours (mammalian reticulocytes, in vitro)
Helper T-Cell	VETIYRATQGAHVGV	15	KP, EC	1600.79	-23.95	97.33	0.1667	6.72	100 hours (mammalian reticulocytes, in vitro)
Helper T-Cell	GTVETIYRATQGAHV	15	KP, EC	1602.77	-23.95	78	-0.16	6.75	30 hours (mammalian reticulocytes, in vitro).
Continue B-Cell	TFAPRHHQLTN	11	KP, EB	1321.46	34.81	44.55	-1,136	9.47	20 hours (mammalian reticulocytes, in vitro)
Continue B-Cell	HVLHQRDPALDLR	13	KP, EB, SE	1569.79	38.86	120	-0.77	6.92	3.5 hours (mammalian reticulocytes, in vitro)
Continue B-Cell	PSGASFTGE	9	KP, SF, EC, EB	851.87	3.04	11.11	-0.4	4	5.5 hours (mammalian reticulocytes, in vitro)

Notes:
*Query Cover, Identity 100 %, **Instability Index**, ***Aliphatic Index,**
MW: Molecular Weight, KP: *Klebsiella pneumoniae*, PR: *Providencia rettgeri*, EC: *Escherichia coli*, EB: *Enterobacter*, SE: *Salmonella enterica*, SF: *Shigella flexneri*.

Microbial Agents in Terrorism, Biomarkers, and Public Health Challenges

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ABSTRACT

Bioterrorism agents are mostly microorganisms with the capacity to deal explosive and lethal harm to humans, animals, and food crops. These microorganisms spread in the form of gases, whole organisms, or products of secondary metabolism of microorganisms. Classification of the agents is into three categories based on the ease of dissemination and end effects on a living population. While most health institutions are equipped to take care of sick people and treat suspected cases of infections, these institutions lack basic means of identifying bioterrorism acts. Special diagnostic equipment to identify causal organisms or agents is not available. Lack of training on what to do when terrorists strike using biological agents can cumulatively increase the lethal effects of such agents. Molecular techniques of identifying microorganisms to species level are as promising as they are time-consuming, while technical expertise and a conducive environment for managing such equipment are mostly not available in the African setting. The governments in Africa as a matter of urgency should provide an atmosphere where the teaming population of people without jobs are employed, while hospitals are adequately equipped, and training of health workers on what to do immediately after cases of terrorism are reported. The review highlights these agents and the diagnostic tools necessary to facilitate early response to bioterrorism.

Keywords: Bioagents, Bioterrorism, Classification, Identification, Spread.

INTRODUCTION

Bioterrorism is the deliberate release or threat of release of a biological weapon to a civilian population with the intent of causing serious illness or death to animals or humans and destruction to food crops [1]. Bioweapons, which could be insects, microorganisms, or toxins, are engineered with the ultimate aim of influencing Government conduct or policy; this could be due to religious, political, or ideological reasons with the ultimate goal of spreading fear and panic within the population. Bioterrorism is a planned threat of discharge of pathogenic microorganisms and their products (bacteria, fungi, toxins, and viruses) to cause morbidity and mortality among the designated human population, food crops, and livestock. Microorganisms are target agents of bioterrorism and have proven to be effective due to their potential of producing disease responses with significant clinical consequences that may lead to death or illness in the target host. The resultant effects are to create an atmosphere of fear, anxiety, and panic among the public [2]. Bioterrorism agents are weapons of mass destruction because of their potential to spread within a short time and create devastating consequences. These microorganisms are directly employed or modified to increase their virulence (causing disease conditions in man and animals) and resistance (against anti-bioterrorism agents) [3].

Classification of bioterrorism agents is according to the risk they pose to State and National security. The risks are defined based on the ability of these agents to be easily transmitted from person to person or from their source to the intended target. Classification of the agents is also on their public health impact to cause high mortality, creating panic among people, and the level of public preparedness required for mitigating it. Microorganisms adapted for bioterror attacks are pathogenic and weaponized. These agents differ in the level at which they cause infections, morbidity, and mortality as consequences of exposure to the organisms. Microorganisms could be genetically altered and conferred with significantly higher virulence and capacity of thriving and maintaining themselves in the environment indefinitely with potential ease of transmission and spread in the population within a short period after release. Other bioterrorism agents include products of the metabolism of microorganisms that kill or incapacitate hosts. The most targeted host of bioterror attacks are humans, commercial plants, and environmental systems.

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Microorganisms are manipulated for bioterrorism because the cost of production is usually low, making it readily available, and the relative ease of isolation and mass production within a short period. Other reasons are ease of transportation from place to place without detection by the routine security system; ease of spread over large areas by wind, water, insects, animal, and humans; and ease of the agent to establish a viable community in the target area for a long time. Bioterrorism agents are transmitted in aerosols and incorporated into food and water as poison. In Africa, little is done to mitigate the problem of terrorists due to varying factors relating to religion, ethnicity, corruption, dearth of infrastructures, incompetence, and other such factors. The review is to highlight bioterrorism and its related challenges to healthcare provision in Africa.

CATEGORIES OF BIOLOGICAL AGENTS OF BIOTERRORISM

Microorganisms used in terrorism attacks are categorized into three groups labeled A, B, and C based on the relative ease of dissemination. The level of risk resulting from exposure to the agent is determined by morbidity and mortality rates in the target hosts.

I. Category A Agents - are the most lethal agents presenting the highest risk to national security and public health, characterized by hemorrhagic fever and its associated syndrome. They spread effortlessly, resulting in death and threat to public safety. Their effects result in general fear among the public and require measures for public health alertness [4]. Microbial agents in this group are *Francisella tularensis* (Tularemia), *Bacillus anthracis* (anthrax gas), Smallpox (Variola major), *Yersinia pestis* (Plague), *Clostridium botulinum* (toxin), Viral hemorrhagic fevers, Lassa virus.

II. Category B Agents - in this category, microbes or microbial products are at moderate risk of exposure to humans and animals. They cause moderate morbidity and low mortality but can spread easily from host to host. Agents in this class are Brucella species (Brucellosis), Epsilon toxin of *Clostridium perfringens*, food poisoning caused by *Salmonella* species, *Escherichia coli* 0157:H7, *Shigella*, and *Staphylococcus aureus*.

III. Category C Agents - are classified as emerging infectious disease agents, mostly with zoonotic attributes. They are relatively lower risk agents compared to categories A and B agents. Hantavirus, Coronavirus, MERs, influenza pandemic, and Nipah virus are examples of category C agents [5].

Characteristics Peculiar To Bioterrorism Agents

The characteristics that define the hazardous potential of bioterror agents are that; the agent should be highly infective at low doses to establish a disease condition with the desired effect [6]; the capacity of the organism to cause disease conditions by evading the host's innate defense system (pathogenicity); ability to easily infect a healthy individual from a diseased patient. The agent should be resistant to treatment remedies and possess relative ease of mass production. These agents should be stable and viable in any environment dispersed. Dispersal should be relatively easy and efficient without altering the stability of the agent and must be virulent, toxic, and lethal at low concentrations or doses.

MANIPULATING MICROORGANISMS FOR TERRORISM

Microorganisms have the attribute of easily being manipulated hence, their use in terrorism. One of the best progression and accomplishments in gene manipulation using biotechnological techniques opened the way for effective adjustment of microbes into new microbes with lethal attributes that are untreatable and wild. The knowledge of molecular biology and biotechnology made it possible for genetic manipulation of biological agents to resist treatment to be able to cause harm to life. Genetic engineering involves a deliberate intervention to transfer genes (DNA) between different/same biological entities to create a new organism with novel characteristics. The new organism created with new characteristics has a higher degree of survival, infectivity, virulence, and drug resistance.

Alteration of naturally occurring pathogens into deadly genetically modified pathogens like the insertion of an alien gene into *B. anthracis* constitute a threat to humans as a bioweapon [7]. Genetically engineered bioterrorism agents are classified based on the technique of creation into a binary biological weapon, designer gene and life forms, gene therapy, host swapping diseases, and designer diseases.

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I. Binary Biological Weapons: are a two-part component system made-up of an autonomous protected part and another part that exists separately but works better to produce a greater result. An example is a blend between Hepatitis B and D. Hepatitis D after infection of Hepatitis B uses the proteins expressed by hepatitis B to increase the severity of the infection, as it alone cannot cause disease. Binary weapons have great possibilities for future application due to their benign properties, making them easy to be preserved and to be manipulated. Transportation from place to place is easy since the parts are not separately dangerous. It additionally makes tracing more troublesome due to its potential and capacity to be stored secretly away for a long time [8].

II. Designer Genes: Available data of already sequenced viruses, plasmids, bacteria, fungi, and animals could now be engineered using recombinant DNA gene-splicing techniques to alter an organism's genetic properties. These data and information in the wrong hands are prone to abuse for the creation of genetically designed microorganisms that are intolerant to antimicrobials and vaccines and increase virulence suited for bioweapons. With these data, it would be feasible to make sicknesses utilizing create agents that could clear out a whole populace. Designer gene is one of the greatest breakthroughs in biotechnology, as adopted strategies are refined [8]. Despite the advantages of this biotechnology, the downsides cannot be ignored because quality can be customized into an irresistible expression that could undoubtedly be changed into a biological weapon [8].

III. DNA/multigene Shuffling: *In-vitro* atomic development encouraged research on the effectiveness with which a wide variety of genetic successions can be determined. The capacity to create new DNA successions was an aid to deliver enormous cascades of DNA that are exposed to evaluation or determination for the scope of wanted characteristics. The technique aided antibiotic production from bacteria and other microorganisms [9].

IV. Gene Therapy: a very important concept in treating patients with genetic abnormalities. Transfer of healthy genes requires a vector, usually a virus that is modified which predisposes the technology to abuse. Gene therapy was utilized in animal and human clinical preliminary examinations with promising outcomes [10]. Modification and enhancement will continue as the technology gain more acceptance and could be adopted for creating bioweapon [8].

V. Stealth Viruses: the introduction of a viral agent into the body system using a vector. The viral agent is dormant until set off by an inward or outward trigger for it to cause disease or harm to the target host. With this innovation, a malignant (cancer) growth causing the embedded infection into humans made lethargic until set off to trigger the disease condition. Whenever the sign is initiated, the cells become unusual and could quickly produce strange cell development prompting growth and eventual death. Stealth viruses can become a candidate bioweapon [11].

VI. Host Swapping Diseases: Instances abound where a pathogenic agent peculiar to a reservoir or host switches to another host. They become resistant and deadly, creating a new emerging threat. Disruption of the harmony between the host and resident pathogen could create viruses that are destructive or harmless, and this occurs when a virus leaps out and move to an alternate host animal where it can make or become new viruses by transforming or getting different genes unintentionally [11].

BIOMARKERS AS RELEVANT TOOL FOR EVALUATING EFFECTS OF BIOTERRORISM

Biomarkers are evaluated based on their roles as descriptors of the measurement of biological systems [12]. Biomarkers are tools that can widen our understanding of prediction, cause, diagnosis, pathogenic processes, or pharmacological responses to medication. For a biomarker to be useful in the identification of biological agents, it should be easily obtainable from blood, saliva, urine, or any other body fluid or tissue. The biomarker test should be fast, and results should be available within minutes. Another factor considered is the method of detection, which should be accurate and easy to carry out. Biomarkers for the detection of bioterrorism agents should be sensitive and specific to the organism in question. It should be consistent between different strata and environments. Biomarkers are organic particles found in blood and body liquids or tissues, resulting from typical or unusual interactions,

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conditions, or illnesses [13,14]. Biomarkers are grouped based on the suitability of the classifying body and applicability. Accordingly, any phase from the beginning to recuperation or severity of illness is fitting to a particular distinguishing marker.

I. Diagnostic Biomarker: These classes of markers affirm the presence of an illness or ailment. These detect specific biomolecules related to a specific disease. They aid in the identification of people with a type of disease. An example is a rheumatoid factor in serum as a diagnostic marker to diagnose and differentiate rheumatoid arthritis from other types of arthritis. Beyond the vital role of diagnostic tools, these markers are useful in prognosis and prediction of the outcome of treatment [15].

II. Monitoring Biomarkers: These evaluate the presence, status, or degree of sickness or ailment [16]. They assess the impact of clinical or environmental agent exposure in a targeted host. It is adopted in a known disease condition to monitor the effect of medical intervention. This biomarker overlapped with other types of biomarkers. It also surveys the restorative reaction by contrasting the progressions in biomarker articulation or fixation when treatment has been administered.

III. Pharmacodynamic/Response Biomarkers: They verify and evaluate the dosing regimen, checking whether a medication follows up on its key objective. They are also referred to as drug activity biomarkers whose role is to measure the effect of the therapeutic agents. They are classified as efficacy biomarkers, mechanism biomarkers, and toxicity biomarkers, which all indicate therapeutic effect, mechanism of action of drugs, and toxicological effect, respectively [16]. Response biomarkers assume a fundamental part in clinical preliminary decisions taking processes giving pertinent data about the clinical advantage of the medication required.

IV. Predictive Biomarkers: These identify susceptible individuals based on associated risk factors.

V. Prognostic Biomarkers: These biomarkers show disease progression without drug intervention. Prognostic markers help to foresee the event of clinical occasion like death, sickness movement, infection repeat, or development of another ailment [17].

VI. Susceptibility or Risk Biomarkers: The primary contrast between these classes of markers is the way susceptibility/risk markers estimate in people without introducing sickness. In this manner, these markers identify well before the presence of illness and are not valuable to portray the reaction to a particular therapy [18]. The choice of biomarkers for determining the effects of terrorism is based on the following criteria:

- Providing reliable and consistent outcomes or results
- Obtained results must be accurate and dependable
- Markers should be sensitive at very low concentrations
- The method and results should be reproducible
- Ease of sampling
- The test/marker should be reproducible

EPIDEMIOLOGICAL IMPORTANCE OF BIOMARKERS

The utilization of biomarkers is the key in epidemiological examinations because the data are used to foresee the advancement of sickness and to carry out infection control programs. Biomarkers estimate reactions taking place in human and animal hosts due to the introduction of environmental or manipulated stress inducers. In environmental epidemiology, disease transmission is monitored and tracked using markers, which address subclinical/reversible changes.

Appropriately, biomarkers in the epidemiology of disease transmission are going through a fast turn of events and development and are becoming one of the most encouraging areas of environmental examination [19]. Biomarkers can signal the impact of therapy on diet, confirm the presence of sickness, and determine how an infection might start in a singular case, no matter what the sort of therapy (prognostic marker). Among the many limitations of biomarkers is that only microorganisms and toxins are translated into weapons to induce fear and threat [20].

Mitigating biological threats requires the adoption of proficient preventive measures with quick and precise techniques to identify the threat/agent amongst environmental samples in the targeted location [21]. The result of the challenge of separating agents from environmental and clinical specimens is the need to design probes for specific biomarkers to remove the burden of

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large sampling sizes and produce biomarkers that are reproducible and specific to bioterrorism agents [22,23]. In the event of a bioterrorism attack, the first and most important step is to identify the agent used for the attack. Closely, it is followed by epidemiologic surveillance, management of the affected individuals, and prevention of attack. Biomarkers are very important in epidemiology when they relate to disease distribution and risk determination. It is a mediator of disease and could be targeted to prevent and treat diseases, which is very important in epidemiology [24]. The epidemiological study design usually assists in identifying and characterizing the bioterrorism treads.

TYPING OF AGENTS OF BIOTERRORISM

Diagnosis to determine agents of bioterrorism involves the use of throughput technology. Typing techniques depend on certain features when special machines investigation, for example, metabolites are adopted. A technique or method for evaluating a type of system should possess the following characteristics. I) Typeable: ability to obtain a clear positive result for the isolates tested. II) The typing tests must give repeatable, unambiguous, clear findings that are simple to understand. III) Reproducible: capacity to provide the same result when the same strain is tested or when the same strain is repeatedly tested. IV) High power of discrimination: distinguishing between unrelated strains. V) Ease of use: a typing method should be extensively helpful and broadly relevant to a variety of microorganisms, as well as simple to conduct and readily available (inexpensive). Typing methods employed in the diagnostic laboratory differ and are classified as follows.

I. Phenotypic Typing Methods

These methods are for detecting qualities that a microbe exhibits or expresses. The parameters include biochemical properties, size, staining qualities, shape, and antigenic properties are all phenotypic characteristics that are independent of the genome. Phenotyping is sensitive to environmental changes in an isolate. Phenotyping group organisms based on their similar characteristics. Common phenotypic methods employed are:

a. **Multilocus Enzyme Electrophoresis (MLEE):** evaluates differences in electrophoretic motilities of a collection of metabolic enzymes in isolates. It is simple to use and offers a high level of repeatability. Most strains are classified using this technique though it has limited discriminating power.

b. **Serotyping**: it gives reliable, reproducible results when employed. Stable testing conditions and preparation methods are very important. Commonly used serological techniques are the complement fixation test, serum agglutination test, and Rose Bengal test [25]. Serotyping is easy to replicate and interpret, as the majority of strains are typeable. Some serotyping methods are difficult to master, and some strains that are autoagglutinable (rough) are untypeable because of the vast number of serotypes and antigen cross-reactions. It has limited discriminatory power [26].

II. Genotypic Typing Methods

These are procedures adopted to determine and evaluate the composition and homology of DNA samples. The techniques are also useful in identifying the presence or absence of target genes and plasmids in DNA samples. Genotypic typing technique measure disparity in bacterial isolates genetic makeup of an individual organism by matching it with another organism sequence. This method shows different alleles a human being inherits from their parents.

a. **Nucleotide Sequence Analysis** - DNA (or RNA) nucleotide-base sequences are for determining genotypic information about an organism. PCR DNA-based methods are species-specific techniques that utilize primers from specific polymorphic sections on the DNA. Sequencing of RNAs is either by converting them to DNA or by sequencing the DNA gene that produced the RNA. The results are repeatable and easy to interpret, and the technique works on any strain, though it is labor-intensive and costly.

b. **Southern blot analysis of RFLPs** - Southern blot assays, unlike restriction endonuclease analysis (REA) of DNA, only identifies a single restriction fragment. The endonuclease digests the DNA, and gel electrophoresis is used to separate the fragments, which are then transferred to

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nitrocellulose membranes. Labeled DNA probes detect the fragments carrying certain sequences. If the DNA sequences of organisms exhibit less than 98 percent homology, it is classified as distinct species, and if there is less than 93 percent identity between the sequences, they are classified as different genera. The 16SrRNA gene is extremely valuable due to its high conservation. They are repeatable, easy to read, and typeable for all strains. It is expensive, labor-intensive and the discriminatory power dependent on the probes used.

c. **Multiplex PCR typing** – to identify species and biovar levels exploiting polymorphism that arises from species-specific localization of the genetic element. Multiplex PCR simultaneously amplifies multiple primer sets of different targets in a single reaction tube to obtain amplicons with different DNA sequences [27]. The technique targets more than one specific DNA sequence in an isolate from the resulting amplicons obtained from the PCR mixture. The design of the primers is specific to the isolate and determines the success of the multiplex PCR co-amplification.

d. **RAPD-PCR (Random Amplified Polymorphic DNA)** - RAPD uses primers composed of short sequences (oligonucleotides) to identify complementary sites on the genome of the target DNA. The procedure is easy. The nucleotides (size range of 8 to 15 in length) primers hybridize to multiple regions in an organism's chromosomal DNA, thus aiding rapid detection of genomic polymorphism. It has poor reproducibility from place to place. It has low stringency that allows the oligonucleotides to anneal and eventually give rise to heterogeneous DNA products that are strain-specific [28].

e. **Real-time PCR** - known also as quantitative PCR, is a rapid and sensitive method for detecting, quantifying, and typing microorganisms. It reduces incidences of contamination and false-positive result [29]. It amplifies and identifies the target sequence; as the reaction mix is run using interacting fluorescent dye or fluorescence-labeled probe. It has the capacity of detecting point mutations and high throughput while reducing contamination. The machine is costly and requires well-trained personnel.

f. **Restriction Fragment Length Polymorphism (RFLP)** - The technique identifies by analyzing specific variations in the DNA molecules in a chromosome. It uses restriction endonucleases, which target restriction sites on the DNA and cleaving to them. The method helps in differentiating specific strains, diversity, and relatedness of microorganisms using the pattern of DNA fragments [30]. It is easy to use, has high consistency, and is quick.

g. **Single nucleotide polymorphisms (SNPs) typing** - it accurately probes the phylogenetic framework of an isolate at the polymorphic region [31]. Single nucleotide polymorphisms (SNPs) are for analyzing single-nucleotide base variation in organisms at the subspecies level. SNP are base pair variations that can alter a nucleotide by replacing the base pair in a genome; hence, one SNP is a difference in a single nucleotide.

h. **PFGE TYPING (Pulsed-field gel electrophoresis)** - It is a discriminating and repeatable typing technique commonly used for characterizing bacterial isolates in outbreaks. It is a low-cost method with high type ability and intra-laboratory reproducibility. It is time-consuming, tedious, and does not give a good resolution of bands [32].

CHALLENGES OF BIOTERRORISM AGENTS TO PUBLIC HEALTH

Bioterrorism constitutes a major threat to public health worldwide. In the natural setting, infectious diseases are one of the leading causes of morbidity and mortality both in humans and animals [33]. Consequently, the intentional release of highly virulent pathogenic microorganisms to cause disease and death within the human, animal, and plant populations is of great concern that calls for concerted efforts and deliberate preparedness [34]. The impact of bioterrorism spreads across all spheres of human endeavors. When outbreaks of known or unknown diseases occur within a defined geographical location, it is pertinent to investigate the source, as it could be the initial stage of a bioweapon attack [35]. The adoption of biological

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agents as a weapon is on the ease of obtaining and manipulating them without detection. The technology adopted for mass production of these deadly agents is the same as those used in the mass production of other medical and household everyday substances for human use and survival.

The limitation of biomarkers in forestalling bioterrorism in a population is that bioterrorism agents are not easily distinguishable from normal biological microbes causing infection. Anytime there is an attack, it takes some time to design the probes and develop specific biomarkers during which casualties would have been recorded. The cost implication of producing biomarkers and huge sampling size constitute issues as they lead to incurring huge expenses. Another factor limiting the use of biomarkers relates to acceptability. Acceptability borders are on ethical considerations, beliefs, and convictions of a people and the target human host. The Covid vaccine rejection by different religious sects is a ready example. The incubation period and the inception of symptoms provide a terrorist with the window period of escape [36]. It necessitates high throughput technology and early warning and rapid detection systems that can detect aerosolized bio-agents as early as possible [37].

Bioterrorism agents are transmitted through water, air, or food, which poses a challenge as they cannot easily be detected, consequently causing illness after the initial exposure until too late [38]. Inadequate training of the first responders and other healthcare workers to recognize and react to diseases caused by biological agents is another challenge. Unpreparedness by different first responder agencies was charged with taking action when bioterrorism acts take place. These agencies sometimes lack improved detection and data gathering equipment and cannot provide contingencies when it matters most. From a public health perspective, it is vital for timely surveillance, media awareness, and publicity.

PUBLIC HEALTH COUNTER-TERRORISM RESPONSE

Counterterrorism is political and military efforts carried out or aimed to prevent or deter terrorist acts. It encompasses law enforcement and intelligence agencies that employ techniques, policies, and strategies to combat terrorism. Responding immediately to biological weapon strikes in combatting terrorism and biological warfare is critical in protecting life against lethal disease outbreaks. Surveillance of infectious diseases is a major task of any public health institution [35]. Hence, steps employed by public health agencies to prevent emerging infectious diseases are applicable in the prevention of bioterrorism agents. An effective surveillance system such as the syndrome surveillance requires an effective communication system, and adequate epidemiological and laboratory provisions to give timely discovery of outbreaks by exploring existing health data to alert public health agencies [35,39]. Local and state-level developmental plans (such as immunization) against bioterrorism agents will help in saving lives and reduce costs [40]. As part of readiness to counter bioterrorism, health departments will require up-to-date laboratory facilities and competent health workers.

To address the bioterrorism challenge from a public health perspective, it is needful to adopt a broad-based approach and tackle the problem from its root. Bioterrorism is a threat to our corporate existence, and therefore all hands must be on deck to counter the menace. It is pertinent to analyze the social determinant associated with terrorism, such as the high rate of joblessness, political isolation, poverty, incorrect philosophy, and inequality. These are factors driving young people into crime. Hence, the provision of a meaningful standard of living to the growing youth population is fundamental to avoiding radicalization and the tendency to become victims of social vices. Therefore, there must be global concerted efforts through a partnership among public health experts, law enforcement agencies, and redirection of government policy towards the universal basic income concept/proposal to lift many out of poverty, promote human dignity and avert the evil consequences of bioterrorism acts [41]. Furthermore, to counter bioterrorism, it is pertinent to invest more in public health infrastructure to aid in rapid detection and prompt diagnosis of agents of bioterrorism [36,42].

CONCLUSION

Activities of terrorist groups are increasing all over the world with ferocious sophistication in their methods of operation. Countries with civil wars reportedly use chemicals as a means of putting out perceived sovereign enemies. The threat of the use of biological agents by terrorists is real and

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concerted efforts at frustrating it needed be put in place. Considering the advantages these agents possess, managing them, storing and transporting them make it easy to be deployed at the least expected of places with ease. To this end, the best method of preventing serious carnage and destruction is to adequately educate the first-line responders in the health sector in communities, and secondly, the people. Training on what to do should be paramount while the government provides a conducive environment that can deter people from joining terrorist groups.

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