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Microbial Isolation and Characterization of Animal Feed Additives Formulated Using Coconut Water and Rice Water

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DECLARATION

I declare that this thesis entitled **Microbial Isolation and Characterization of Animal Feed Additives Formulated Using Coconut Water and Rice Water** is the results of my own research except as cited in the references.

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Microbial Isolation and Characterization of Animal Feed Additives Formulated Using Coconut Water and Rice Water

ABSTRACT

Animal feed additives are rooted in the need to address challenges such as inefficient feed utilization, suboptimal animal growth, and health issues. These issues underscore the importance of developing and implementing effective feed additives to enhance digestibility, improve animal performance, and ultimately ensure the production of high-quality animal products while positively influencing the environment. This study aimed to isolate the microbial populations and characterize the animal feed additives produced from coconut water and rice water. The sources can be easily obtained from the surroundings and used as the substrate for the fermentation process. Microbial isolation and characterization were performed using various techniques such as serial dilution, spread plating agar, streaking, and gram staining. The fermentation broths were tested for their biochemical properties. The result of biochemical properties showed that protein content 0.092mg/ml which is higher in animal feed additives produced from coconut water compared to animal feed additives produced from rice water. The fermentation process took around 14 days approximately until constant pH was achieved. A glucose concentration test was also conducted and showed that both solutions have approximately the same glucose content, but animal feed additives produced from coconut water have slightly higher glucose content. In addition, antimicrobial tests were carried out and showed that the solutions have activity towards *E. coli.*

Keywords: Animal feed additives, broths, fermentation, isolation, substrate,

Pemencilan Mikrob dan Pencirian Bahan Tambahan Makanan Haiwan Diformulasikan Menggunakan Air Kelapa dan Air Beras

ABSTRAK

Aditif makanan haiwan berakar umbi dalam keperluan untuk menangani cabaran seperti penggunaan makanan yang tidak cekap, pertumbuhan haiwan yang tidak optimum dan isu kesihatan. Isu-isu ini menekankan kepentingan membangunkan dan melaksanakan bahan tambahan makanan yang berkesan untuk meningkatkan kebolehcernaan, meningkatkan prestasi haiwan, dan akhirnya memastikan pengeluaran produk haiwan berkualiti tinggi sambil mempengaruhi alam sekitar secara positif. Kajian ini bertujuan untuk mengasingkan populasi mikrob dan mencirikan bahan tambahan makanan haiwan yang dihasilkan daripada air kelapa dan air beras. Sumbernya boleh didapati dengan mudah dari persekitaran dan digunakan sebagai substrat untuk proses fermentasi. Pemencilan dan pencirian mikrob dilakukan menggunakan pelbagai teknik seperti pencairan bersiri, kaedah spread plate, coretan, dan pewarnaan gram. Cecair fermentasi telah diuji untuk sifat biokimianya. Hasil daripada sifat biokimia menunjukkan kandungan protein 0.092mg/ml yang lebih tinggi dalam bahan tambahan makanan haiwan yang dihasilkan daripada air kelapa berbanding dengan bahan tambahan makanan haiwan yang dihasilkan daripada air beras. Proses penapaian mengambil masa kira-kira 14 hari sehingga pH malar dicapai. Ujian kepekatan glukosa juga telah dijalankan dan menunjukkan bahawa kedua-dua larutan mempunyai kandungan glukosa yang lebih kurang sama, tetapi bahan tambahan makanan haiwan yang dihasilkan daripada air kelapa mempunyai kandungan glukosa yang lebih tinggi sedikit. Di samping itu, ujian antimikrob telah dijalankan dan menunjukkan bahawa larutan mempunyai aktiviti terhadap *E. coli.*

Kata kunci: Aditif makanan haiwan, larutan, penapaian, pengasingan, substrat,

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Animal feed additives are necessary in formulating feeds additives are non-nutritive products added to basic feed mix to enhance growth or other productive function, increase efficiency of feed utilization, preserve feed, or benefit animal health and metabolism. Feed additives in animal feed can improve productivity and performance by enhancing digestibility, maintaining, and stabilizing beneficial microflora in the gut and finally can improve quality of animal products and positively influence the environment. Feed additives are products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health, e.g., providing enhanced digestibility of the feed materials.

In this feed additive, there are specific bacteria or probiotics microorganism that influence the efficiency of this product. The bacteria are called *Streptomyces* and *Lactobacillus* (probiotic). Streptomyces have the capacity to release hydrolytic exoenzymes that enhance the digestive tract of livestock's amylolytic and proteolytic activities for more effective utilization of the feed, ultimately resulting in improved growth performance of the livestock (Tan et al., 2016). Meanwhile, lactobacillus (probiotics) can lessen the spread of pathogens and their emission during infection, decrease gut permeability, improve clinical symptoms in livestock, increase immunity, and enhance disease resistance and general health (Lambo et al., 2021).

The study was conducted by using two sources of probiotic bacteria which were coconut water and rice water. Research found out that there were 7 strains of Lactic Acid Bacteria (LAB) were found in coconut water (Camargo Prado et al., 2015). It turned out that coconut water and rice water are good natural sources of probiotic microbes.

Probiotic supplements (live yeast or bacteria) have been shown to improve the health and performance of broiler chickens, as well as their resistance to infections by *C. perfringens,*

E. coli, and Salmonella. Probiotics can boost laying hen productivity and feed efficiency while also improving egg quality (lower yolk cholesterol, thicker shells, and heavier eggs) (Chaucheyras-Durand & Durand, 2010). The bacterial probiotics in monogastric can help lower the pH of the gut, improve the ecological conditions for the resident microbiota, and lessen the chance of pathogen colonization by producing organic acids (lactic or acetic acid) (Servin, 2004). It has been shown that harmful bacteria can't grow when antimicrobial peptides like bacteriocins are released, or that enzymes that can hydrolyze bacterial toxins are produced (Buts, 2004).

1.2 Problem Statement

Food additives are common matters in farming where farmers often create or produce their own livestock's foods additives by using common and affordable ingredients from the nearby store. Hence, the food produced has no scientific research or data. So, people or farmers would not know what they actually supply to the livestock. Thus, the cost of supplying food to livestock is expensive depending on the amount of the livestock (Woyengo et al., 2014). The nutritional contents of the food supplied are not fully digested (Ørskov, 1977). It can be concluded that the food supplied is expensive and the nutritional content is not being used 100% without the presents of food additives. Farmers need to acknowledge the importance of food additives for animals such as enhancing feed efficiency. Feed additives are often used to improve feed efficiency and conversion rates. Understanding their nutritional value allows farmers to select additives that complement the existing diet and maximize the utilization of feed, ultimately leading to better growth performance and cost savings. Next, food additives are able to maintain animal health. Certain nutrients provided by feed additives can have direct benefits for animal health. For example, additives containing vitamins, minerals, or probiotics can support immune function, gut health, and overall well-being. Knowing the nutritional content enables farmers to choose additives that address specific health challenges or deficiencies in the animal's diet. Last, farmers can ensure product quality. The nutritional quality of animal feed directly impacts the quality of animal products such as meat, eggs, and dairy. By selecting feed additives with appropriate nutritional profiles, farmers can improve the nutritional content and overall quality of these products, meeting consumer demands for healthier and more sustainably produced food.

1.3 Objectives

1.To formulate animal feed additives using coconut water and rice water

2.To isolate microorganisms from animal feed additives formulated.

3.To determine the glucose and protein content of the formulated animal feed additives.

1.4 Scope of Study

In this study, two sources of probiotics were used mainly were coconut and rice water. The formulations of feed additives were produced using coconut water and rice water. In this study, microbial isolation was carried out to identify the microbes that are present in the animal feed additives. Next, media preparation was conducted with 2 type of media which are Potato Dextrose Agar (PDA) and De Man, Rogosa and Sharpe Agar (MRS). Other than that, microbial isolation from animal feed additives was carried out by spread plate method. The spread plate method was more exact and accurate at counting the heterotrophic plate count population than the pour plate method (Taylor et al., 1983). After that, bacteria identification such as Gram staining was conducted to identify the microorganisms by morphology. Next, protein concentration and glucose tests were conducted to characterize and identify the biochemical properties of the formulated animal feed additives. Lastly, the formulated solution underwent an antimicrobial test to identify microbial activity.

1.5 Significances of Study

This study provided scientific data on the animal feed additives formulated. Also, the feed additives lessened the cost of farming and improved the quality production in the farming sector. The significance of studying animal feed additives formulated lies in understanding the microbial activity and how microbes work in animal digestive system and how the microbes affect the health of livestock.

This study exposed the importance of microbes for animal livestock in order to improve their digestive system as a lot of farmers tend to create their own food for their livestock which lack nutrition and fiber. Therefore, the husbandry industry has been compelled to think about including alternate feedstuffs in the diet due to sustained price increases for standard cereal grain and protein meal feed commodities (Woyengo et al., 2014).

CHAPTER 2

LITERATURE REVIEW

2.1 Production of Animal Feed Additives

Animal feed additives contain probiotics which are important for animals which can change the gut microbiota in a way that reduces pathogen shedding and illness symptoms, boosts gut immunity, and improves disease resistance and overall health. (Arsène et al., 2021).

Animal feed additives also contain yeast which undergo fermentation process to generate more nutrients in the animal feed. In generally, live yeast (direct fed microbial; DFM; probiotic), yeast cell wall components (mannan-oligosaccharides, -glucans, and nucleotides), ethanol co-products (DDGS, specialty high protein distillers grains), or a mix of these are fed to animals as yeast and yeast-based products (Shurson, 2018).

Among the probiotics microbes that have been used in animal feed are Bacillus *velezensis.* A bacteria called *Bacillus velezensis* that encourages plant development can also prevent plant diseases from developing. But because of its characteristics, it is starting to appear in animal feed as a probiotic. Different *B. velezensis* strains isolated from various sources were discovered to be able to produce antimicrobial substances and have a positive impact on the gut microbiota, with the potential to be a candidate probiotic in the animal feed business (Khalid et al., 2021).

Some strains of lactic acid bacteria (LAB), Bacillus, and yeasts, which have been created in many kinds of animal feeds, are the most widely utilised probiotics. However, probiotics may have a greater favourable impact on the effectiveness of animal production systems when coupled (Agregán-Pérez et al., 2021).

The terms lactic acid bacteria (LAB) refer to a group of gram-positive, non-spore-forming bacteria who’s primary by product of fermented sugar is lactic acid. Due to their positive impacts on the host's health and high efficacy in the treatment of human and animal ailments, LAB are regarded as a form of probiotic. LAB are a family of microbial agents that are

frequently utilised in the breeding of livestock and poultry. They are also regarded as the greatest alternatives to antibiotics for enhancing animal health (Deng et al., 2021). Because of their dynamic nutritional value, which confers many health advantages, modulates host immunological responses, and inhibits the growth of hazardous food-borne pathogens, lactic acid bacteria (LABs) are thought to be the most researched probiotic microorganisms. Fermented foods and drinks, including fermented milk products, are frequently found to include LABs (J. Reis et al., 2012).

2.2 Application of Animal Feed Additives

Probiotics and fungi have tremendous industrial applications, especially in the husbandry sector. Animal feed additives can assist the digestion of livestock including chicken and poultry. They contain yeast like Saccharomyces cerevisiae as well as bacteria like Lactobacillus and Bifidobacterium. By encouraging beneficial gut bacteria, these additions can enhance gut health, lower the frequency of digestive diseases, and increase immunological function in animals (Kim et al., 2019).

Animal feed additives can also be used to reduce water pollution. In freshwater fish industry, the ponds tend to become contaminated due to the fish feed residue etc. Waters containing more microorganisms have more bacteria, which make up the base of the ecological pyramid. The pyramid itself expands as the base itself does, leading to increased ecosystem diversity and a larger bottom. This will enhance ponds' capacity for self-purification and aid in their restoration to their former state of cleanliness and beauty (Himangini et al.,2019).

Animal feed additives are used worldwide for a variety of livestock, including poultry, for a number of purposes, including to provide critical nutrients, improve feed palatability, enhance growth performance, and maximize feed utilization. Animals with high growth rates need to maintain a high level of health, and in these situations the use of appropriate products frequently appears as a basis. There is more pressure on the industry to find more natural and non-residual substitutes for the conventional feed additives used up until recently as animal feed

products due to rising industry standards, consumer awareness, and the desire for healthier food items of animal origin (Pandey et al., 2019).

The best alternatives for animal feed additives are determined primarily by consumer and animal health considerations. Probiotics, prebiotics, enzymes, and herbs are a few alternatives that are being considered for use as animal feed additives. This decision to use feed additives is supported by scientific and empirical research on these alternatives, as it has been discovered that herbs and their extracts (botanicals) have a variety of activities that not only stimulate feed intake but also stimulate endogenous secretions or have antimicrobial, coccidiostat, or anthelmintic activity (Pandey et al., 2019).

2.3 Probiotics

Probiotics are beneficial bacteria that can improve gut health and overall well-being in animals. When used as feed additives, they can offer several advantages such as enhanced digestion, improved nutrient absorption, strengthened immune system, and even reduced incidence of diseases. From this study, the animal feed additives were produced from fermentation process. Probiotic fermentation products are derived from the fermentation process of probiotic bacteria or their metabolic byproducts. During fermentation, probiotic bacteria break down organic substrates such as carbohydrates, proteins, and fats, producing a range of compounds that can have beneficial effects on gut health and overall well-being in animals. Probiotic fermentation often results in the production of organic acids such as lactic acid, acetic acid, propionic acid, and butyric acid. These organic acids create an acidic environment in the gut, which can inhibit the growth of pathogenic bacteria while promoting the growth of beneficial microbes. Additionally, organic acids help lower the pH of the gastrointestinal tract, which can enhance nutrient absorption and digestion efficiency.

On the other hand, animal feed additives can be categorized as vinegar as it has low pH value. Acetic acid, also known as ethanoic acid, is a simple organic compound with the chemical formula CH_3COOH. It is a weak acid that occurs naturally in various foods and beverages, including vinegar, which is a dilute solution of acetic acid. Acetic acid is produced through

fermentation processes, where carbohydrates such as sugars or ethanol are metabolized by certain microorganisms, including acetic acid bacteria.

2.4 Acetic Acid Bacteria

Meanwhile Acetic acid bacteria (AAB) are a group of Gram-negative bacteria that belong to the family Acetobacteraceae. These bacteria are aerobic, meaning they require oxygen to grow and metabolize substrates. Acetic acid bacteria are commonly found in natural environments such as soil, water, and air, as well as in fermented food and beverages. They play a crucial role in the production of vinegar and other fermented products through the oxidation of ethanol or other organic compounds to acetic acid.

Acetic acid bacteria are known for their ability to produce acetic acid through fermentation. In animal feed, AAB can be utilized to ferment feed materials, leading to the production of acetic acid. Acetic acid has antimicrobial properties and can help preserve feed by inhibiting the growth of spoilage organisms and pathogens, thereby extending the shelf life of feed. Since AAB are mostly found in vinegar which people used vinegar as food preservative (Solieri & Giudici, 2009).

Acetic acid produced by AAB can also have beneficial effects on gut health in animals. When animals consume feed treated with AAB-produced acetic acid, it can promote the growth of beneficial gut microorganisms while inhibiting the proliferation of harmful bacteria. This can contribute to improved digestion, nutrient absorption, and overall gut health in animals. Acetic acid produced by AAB has been shown to have antifungal properties. In animal feed, mycotoxins produced by molds can pose serious health risks to animals. By inhibiting the growth of molds and detoxifying mycotoxins, AAB-produced acetic acid may help mitigate the negative effects of mycotoxin contamination in feed.

AAB can be applied to animal feed through various methods, including direct inoculation of feed materials with AAB cultures or by using AAB-rich substrates as feed additives. The effectiveness of AAB in preserving feed and promoting gut health may vary depending on

factors such as the concentration of acetic acid produced, the feed matrix, and environmental conditions during fermentation.

2.5 Lactic Acid Bacteria

LAB are widely utilized in food fermentation processes due to their ability to convert sugars into lactic acid. This acidification lowers the pH of the food, creating an environment that inhibits the growth of spoilage bacteria and pathogens, thus extending the shelf life of the product. Foods such as yogurt, cheese, sauerkraut, pickles, and sourdough bread rely on LAB fermentation for preservation.

Some strains of LAB are considered probiotics, which are live microorganisms that confer health benefits when consumed in adequate amounts. Probiotic LAB, such as certain strains of *Lactobacillus* and *Bifidobacterium*, can survive passage through the digestive tract and exert beneficial effects on gut health, immune function, and overall well-being. Probiotic LAB are commonly added to yogurt, kefir, fermented milks, and other functional foods. Other than that, it is most likely that LAB can supress or inhibit pathogens activity by producing antimicrobial compound (Soomro et al., 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation Of Animal Feed Additives

In this study, two animal feed additives were formulated by using two sources of probiotics from coconut water and rice water. The ingredients for producing animal feed additives are shown below.

Table 3.1: Preparation of Animal feed additives (Formulation 1)

Ingredient/material	Composition
Coconut water	100 ml
Palm sugar	100 g
Distilled water	Up to 1000 ml

Table 3.2: Preparation of Animal feed additives (Formulation 2)

Ingredient/material	Composition
Rice water	100 ml
Palm Sugar	100 g
Distilled water	Up to 1000 ml

The preparation of Formulation 1 was conducted by using 100 ml of coconut water, 100 g of palm sugar was mixed up to 1000ml of distilled water and kept in airtight container and stored away from sunlight. The solution underwent fermentation process until the solution becomes dark brown color and produces sweet sour odor. Meanwhile for the preparation of Formulation 2, 100 ml of rice water solution was used, 100 g of palm sugar was mixed up to 1000 ml of fresh milk. The solution was kept in an airtight container for fermentation process. The pH of the solutions was recorded every two days until a constant level of pH was achieved.

3.2 Isolation Of Acetic Acid Bacteria (AAB) And Lactic Acid Bacteria (LAB) From Animal Feed Additives.

3.2.1 Preparation of media

In this study, the isolation method was conducted using Potato Dextrose Agar (PDA) and De Man, Rogosa and Sharpe agar (MRS). The ingredients for both media productions are listed below.

Table 3.3: Preparation of media (PDA agar)

Ingredients	Quantity
Potato Dextrose Agar (PDA)	32.5g
Bromocresol Purple	0.030g
Ethanol	20 ml
Distilled water	Up to 500ml

The preparation of 500mL of PDA media was conducted by using distilled water following the instruction manual, 32.5g of commercial PDA powder was added with 500 ml of distilled water. Next, 0.030g of bromocresol purple and 20 ml of ethanol was added in the Schott bottle. The media was mixed before putting it for autoclave at 120°C for 15 minutes. The media was poured on the plate under sterile condition.

The preparation of MRS agar was conducted by following the instructions given from the MRS agar labelling. MRS agar powder was weight 31g and mixed with distilled water up to 500 ml in a 500ml Schott bottle. The solution was mixed before putting it for autoclave at 120°C for 15 minutes. The media was poured on the plate under sterile condition.

Table 3.4: Preparation of media (MRS agar)

Ingredients/ material	Quantity
De Man, Rogosa and Sharpe agar (MRS).	31 g
Distilled water	Up to 500ml

3.2.2 Isolation of Microorganism from animal feed additives

The spread plate technique was used to isolate and enumerate the microbes from the animal feed additives. First, from the sample, a dilution series was made. Next, 0.1ml of the dilution series was pipetted out onto the center of the surface of an agar plate. After that, the L-shape glass spreader was dipped into alcohol and slightly flamed 1-3 times over a Bunsen burner.

After that, the sample was spread evenly on the surface of MRS and PDA media using sterile glass spreader. Next, the plate underwent an incubation process at 37°C for 24 hours.

3.2.3 Bacteria Identification and Gram Staining

A drop of bacterial colonies solution was applied on to a slide. Next, 5 drops of Hucker's crystal Violet were added to the culture. After a few minutes, the bacteria stain turned to purple. After that, the slide was rinsed with water. Next, 5 drops of iodine solution added to the culture. The slide was left for about 30 seconds, and then was rinsed with water to remove excess. Next, the slide was tilted and decolorized with solvent (acetone-alcohol solution) until purple colour stops running. Then the slide was washed for about 5 seconds with water and shake off to remove the excess. The culture was examined under a microscope at 400x and 1000x oil immersion.

3.3 Biochemical Characterization of Animal Feed Additives

3.3.1 Determination of Protein Concentration

From the animal feed additives solution, a serial dilution was performed. The known series concentration used for this was Bovine Serum Albumin (BSA). Five minimum standards were used to make the calibration curve and 5 of the microtube were labelled as (T1, T2, T3 and T4), and one ml of distilled water was placed in fourth microtube label 'blank'. Next, the required volume was pipetted into the first flask or microtube. The process was repeated by pipetting the previous solution into a new microtube with added solvent. Each microtube were received 3 ml Bradford reagent and was allowed to stand for 5 minutes.

After dilution series was complete, the samples and unknowns were prepared. The standards were transferred to cuvettes. Next, the standards and samples were put in the spectrophotometer. The absorbance at 595 nm was determined using UV-VIS Spectrophotometer. From BSA standard curve, the absorbance was used to compute protein concentration.

3.3.2 Determination of Glucose Concentration

Glucose concentration was determined by using the colorimetric and Dinitro salicylic acid (DNS) procedure. First, 1mg/ml of glucose stock solution was prepared by solving 100 mg of glucose with 100 ml of distilled water. Next, six sterilized test tube were prepared containing 0, 0.5,1.0,1.5,2.0,2.5 ml of glucose stock solution. Then, distilled water was added until each test tubes filled up to 3ml.

DNS solution was prepared by mixing 1.00g of DNS into a beaker. Added by 20 ml of 2.0 NaOH with 50 ml of distilled water. Next, 30g of Potassium sodium tartrate was added into the beaker. The solution was heated until fully dissolved and filled up to 100 ml with distilled water.

For the sample, 3ml of animal feed additive solution was mixed with 4 ml of DNS solution and kept in water bath at 70˚C for 15 minutes together with the standards until the solution of each test tubes developed into red-brown colour.

After 10 minutes, the test tubes were left to cool down and the absorbance of each standard was measured at 540 nm that is specific to glucose. After that, the spectrophotometer was set to 540 nm which is the maximum absorption wavelength of glucose with the standards and samples.

3.4 Determination of Antimicrobial activity

The antimicrobial test was conducted by using two bacteria strains which were gram negative *Escherichia coli* and gram-positive bacteria, *Bacillus subtilis*. Both bacteria strains were culture into 10 ml of nutrient broth. First. a sterile cotton swab was immersed into the *E. coli* cultured media and evenly swabbed on the nutrient agar plate Next, 4 sterile paper discs were placed on the nutrient agar plate by using sterile forceps. Then, 10µl of 0.025 mg/ml of chloramphenicol as positive control and sterile distilled water as negative control was dropped onto two paper discs. Next, 10µl of Formulation 1 and Formulation 2 was dropped onto the other two remaining paper discs. Next, the plates are kept in incubator for incubation process. The result was obtained by observing and measuring the inhibition zone.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production of Animal Feed Additives

Animal feed additives (Formulation 1) were produced from 50 ml of coconut water mixed with 150g of brown sugar with 100 ml of distilled water. The solution was stirred until fully dissolved. Then, the solution was poured into 1000ml of Schott bottle and was filled up to 1000ml of distilled water.

Meanwhile Formulation 2, 100g of rice was weight and rinsed with 100 ml of distilled water. The rice water was poured into a 1000ml Schott bottle. Then 150g of brown sugar was added into the Schott bottle. The bottle was filled with up to 1000 ml of distilled water.

Formulation 1 and 2 were kept in 2 closed Schott bottles away from the sunlight for 2 weeks as the solution turned into dark brown color and developed sweet sour smell as a result of fermentation. The pH of the solutions was recorded every two days until a constant pH value was achieved.

4.2 Isolation Of Acetic Acid Bacteria (AAB) And Lactic Acid Bacteria (LAB) From Animal Feed Additives.

4.2.1 Preparation of Media

In this study, Potato Dextrose Agar (PDA) and De Man, Rogosa and Sharpe Agar (MRS) were used to conduct isolation of AAB and LAB bacteria.

PDA was used to isolate AAB bacteria as it has properties which allows AAB to grow which is low pH level media due to low pH culture media are recommended for the routine

isolation of Acetobacter from natural or artificial habitats (Hommel & Ahnert, 1999). The preparation of PDA agar was mixed with 0.015g of Bromocresol Purple. PDA is able to cultivate a wide range of soil-found bacteria and fungus. This agar can be used to stop the growth of bacteria and/or fungi by adding acid or antibiotics. Dibromo-o-cresolsulfonphthalein, another name for bromocresol purple, is an acidic dye that is a member of the phthalein and sulphonphthalein dyes family. At pH 5.2–6.8, the dye color shifts from yellow to violet.

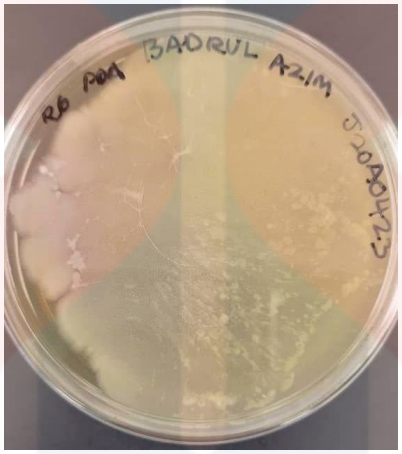


Figure 4.1: Formulation 1 on PDA plate + 0.015g Bromocresol purple

Figure 4.2: Formulation 2 on PDA plate + 0.015g of Bromocresol purple

From the observations, 0.015g of bromocresol purple was not sufficient to inhibit fungi from these formulations. As shown in Figure 4.1 and 4.2, 0.030g of bromocresol purple was used to inhibit the growth of fungi developed from these formulations to isolate AAB bacteria. Figures 4.3 and 4.4 below show no mycelium growth on either Formulation on PDA surface.

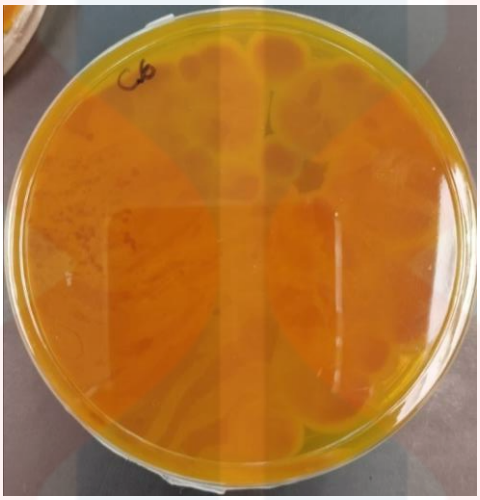


Figure 4.3: Formulation 1 on PDA plate + 0.030g Bromocresol purple

Figure 4.4: Formulation 2 on PDA plate + 0.030g Bromocresol purple

MRS agar is mainly designed to cultivate Lactobacilli from different sources. The entire class of lactic acid bacteria is cultivated with it ("de man, rogosa and sharpe (MRS) agar," 2003).

All Lactobacilli strains can grow more abundantly in the MRS medium, but strains like *L. brevis* and *L. fermenti* that have a slow and difficult growth rate can grow even more abundantly. *Lactobacillus bulgaricus* in yoghurts can be counted using MRS Agar after it has been acidified to pH 5.4. The MRS agar has a transparent amber color. The dehydrated medium ought to be beige in color, uniform, and free flowing.

4.2.2 Isolation of Microorganism from Animal Feed Additives

The isolation technique to isolate AAB and LAB bacteria from Formulation 1 and Formulation 2 was spread plate technique. One of the reasons was the spread-plate approach creates colonies that are uniformly scattered over the agar medium's surface. Individual colonies' cells can be separated and utilized for further experimental manipulations (Sanders, 2012).

This process was carried out in laminar flow to prevent contamination. In cell culture research, a sterile atmosphere is created in a laminar air flow cabin, which also secures the personnel and the samples (Uysal et al., 2018).

After the spread plate was conducted, the plates were kept in incubator 37˚C for 24 hours. Then, selected plates which showed clear colony formation were cultured again in new PDA and MRS plate.

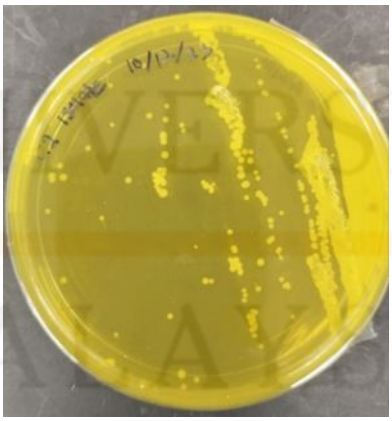


Figure 4.5: Isolation of AAB from Formulation 1 on PDA

As shown in Figure 4.5 and 4.6, 2 colonies were found in the plate. The colonies from Formulation 1 showed pale yellow color with scattered colony. The colonies are distributed in various patterns across the agar surface. Some are isolated and well-defined, while others form

lines or streaks, indicating that they may have been spread using a streaking method for isolation. These lines of colonies show a gradient of density, with individual colonies being more distinct at one end and becoming denser and confluent towards the other end, which is a common result of the streak plate technique used to isolate individual bacterial colonies.

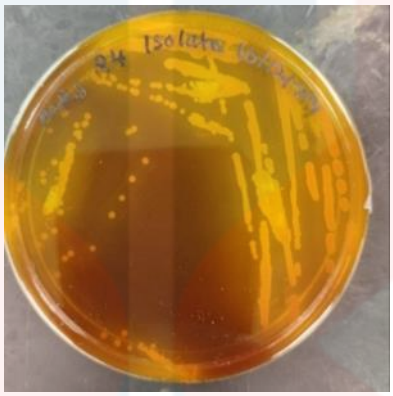


Figure 4.6: Isolation of LAB from Formulation 2 on MRS

Meanwhile, Figure 4.6 shows a dark, amber-colored agar medium and bacterial colony that are bright yellow in color. The colonies are primarily linear in arrangement, suggesting that a streaking technique was used for isolation. This technique often results in a pattern where the colonies become more isolated and less confluent as the streaking progresses, which is visible here.

The texture of the colonies appears smooth, which is common for many types of bacterial growth on agar. The colonies are mostly elongated along the streak lines, with some individual colonies that are round or oval in shape, indicating that they have grown from a single bacterial cell or a small cluster of cells.

The shape of the colonies along the streak lines is somewhat irregular, with varying widths and lengths. Some of the lines of colonies are broken, with gaps between growth, while others are continuous. The individual colonies that are not part of the streak lines are more uniform in shape, typically round, which is characteristic of many bacterial colonies when they have space to grow without merging with adjacent colonies.

The bright yellow color of the colonies stands out against the dark background of the agar, which may indicate the production of a pigment by the bacteria or could be a natural coloration of the bacterial species being cultured. The overall appearance of the plate suggests

that it was inoculated with the intention of isolating and identifying specific bacterial colonies from a sample.

4.2.3 Bacteria Identification and Gram Staining

In this study, Gram staining method was carried out to identify the microorganisms that contain in the animal feed additives. This process was conducted on each of the selected isolated plate from PDA and MRS.

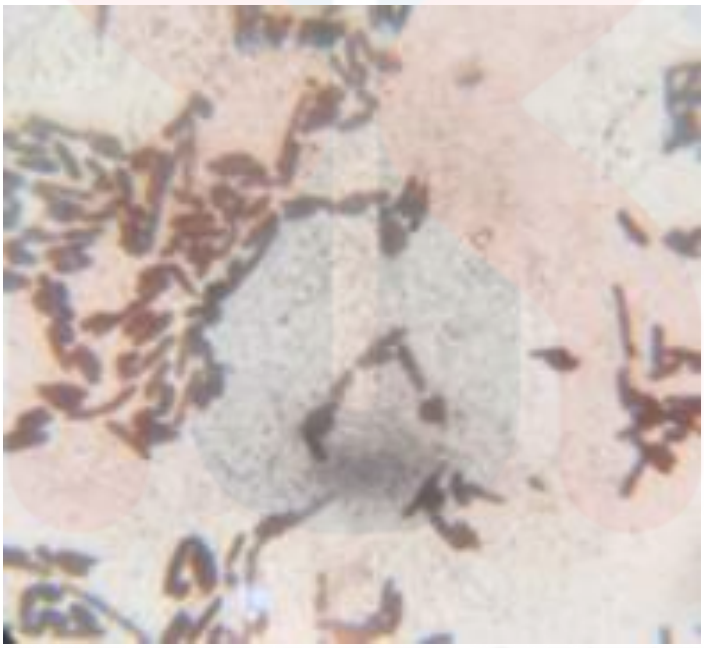


Figure 4.7: Gram staining for AAB (1000x)

Figure 4.7 shows the result of gram staining for bacteria isolated from PDA + bromocresol purple plate. When observed under microscope, the cellular morphology appeared to be red-pink color. The colour of gram-negative organisms is either red or pink (Tripathi & Sapra, 2023).

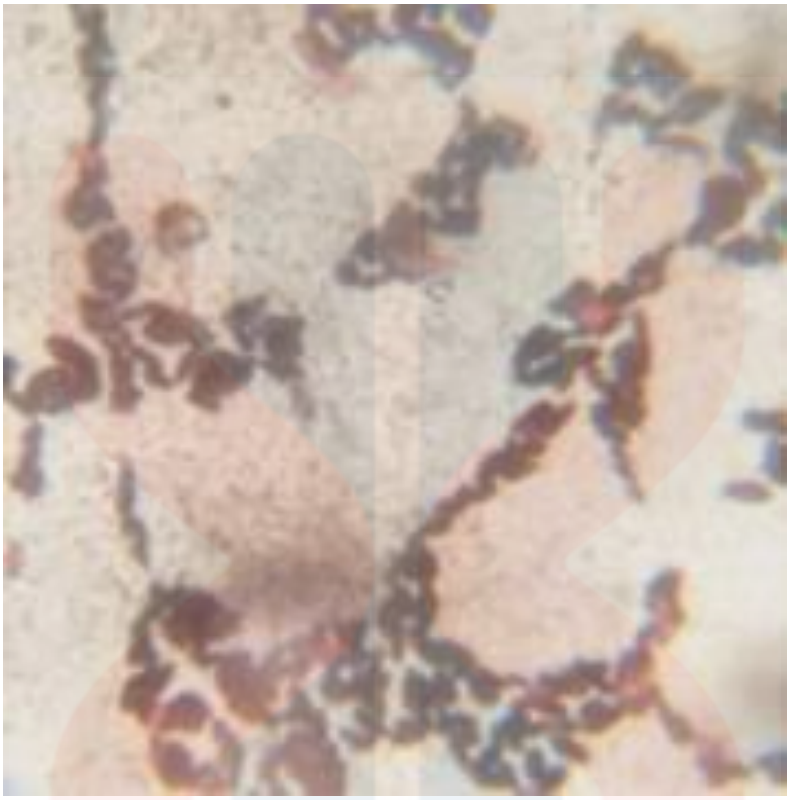


Figure 4.8: Gram staining for LAB (1000x)

The ability of the bacterial cell wall to hold onto the crystal violet dye after being treated with a solvent is the fundamental idea behind gramme staining. Gram-negative bacteria have a higher lipid content, while gram-positive bacteria have a higher peptidoglycan content.

All bacteria initially absorb crystal violet dye; however, gram-negative species' lipid coating dissolves when a solvent is used. Gramme negatives lose the primary stain when the lipid layer dissolves. On the other hand, the solvent causes the gram-positive cell walls to become dehydrated, which closes the pores and stops the violet-iodine combination from diffusing, leaving the bacteria marked. In gramme staining, the duration of decolorization is crucial since an extended exposure to a decolorizing agent can eliminate all of the stains from both kinds of bacteria (Tripathi & Sapra, 2023).

4.3 Measurement of pH

From the Table 4.1, both formulations start with the same pH of 6.3 on Day 0.The pH levels for both formulations remain stable at 6.3 on Day 2.Starting from Day 4, there is a noticeable decrease in pH for both formulations, with Formulation 1 at 4.4 and Formulation 2 at 4.3.The pH continues to decrease for both formulations until Day 10, where they both reach a pH of 3.5.On Day 12, Formulation 1 shows a slight increase in pH to 3.7, while Formulation 2 remains at 3.5.From Day 14 to Day 18, both formulations maintain a pH of 3.5.

Overall, the data indicates that both formulations experience a significant drop in pH over the first 10 days, followed by a period of relative stability in acidity levels. The slight increase in pH on Day 12 for Formulation 1 could be due to measurement variability or some other factor affecting the pH. The consistency in pH from Day 14 onwards suggests that the formulations have reached a stable acidic condition.

Table 4.1: The pH of the Animal Feed Additives for two weeks

Day	Formulation 1	Formulation 2
0	6.3	6.3
2	6.3	6.3
4	4.4	4.3
6	4.2	4.1
8	4.0	4.0
10	3.5	3.5
12	3.7	3.5
14	3.5	3.5
16	3.5	3.5
18	3.5	3.5

The graph in Figure 4.9 plots the pH value of two formulations of animal feed additives over an 18-day period. The x-axis represents the days, ranging from 0 to 18, and the y-axis represents the pH value, ranging from 0 to 7. The graph has two lines representing Formulation 1 (in orange) and Formulation 2 (in Gray), which show the following trends. Both formulations

start at a pH of 6.3 on Day 0, which indicated by the labels on the graph. The pH levels for both formulations remain constant at 6.3 on Day 2. From Day 4 onwards, there is a sharp decline in pH for both formulations, with Formulation 1 reaching a pH of 4.3 and Formulation 2 reaching a pH of 4.1 by Day 6. The decline continues until Day 10, where both formulations level off at a pH of 3.5.

From Day 12 to Day 18, the pH of both formulations remains constant at 3.5. The graph visually demonstrates the decrease in pH over time for both formulations, with a rapid decline in the first 10 days followed by a plateau. The lines for both formulations are remarkably close together, indicating that the pH trends for Formulation 1 and Formulation 2 are similar throughout the two-week period. This suggests that both formulations have a similar acidification process over the duration of the experiment.

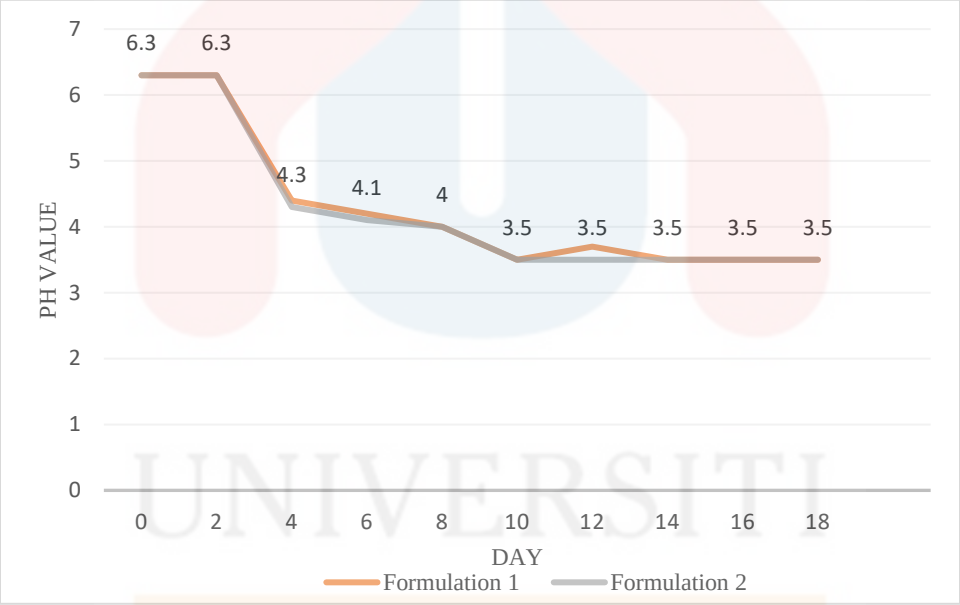


Figure 4.9: pH of the Animal Feed Additives From (-) Formulation 1 and (-) Formulation 2 for two weeks.

4.4 Biochemical Characterization of Animal Feed Additives

4.4.1 Determination of Protein Concentration

Based on the standard curve shown above, the protein concentration in mg/ml was found to be y=7.561x with a R^2 value of 0.9533. here, y-axis is the absorbance and x-axis are the BSA concentration.

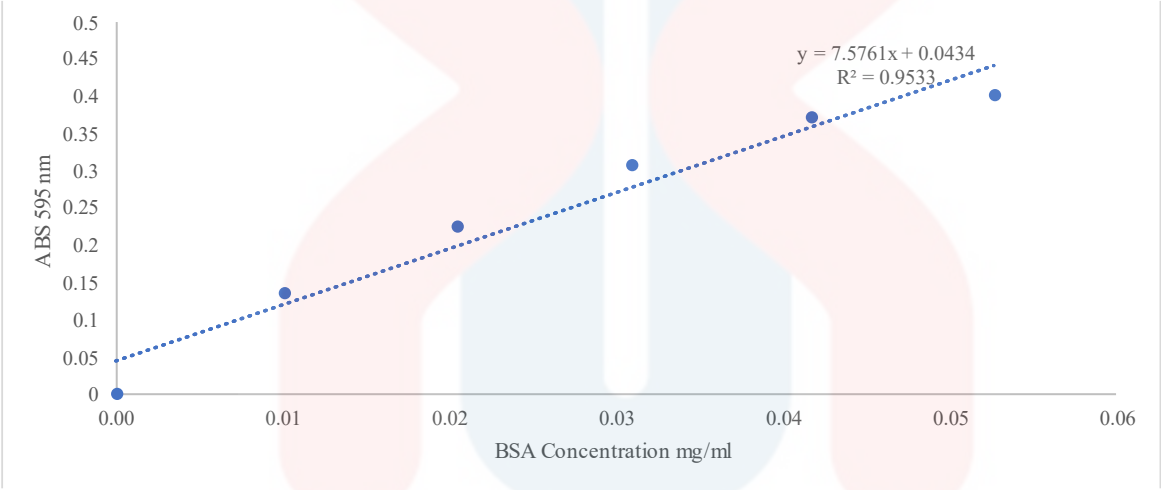


Figure 4.10: Relationship between absorbance at 595 nm and Concentration of BSA (mg/ml)

Table 4.2: The average value sample of absorbance at 595nm.

Sample	Absorbance 595nm	Average
Formulation 1	0.263	0.200
	0.261	
	0.276	
Formulation 2	0.277	0.270
	0.258	
	0.274	

The protein concentration test was carried out to measure the exact amount of protein contained in the animal feed additive produced from two probiotic sources, coconut water and rice water. Based on Figure 4.10, the protein standard curve achieved linear line which means that the protein content is best. A standard curve is used to accurately determine the concentration of sample from the signal generated by an assay. This test was carried out by using Bradford reagent. Protein concentrations can be quickly and rather sensitively determined using the Bradford test. It is predicated on the Coomassie Brilliant Blue G-250 dye's maximum absorbance shifting from 465 to 595 nm after binding to denatured proteins in solution (Kielkopf et al., 2020). The absorbance of the protein was measured at 595nm to obtain a linear line. Certain compounds or contaminants in the sample may interfere with the assay, leading to inaccurate readings. For instance, the presents of bubbles in cuvette and less accurate BSA concentration can interfere with the protein assay reagents, affecting the absorbance measurements. To overcome this issue, the cuvette must be cleaned and wiped carefully to prevent any presents of bubble with tissue. Next, the BSA stock solution must be prepared accurately to produce the correct solution concentration.

The test was conducted by preparing the samples, 1mg/ml of BSA solution. The BSA solution was prepared by weighing 0.1g of BSA powder and 100 ml of distilled water. The mixture was stirred gently to prevent any bubble from occurring until fully dissolved.

Table 4.3: Preparation of Protein concentration

BSA Concentration (mg/ml)	BSA (µl)	Pbs (µl)	Abs 595nm
0.00	0.00	500.00	0
0.01	5.00	495.00	0.135
0.02	10.00	490.00	0.225
0.03	15.00	485.00	0.307
0.04	20.00	480.00	0.371
0.05	25.00	475.00	0.402

From Table 4.3 above, six samples were prepared including blanks. 3ml of Bradford reagent was added into each test tube. After that, the tubes were left for 5 minutes to let the reaction occur. Then, the absorbance was measured at 595 nm.

The calibration process in a protein concentration test is a critical step that allows for the accurate determination of protein concentrations in samples. The absorbance readings obtained from the standards are plotted against the known concentrations of the standards. This created a standard curve, which was a graphical representation of the relationship between absorbance and protein concentration. Once the standard curve is created, a regression analysis was performed to determine the mathematical relationship between absorbance and protein concentration. This analysis helps to define the equation of the line or curve and allows for the interpolation of protein concentrations in unknown samples based on their absorbance readings. From the equations of $y = mx + c$, the protein concentrations can be calculated.

Table 4.4 : Protein Concentration.

Sample	Formulation 1	Formulation 2
Protein Content	0.092 mg/ml	0.02997 mg/ml

From the calculation shown in Table 4.4, Formulation 1 contains more protein compared to formulation 2 which was 0.082 mg/ml meanwhile formulation 2 contains 0.03 mg/ml of protein. Formulation 1 was produced from coconut water meanwhile Formulation 2 was produced from rice water. When measured on either fresh or dry samples, the total protein, Non

Protein Nitrogen (NPN), and true protein levels of young coconut water are higher than those of mature coconut water (Sinaga et al., 2015). Besides, rice water has less amount of protein mainly because Formulation 2 was produced by rinsing rice with water. Hence, rinsed rice water was used as probiotic source for this study. The solution was not edible for humans as it is tasteless, and the texture is cloudy. Meanwhile, a long-standing tropical beverage, coconut water (Cocos nucifera L.) has seen a steady rise in demand in recent years on the global market (Prades et al., 2012). Therefore, coconut water is edible and nutritious to consume and categorized as a beverage.

4.4.2 Determination of Glucose Concentration

Based on the standard curve shown above, the glucose concentration in mg/ml was found to be y=1.7953x with R^2 value of 0.9395. here, y-axis is the absorbance meanwhile x-axis is the concentration.

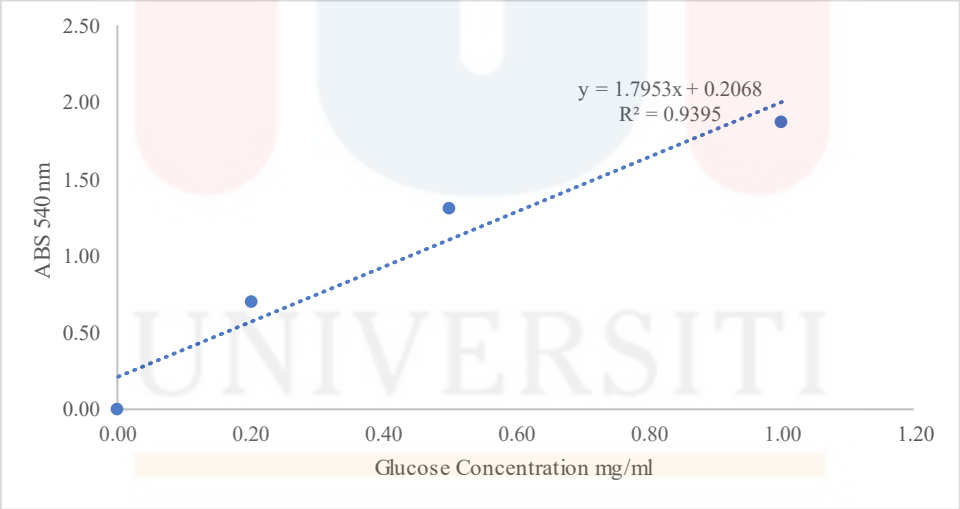


Figure 4.11: Relationship between absorbance at 540 nm and Concentration of Glucose (mg/ml)

Glucose concentration test was conducted to determine the amount of glucose produced from the animal feed additive solution produced from two probiotic sources, coconut water and rice water. From the Figure 4.11 above, a linear line was achieved. The determination of glucose concentration was conducted by using DNS assay. The DNS method is based on the reduction of

3,5-dinitrosalicylic acid (DNS) by reducing sugars such as glucose. In the presence of reducing sugars, DNS is reduced to 3-amino-5-nitrosalicylic acid, which forms a coloured product with an absorbance maximum at around 540 nm. The intensity of the colour is directly proportional to the concentration of reducing sugars in the sample. The concentration of glucose in the sample is determined by comparing its absorbance to the standard curve. Higher absorbance values indicate higher concentrations of glucose in the sample.

The DNS (3,5-dinitrosalicylic acid) method offers several advantages for the detection and quantification of reducing sugars, including glucose. Firstly, it is a relatively simple and rapid assay, making it practical for use in various laboratory settings. The method is also sensitive, capable of detecting low concentrations of reducing sugars, which is particularly useful when analysing samples with low glucose content. Moreover, the reagents required for the DNS method are generally stable and inexpensive, contributing to its cost-effectiveness. Additionally, the DNS method can detect a wide range of reducing sugars beyond glucose, such as maltose and fructose, providing versatility in its applications.

Table 4.5: Preparation of Glucose standard curve

Glucose concentration (mg/ml)	Glucose (µl)	Distilled water (µl)	abs 540 nm
0.00	0	3.0	0.00
0.20	0.5	2.5	0.70
0.50	1.0	2.0	1.31
1.00	1.500	1.5	1.87

From the Table 4.5, 4 test tube were prepared and each tube was added 3ml of DNS assay. the composition of DNS reagent consisted of 1.0 g of Dinitrosalicyclic Acid, Rochelle salt, 20 ml of 2.0 NaOH and 30g of Potassium Sodium Tartrate. Then, the solution was diluted with 100 ml of distilled water. Hence, samples for glucose concentration consisted of 3 ml of Formulation 1 and 2 added by 4 ml of DNS reagent.

Table 4.6: The average value sample of absorbance at 540nm.

Sample	Absorbance 540nm	Average
Formulation 1	2.410	2.394
	2.378	
Formulation 2	2.420	2.444
	2.467	

Table 4.7: Glucose Concentration.

Sample	Formulation 1	Formulation 2
Glucose Content	1.22 mg/ml	1.25 mg/ml

From the Table 4.7, both formulations contained approximately same glucose concentration, Formulation 1 has 1.22 mg/ml of glucose meanwhile Formulation 2 has 1.25 mg/ml of glucose which was slightly high. During fermentation process, microorganisms use glucose as a substrate for energy production. Glucose is broken down through a series of enzymatic reactions in pathways such as glycolysis or the pentose phosphate pathway. These pathways convert glucose into intermediate metabolites, which are further metabolized to generate ATP (adenosine triphosphate), the cell's primary energy source. As glucose is metabolized through fermentation pathways, various byproducts are produced depending on the microorganism and the environmental conditions.

While fermentation primarily involves the breakdown of glucose, some microorganisms have the ability to regenerate glucose through metabolic pathways such as gluconeogenesis. Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors such as amino acids, glycerol, or lactate. This process allows certain microorganisms to replenish glucose reserves and maintain energy production during prolonged periods of nutrient deprivation. When there is not enough hexose available to the bacteria, some of which are E. coli, they use gluconeogenesis to synthesise glucose from non-sugar C2 or C3 molecules or the intermediates of the tricarboxylic acid (TCA) cycle (Anderson & Cooper, 1969).

4.5 Antimicrobial Activity

This test was conducted to determine whether animal feed additives solution able to inhibit the growth of the bacteria or fungi.

Two strains of bacteria were used in this test which are *Escherichia coli* and *Bascillus Subtilis. B. subtilis* is a rod-shaped, aerobic, Gram-positive bacterium that grows quickly. Its cells are usually 1-6 μm long and slightly less than 1 μm in diameter. About 30 to 35 C is the ideal growing temperature, resulting in a doubling time of as low as 20 minutes (Errington & Aart, 2020). Also, Gram-negative *Escherichia coli* is a rod-shaped bacterium that belongs to the Gammaproteobacteria class and family Enterobacteriaceae. One of the bacteria that has been thoroughly examined is Escherichia coli. Under ideal growth conditions, Escherichia coli can grow quickly, replicating in about 20 minutes (Jang et al., 2017).

The antimicrobial test for this study was carried out by using 0.01 ml of 0.025 mg/ml of Chloramphenicol as the antibiotic (positive control) and 0.01ml of sterile distilled water (negative control). Meanwhile 0.01ml of samples (Formulation 1 and 2). *E. coli* and *Bacillus Subtilis* were cultured in nutrient broth.

This process was conducted under laminar flow to minimize the risk of contamination occur. By using sterile cotton swab, the swab was dipped into the *E. coli* culture and spread evenly onto the nutrient agar surface. After that, sterile paper discs were placed using forceps. 0.01ml of each sample and controls were placed to prevent over-flow at the paper disc.

Antimicrobial properties refer to the ability of a substance to inhibit the growth of microorganisms or to kill them outright. These properties are valuable in various contexts, including medicine, food preservation, agriculture, and personal care products. From this study, AAB and LAB that presents in coconut and rice water has the ability to inhibit microbial growth, E. coli. From Table 3.5 and 3.6 above, inhibition zone of Formulation 1 and 2 shows 0.8 and 0.9 cm approximately on bacteria strain, *E. coli.*

Table 4.8: Antimicrobial activity of Formulation 1

Sample	**Test organism**	
	E. coli	***B. subtilis***
Positive control	3.75 cm	3.15 cm
Formulation 1	0.8 cm	-

Table 4.9: Antimicrobial activity for Formulation 2

Sample	**Test organism**	
	E. coli	***B. subtilis***
Positive control	3.75 cm	3.15 cm
Formulation 2	0.9 cm	-

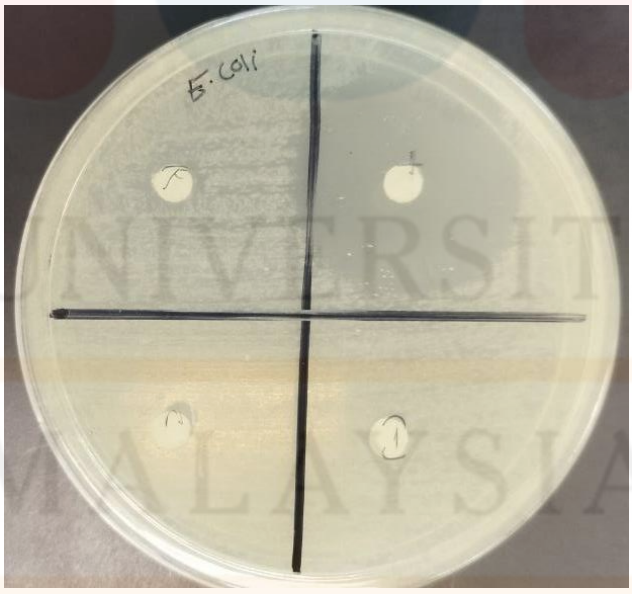


Figure 4.12: Antimicrobial test on *E.coli* strain.

These antimicrobial characteristics of LAB resulted from competition for nutrients and the synthesis of one or more antimicrobial active metabolites, including hydrogen peroxide, organic

acids (mostly lactic and acetic acid), and other substances including bacteriocins and antifungal peptides. Because of their ability to prevent foodborne pathogens and spoiling while extending the shelf life of food, probiotic LAB has been receiving a lot of attention for their significant contribution to food preservation (J. A. Reis et al., 2012).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

On the basis of the results, it is concluded that animal feed additives can be formulated by using coconut and rice water. This study also succeeded in providing insight into microbial populations responsible for producing animal feed additives from coconut and rice water thus demonstrating the potential of acetic acid bacteria as a candidate for industrial applications. Protein and glucose content were also determined. The results proved that Formulation 1, animal feed additives from coconut water contain more protein compared to animal feed additives from rice water which is Formulation 2. The findings of this study can be developed into more efficient and cost-effective methods which can be used in producing or processing animal food. However, further research is needed to optimize the condition of animal feed additives production.

5.2 Recommendations

Further research is needed to improve the production of animal feed additives. It would be beneficial to study the potential of other sources of substances in producing animal feed additives. This may include the usage of other waste materials such as left over fruit, bamboo leaves and dried leaves. More study is needed to study the stability, shelf life and safety of animal feed additives. This can be achieved by studying the formulations activity over time and analyzing the potential risk associated with using the enzymes in industrial applications.

REFERENCES

Agregán-Pérez, R., Alonso-González, E., Mejuto, J. C., & Pérez-Guerra, N. (2021). Production of a Potentially Probiotic Product for Animal Feed and Evaluation of Some of Its Probiotic Properties. *International Journal of Molecular Sciences, 22*(18), 10004. https://www.mdpi.com/1422-0067/22/18/10004

Anderson, A., & Cooper, R. A. (1969, Jul). Gluconeogenesis in Escherichia coli The role of triose phosphate isomerase. *FEBS Lett, 4*(1), 19-20. https://doi.org/10.1016/0014-5793(69)80184-5

Arsène, M. M. J., Davares, A. K. L., Andreevna, S. L., Vladimirovich, E. A., Carime, B. Z., Marouf, R., & Khelifi, I. (2021, Feb). The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics. *Vet World, 14*(2), 319-328. https://doi.org/10.14202/vetworld.2021.319-328

Buts, J. (2004). Exemple d'un médicament probiotique: Saccharomyces boulardii lyophilisé. *Flore microbienne intestinale. John Libbey Eurotext, Montrouge, France*, 221-244.

Chaucheyras-Durand, F., & Durand, H. (2010). Probiotics in animal nutrition and health. *Beneficial microbes, 1*(1), 3-9.

de man, rogosa and sharpe (MRS) agar. (2003). In J. E. L. Corry, G. D. W. Curtis, & R. M. Baird (Eds.), *Progress in Industrial Microbiology* (Vol. 37, pp. 511-513). Elsevier. https://doi.org/https://doi.org/10.1016/S0079-6352(03)80066-8

Deng, Z., Hou, K., Zhao, J., & Wang, H. (2021, 2021/12/14). The Probiotic Properties of Lactic Acid Bacteria and Their Applications in Animal Husbandry. *Current Microbiology, 79*(1), 22. https://doi.org/10.1007/s00284-021-02722-3

Errington, J., & Aart, L. T. V. (2020, May). Microbe Profile: Bacillus subtilis: model organism for cellular development, and industrial workhorse. *Microbiology (Reading), 166*(5), 425-427. https://doi.org/10.1099/mic.0.000922

Hommel, R. K., & Ahnert, P. (1999). ACETOBACTER. In R. K. Robinson (Ed.), *Encyclopedia of Food Microbiology* (pp. 1-7). Elsevier. https://doi.org/https://doi.org/10.1006/rwfm.1999.0005

Jang, J., Hur, H.-G., Sadowsky, M. J., Byappanahalli, M. N., Yan, T., & Ishii, S. (2017). Environmental Escherichia coli: ecology and public health implications—a review. *Journal of Applied Microbiology, 123*(3), 570-581. https://doi.org/https://doi.org/10.1111/jam.13468

Khalid, F., Khalid, A., Fu, Y., Hu, Q., Zheng, Y., Khan, S., & Wang, Z. (2021, 2021/07/01). Potential of Bacillus velezensis as a probiotic in animal feed: a review. *Journal of Microbiology, 59*(7), 627-633. https://doi.org/10.1007/s12275-021-1161-1

Kielkopf, C. L., Bauer, W., & Urbatsch, I. L. (2020, Apr 1). Bradford Assay for Determining Protein Concentration. *Cold Spring Harb Protoc, 2020*(4), 102269. https://doi.org/10.1101/pdb.prot102269

Kim, S. K., Guevarra, R. B., Kim, Y. T., Kwon, J., Kim, H., Cho, J. H., Kim, H. B., & Lee, J. H. (2019, Sep 28). Role of Probiotics in Human Gut Microbiome-Associated Diseases. *J Microbiol Biotechnol, 29*(9), 1335-1340. https://doi.org/10.4014/jmb.1906.06064

Ørskov, E. R. (1977). Capacity for Digestion and Effects of Composition of Absorbed Nutrients on Animal Metabolism. *Journal of Animal Science, 45*(3), 600-608. https://doi.org/10.2527/jas1977.453600x

Pandey, A. K., Kumar, P., & Saxena, M. J. (2019). Feed Additives in Animal Health. In R. C. Gupta, A. Srivastava, & R. Lall (Eds.), *Nutraceuticals in Veterinary Medicine* (pp. 345-362). Springer International Publishing. https://doi.org/10.1007/978-3-030-04624-8_23

Prades, A., Dornier, M., Diop, N., & Pain, J.-P. (2012). Coconut water uses, composition and properties: a review. *Fruits, 67*(2), 87-107.

Reis, J., Paula, A., Casarotti, S., & Penna, A. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews, 4*, 124-140.

Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012, 2012/06/01). Lactic Acid Bacteria Antimicrobial Compounds: Characteristics and Applications. *Food Engineering Reviews, 4*(2), 124-140. https://doi.org/10.1007/s12393-012-9051-2

Sanders, E. R. (2012, May 11). Aseptic laboratory techniques: plating methods. *J Vis Exp*(63), e3064. https://doi.org/10.3791/3064

Servin, A. L. (2004). Antagonistic activities of Lactobacilli and bifidobacteria against microbial pathogens. *FEMS microbiology reviews, 28*(4), 405-440.

Shurson, G. C. (2018, 2018/01/01/). Yeast and yeast derivatives in feed additives and ingredients: Sources, characteristics, animal responses, and quantification methods. *Animal Feed Science and Technology, 235*, 60-76. https://doi.org/https://doi.org/10.1016/j.anifeedsci.2017.11.010

Sinaga, S. M., Margata, L., & Silalahi, J. (2015). Analysis of total protein and non protein nitrogen in coconut water and meat (Cocos Nucifera L.) by using Kjeldahl method. *Int. J. Pharm. Tech. Res, 8*, 551-557.

Solieri, L., & Giudici, P. (2009). Vinegars of the World. In L. Solieri & P. Giudici (Eds.), *Vinegars of the World* (pp. 1-16). Springer Milan. https://doi.org/10.1007/978-88-470-0866-3_1

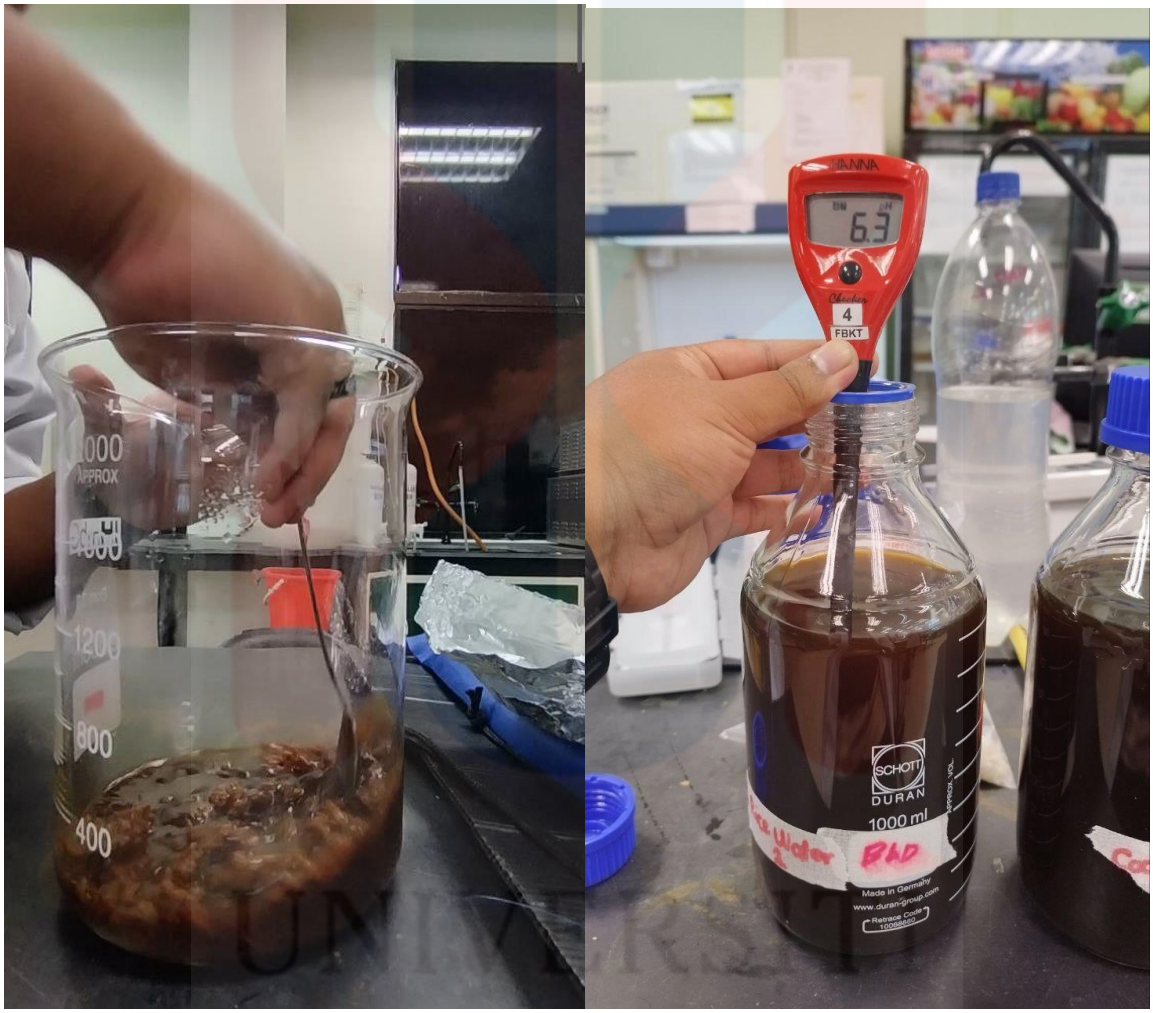
Soomro, A., Masud, T., & Anwaar, K. (2002). Role of lactic acid bacteria (LAB) in food preservation and human health-a review. *Pakistan Journal of Nutrition, 1*(1), 20-24.

Tripathi, N., & Sapra, A. (2023). Gram Staining. In *StatPearls*. StatPearls Publishing

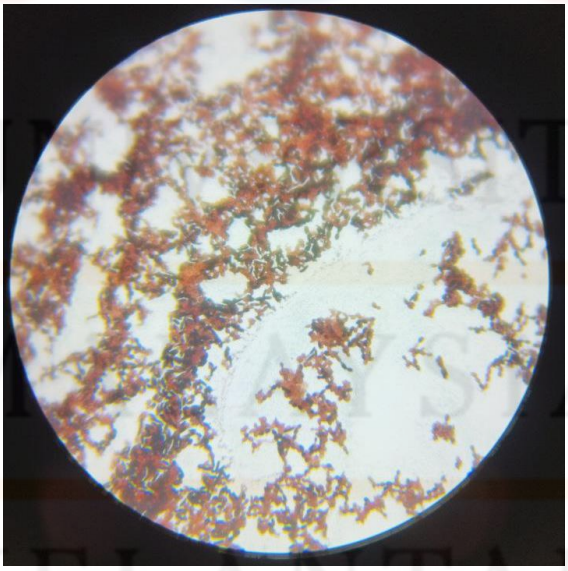
Uysal, O., Sevimli, T., Sevimli, M., Gunes, S., & Eker Sariboyaci, A. (2018). Chapter 17 - Cell and Tissue Culture: The Base of Biotechnology. In D. Barh & V. Azevedo (Eds.), *Omics Technologies and Bio-Engineering* (pp. 391-429). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-804659-3.00017-8

Woyengo, T. A., Beltranena, E., & Zijlstra, R. T. (2014). NONRUMINANT NUTRITION SYMPOSIUM: Controlling feed cost by including alternative ingredients into pig diets: A review1,2. *Journal of Animal Science, 92*(4), 1293-1305. https://doi.org/10.2527/jas.2013-7169

APPENDIX A



APPENDIX B



APPENDIX C