

ANTIMICROBIAL ACTIVITY OF COFFEE HUSK EXTRACTS AGAINST *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM IN MICROBIOLOGICAL MEDIA AND MILK

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ABSTRACT

Pathogenic bacteria pose significant challenges to food safety in the United States and globally. *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* are among the pathogens that frequently trigger foodborne illness outbreaks, leading to substantial economic losses and public health burdens. Consumption of food contaminated by these bacteria, such as raw milk, can result in various gastrointestinal symptoms, ranging from mild discomfort to severe, life-threatening conditions. With the rise of antibiotic-resistant strains, it is crucial to explore alternative antimicrobial agents.

Coffee, particularly the species *Coffea arabica* (yellow) and *Coffea liberica* (red) grown in Hawaii, represents a significant cash crop. The coffee cherries, the fruit of coffee trees, is considered a superfood due to its skin and juice, rich in bioactive compounds. However, the wet coffee seed comprises only approximately 55% of the fruit, leaving the other half of the coffee fruit (coffee husk) underutilized or considered a by-product of coffee production. This study aimed to assess the antimicrobial efficacy and mechanism of action of coffee husk against *S. Typhimurium* and *L. monocytogenes*.

Coffee husk samples were obtained from the Island of Hawai'i and O'ahu, and bioactive compounds were extracted using 80% acidified methanol. The total phenolic content of the extracts was quantified, and other chemical analyses, such as pH, sugar content, titratable acidity, and anthocyanin content, were conducted. The pH values of red and yellow coffee husk extracts were 4.10 and 4.18, respectively. The sugar and titratable acidity ratios for red and yellow coffee husk extracts were 9.60/1.55 °Brix/% and 8.87/1.41 °Brix/%, respectively. The total phenolic content of red and yellow coffee husk extracts was 5058.27 GAEmg/L and 4600.27 GAEmg/L,

respectively. The anthocyanin content of the red coffee husk extract was 22.03 mg/mL, significantly higher than that of the yellow coffee husk extract, which was 0.06 mg/mL.

Subsequent evaluations revealed that the red and yellow coffee husk extracts had a minimum inhibitory concentration (MIC) of 12.5 mg/mL and a minimum bactericidal concentration (MBC) of 50 mg/mL against *S. Typhimurium* and *L. monocytogenes*. Notably, probiotic strains *Lactococcus lactis* and *Lacticaseibacillus rhamnosus* GG grew even in the presence of 50 mg/mL coffee husk extracts. Biofilm assays demonstrated that 6.25 mg/mL (1/2 MIC) red and yellow coffee husk extracts significantly reduced *S. Typhimurium* levels in formed biofilm by 0.53 log and 0.42 log, respectively. However, coffee husk extracts at this concentration did not affect biofilm formation by *L. monocytogenes*.

Both coffee husk extracts at 12.5 mg/mL (MIC) caused significant damage to the cell membrane of *L. monocytogenes* and *S. Typhimurium*. The cell membrane damage to *S. Typhimurium* caused by 25 mg/mL (2 MIC) red and yellow coffee husk extracts was comparable to the positive control treated with 70% ethanol. Additionally, this study demonstrated significant protein leakage in both *L. monocytogenes* and *S. Typhimurium* when treated with red and yellow coffee husk extracts at 12.5 mg/mL. Notably, the intracellular protein leakage caused by 25 mg/mL red coffee husk extract in *L. monocytogenes* and *S. Typhimurium* was significantly higher than that caused by 70% ethanol. This correlation between loss of membrane integrity and leakage of intracellular proteins confirmed the detrimental effects of coffee husk extracts on pathogenic bacterial cells.

Finally, the antimicrobial properties of coffee husk extracts were evaluated in milk artificially contaminated with *L. monocytogenes* and *S. Typhimurium*. Comparing the concentration of bacteria in treated milk with the initial concentration of the control group, neither

red nor yellow coffee husk extract at 25 mg/mL showed a significant effect on *L. monocytogenes* or *S. Typhimurium* in milk stored at 7°C, whereas 12.5 mg/mL red and yellow coffee husk extracts reduced the growth of these pathogenic bacteria in milk at 21°C. Moreover, both coffee husk extracts at 25 mg/mL completely suppressed *L. monocytogenes* and *S. Typhimurium* in milk during storage for up to 72 hours.

In conclusion, coffee husk extracts exhibit strong antimicrobial effects on *L. monocytogenes* and *S. Typhimurium* but not on tested probiotic bacteria. The extracts can damage the cell membrane of these pathogenic bacteria and cause the leakage of intracellular proteins. They can also reduce biofilm formation by *S. Typhimurium*. Coffee husk extracts offer a potent and natural alternative to traditional food preservatives for enhancing the microbiological quality and safety of food.

Keywords

antimicrobial effect; biofilm formation; cell membrane integrity; coffee husk; polyphenols; *Listeria monocytogenes*; milk preservation; protein leakage; *Salmonella Typhimurium*

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Chapter 1

INTRODUCTION

Food safety is a critical global concern, with pathogens such as *S. Typhimurium* and *L. monocytogenes* posing significant health risks. The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* causes 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually. Common symptoms include diarrhea, vomiting, fever, and abdominal cramps (CDC, 2024a). Similarly, *Listeria* infections can lead to severe outcomes, particularly among vulnerable populations such as pregnant women, newborns, the elderly, and immunocompromised individuals. Approximately 1,600 individuals contract listeriosis every year in the U.S., resulting in 260 deaths. Listeriosis usually causes fever, muscle aches, and gastrointestinal issues, with severe cases leading to deadly meningitis and septicemia (CDC, 2024b). For pregnant women, *Listeria* infections can cause miscarriage and stillbirth (Z. Wang et al., 2021).

S. Typhimurium can reside in the intestinal tract of farm animals and be released into various natural environments, such as soil and water sources. About 95% of human infections with *S. Typhimurium* are foodborne (Foley & Lynne, 2008). Therefore, understanding how *S. Typhimurium* appears and spreads among food items is crucial. This pathogen can infect humans through the consumption of contaminated water. Additionally, foods of animal origin, such as meats, eggs, and milk, are common vehicles for this pathogen. *S. Typhimurium* can adhere to vegetables and fruits during their growth, especially when irrigated with contaminated water or fertilized with inadequately composted manure. This pathogen can also contaminate other food products through contact with humans or animals (Fatica & Schneider, 2011). *S. Typhimurium* can grow in both aerobic and anaerobic conditions, making it a resilient pathogen. (Semenov et al., 2011).

L. monocytogenes, on the other hand, is widely distributed in nature. It is notable for its ability to grow at refrigeration temperatures and possesses high salt tolerance, making it a significant concern in ready-to-eat and processed foods (Bonanno et al., 2024). *L. monocytogenes* has a natural advantage over other bacteria due to its cold response mechanisms, allowing it to quickly adjust cell permeability and alter the composition of intracellular solutes at low temperatures. The primary mechanism involves the production of several cold shock proteins (Csps and Caps), which promote the formation of biofilms, thereby increasing its survival under stressful conditions (Tasara & Stephan, 2006). Moreover, *L. monocytogenes* can survive in 7 M NaCl for over 20 hours, posing a significant challenge for ensuring the safety of cured meat products (D. Liu et al., 2005). *L. monocytogenes* can persist in food processing environments due to its ability to form biofilms. This can lead to equipment contamination and repeated outbreaks. Commercially available acidic and alkaline sanitizing solutions and enzyme preparations are ineffective against *L.* biofilms that have grown for four days, with 75% alcohol being the only effective solution. This indicates that once *L.* biofilms form, it is difficult to eliminate them (Fagerlund et al., 2020).

Raw milk is susceptible to contamination by both *S. Typhimurium* and *L. monocytogenes*. These bacteria, present in water, soil, and grass feed, may be transmitted to cows and enter raw milk during dairy production. Despite the risks, retail sales of raw milk are legal in 13 states in the U.S., with many other states permitting raw milk sales on farms (*Raw Milk Laws by State*, n.d.). Foodborne illness outbreaks have been linked to unpasteurized milk, which can harbor these pathogens. Between 2015 and 2019, more than 75% of *L. monocytogenes* outbreaks were associated with contaminated dairy products and fruits (The Interagency Food Safety Analytics Collaboration, 2021). Consuming raw milk or derived soft cheese produced under unsanitary

conditions can lead to severe health issues. Moreover, bacterial resistance to conventional intervention methods poses a significant challenge. Biofilms formed by *L. monocytogenes* can contribute to microbial contamination and infection, necessitating more stringent approaches to sanitation and disinfection (Carpentier & Cerf, 2011). This underscores an urgent need for effective food preservation methods.

Food preservatives are widely used in food production, including antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and citric acid, as well as antimicrobial agents like sorbic acid and nitrite. These preservatives effectively extend the shelf-life of food. For instance, sorbic acid, in concentrations ranging from 0.02% to 0.3%, can inhibit Gram-negative, Gram-positive, aerobic, and anaerobic bacteria (Davidson et al., 2005). However, traditional preservatives are increasingly scrutinized for their potential health impacts. For example, sodium benzoate, deemed safe by regulatory agencies, has been linked to potential food allergies, attention deficit, and brain damage (Hamid et al., 2012). Additionally, the rise of antimicrobial-resistant microorganisms has attracted attention as a major public health threat (Grudlewska-Buda et al., 2023). When antimicrobial agents are used, they create selective pressure on microbial populations. This means that susceptible microorganisms are killed or inhibited, while those with resistance mechanisms survive and proliferate. Over time, this selective pressure can lead to an increase in the proportion of resistant microorganisms in a given environment (CDC, 2024a).

This growing concern has led to a search for natural food preservatives. Grapefruit seed extract has been found to reduce *Salmonella* and *L. monocytogenes* by 2.5 and 2.8 log, respectively (Xu et al., 2007). Ohelo berry juice had a minimum inhibitory concentration and a minimum bactericidal concentration of 12.5% and 50%, respectively, against *L. monocytogenes* (Wu et al.,

2022). Numerous studies have shown that polyphenols, a class of phytochemicals found in various plants, possess significant antimicrobial properties (Daglia, 2012). For example, the extract of the stem of sweet cherry fruit, with 32.49 mg/g polyphenols, can suppress Gram-positive *Staphylococcus aureus* and *Enterococcus faecalis* (Afonso et al., 2020). Polyphenols can inhibit bacterial growth or even kill bacteria through multiple mechanisms. They may disrupt bacterial cell membranes, interfere with cell energy production, alter cell morphology, and inhibit essential enzymes, making them a promising natural alternative for food preservation (Ning et al., 2022).

Coffee is one of the most consumed beverages globally, valued for its refreshing properties and health benefits. Essential components of coffee include caffeine, chlorogenic acid, and diterpenes. Coffee significantly affects the cardiovascular system, carbohydrate, and lipid metabolism. Additionally, coffee consumption is associated with a reduced incidence of diabetes and liver diseases. In neurological disorders, there appears to be a protective effect against Parkinson's disease. The impact of coffee on cancer risk depends on the specific tissue involved, although it generally contributes to a reduced risk (Cano-Marquina et al., 2013).

Hawaii coffee utilized production totaled 19.2 million pounds (cherry basis) in 2023-2024, contributing significantly to the local economy and tourism (USDA, 2024). However, appropriately 4.1 million pounds of coffee cherries were harvested but not utilized for production due to damage caused by weather events or coffee berry borer and other pests. Moreover, coffee production generates a massive amount of by-products, particularly coffee husk. Coffee husk occupies half the weight of the whole coffee fruit and is rich in nutrients and polyphenols (Cangussu et al., 2021). The total polyphenol content in coffee husk ranges from 489.5 GAE/g to 1809.9 GAE/g, depending on the source (Geremu et al., 2016). The primary classes of polyphenols in coffee husk include flavonoids and phenolic acids (Sangta et al., 2021). These polyphenols hold

great antioxidant and antimicrobial potential. Given the microbial hazards associated with raw milk and the limitations of traditional preservatives, exploring the use of coffee husk extracts to control pathogenic bacteria is timely and holds significant promise for food safety. The inhibitory ability of coffee husk extracts varies widely, showing 10-12mm inhibition zones of 300 mg/mL extract against *Staphylococcus aureus* and *Escherichia coli*, while showing no significant antimicrobial activity against *Staphylococcus aureus* and *S. Typhimurium* (Duangjai et al., 2016; N. S. Ismail et al., 2020).

The objectives of this study were to investigate the antimicrobial properties of red (*Coffea liberica*) and yellow (*Coffea arabica*) coffee husks, understand the mode of action of their antimicrobial activity and evaluate their antibacterial efficacy in milk beverages. By focusing on these goals, this research aimed to contribute to developing natural food preservatives that could mitigate the risks associated with foodborne pathogens, particularly in dairy products.

Chapter 2

LITERATURE REVIEW

2.1 Bioactive Compounds in Coffee Cherries

2.1.1 Introduction

Coffee is one of the most popular beverages worldwide, providing significant economic benefits. According to the International Coffee Organization (ICO), the production and consumption of coffee in the 2022-2023 period were 168.2 million and 173.1 million 60-kg bags, respectively (ICO, 2023). Beyond its economic impact, coffee offers numerous health benefits. It is widely appreciated for its refreshing and invigorating qualities.

Coffee cherry contains various bioactive compounds, such as caffeine and polyphenols, which contribute to its health-promoting properties. These compounds are known for their antioxidative effects, helping protect cells from damage caused by free radicals. The antimicrobial properties of coffee cherry can inhibit the growth of harmful microorganisms, thereby reducing the risk of infections and intoxications (Mesfin et al., 2022). Coffee's hypoglycemic effects assist in regulating blood sugar levels, making it potentially beneficial for individuals with diabetes (Martina et al., 2019). Additionally, coffee can help reduce inflammation in the body, lowering the risk of chronic diseases (Nehlig, 2022). Furthermore, the anticancer properties of coffee have been linked to a lower risk of certain types of cancer; for example, research has shown that drinking one cup of coffee brings favorable effects for liver cancer (Alicandro et al., 2017). Coffee can enhance the body's release of anti-inflammation cytokines and protect consumers against various autoimmune diseases. However, patients with autoimmune conditions like rheumatoid arthritis should be aware that coffee could interact with the medications and decrease their efficacy (Sharif et al., 2017). Its cardioprotective effects contribute to regulating vascular tone, improving glucose

metabolism, increasing reverse cholesterol transport, inhibiting foam cell formation, reducing oxidative stress, modulating the immune system, and impacting platelet function (Bøhn et al., 2012). Lastly, the anti-aging properties of coffee help maintain skin health and slow signs of aging (Saewan, 2022).

In Hawaii, coffee is the second most valuable agricultural commodity. Species such as *Coffea liberica* and *Coffea arabica* have thrived after being introduced to Hawaii due to the region's conducive climate and fertile volcanic soil on five islands of Hawai'i, especially Hawai'i Island (the Big Island). The annual yield of coffee in Hawaii is approximately 19.2 million pounds (USDA, 2024), contributing significantly to the local economy. The history of coffee cultivation in Hawaii dates back to the early 19th century, around 1825 (Melillo, n.d.). Today, Kona Coffee, cultivated in the Kona District on the Big Island, has become a cultural symbol and a hallmark of Hawaiian agriculture.

2.1.2 Phytochemicals in Coffee Cherries

Phytochemicals, also referred to as phytonutrients, are bioactive compounds synthesized by plants that are crucial for their growth, development, and defence mechanisms. The primary phytochemicals in coffee cherries are organic acids and phenolic compounds. These chemicals contribute significantly to the flavor, aroma, and potential health benefits of coffee cherries.

2.1.2.1 Organic Acids

Organic acids are a vital class of phytochemicals in coffee cherries, characterized by the presence of one or more carboxyl groups (-COOH). These compounds play a pivotal role in maintaining the internal pH environment of plant tissues, essential for enzymatic activities and metabolic processes. By regulating pH, organic acids contribute to optimal conditions for nutrient absorption and overall plant vitality.

In coffee cherries, organic acids such as citric acid, oxalic acid, malic acid, maleic acid, and tartaric acid in coffee bean contribute to the beverage's distinctive flavor profile (Yeager et al., 2023). Their acidic properties influence the sensory attributes of coffee, imparting tanginess and complexity. Additionally, organic acids possess antimicrobial activity and can inhibit the growth of bacteria, yeasts, and molds, thereby extending the shelf life of coffee products, like the chlorogenic acid in coffee husks (Bondesson, 2015). Through these mechanisms, organic acids enhance both the preservation of coffee quality and the overall consumer experience (Coban, 2020).

2.1.2.2 Polyphenols

Polyphenols are another significant group of coffee cherry phytochemicals known for their chemical structure, which includes multiple hydroxyl (-OH) groups attached to one or more aromatic rings (Xiao & Kai, 2012). These compounds are broadly classified into flavonoids and non-flavonoids, both contributing uniquely to health benefits. The basic structure of non-flavonoids is one phenol ring with a hydroxyl group (Figure 1, a), and flavonoids (Figure 1, b) contain two phenol rings (ring A and ring B), which connect via an oxygen-containing central pyran ring (ring C) (Li & Duan, 2018). Phenolic acid is a non-flavonoid structure with a carboxyl group (-COOH).

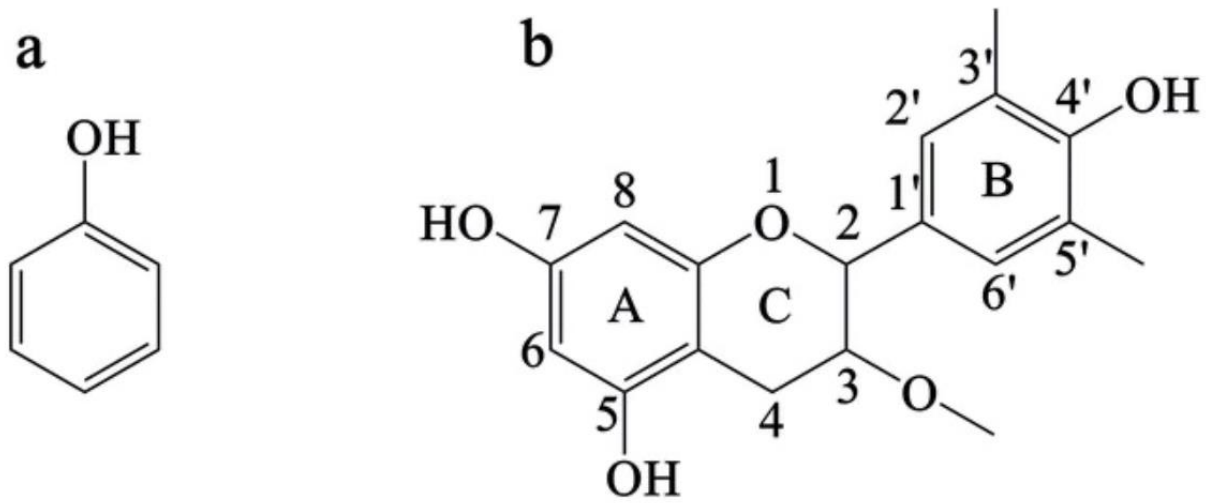


Figure 1 Basic structure of (a) non-flavonoids and (b) flavonoids.

Total polyphenols represent the cumulative content of all polyphenolic compounds in coffee cherry, which include chlorogenic acids, tannins, anthocyanins, and isomers in coffee bean (Farah & Donangelo, 2006) and Chlorogenic and p-coumaric acids in coffee husk (Ramón-Gonçalves et al., 2019). These compounds exhibit a remarkable capacity to combat oxidative stress by neutralizing free radicals, thus reducing oxidative damage. This antioxidant activity is crucial for protecting cells and tissues from the harmful effects of reactive oxygen species (ROS) (Rudrapal et al., 2022). Additionally, polyphenols in coffee cherries demonstrate anti-inflammatory properties by suppressing the production and release of inflammatory mediators (Kim et al., 2020). They also possess antimicrobial properties, effectively inhibiting the growth of various bacteria through multiple mechanisms. For example, tannins can cause instability of the cytoplasmic membrane, alter cell membrane permeability, inhibit extracellular microbial enzymes, and directly affect microbial metabolism (Daglia, 2012).

A specific class of polyphenols, anthocyanins, are responsible for various plant tissues' vibrant red, purple, or blue hues. In red coffee cherries, anthocyanins, like cyanidin-3-rutinoside and cyanidin-3-glucoside, exhibit potent anti-inflammatory properties by modulating inflammation-related signaling pathways and inhibiting pro-inflammatory mediators (Iqbal et al., 2018; Murthy et al., 2012). They also have broad-spectrum inhibitory effects on pathogens, including bacteria and viruses (Ma et al., 2019). Delphinidin-3-O-glucosyl-glucoside, cyanidin-3-O-glucosyl-rutinoside, and other anthocyanins could inhibit viruses like SARS-CoV-2 in molecular docking (Petruskevicius et al., 2023). Moreover, anthocyanins possess antioxidant, cardioprotective, and anticancer properties, making them significant contributors to the health benefits of coffee (Rechner & Kroner, 2005; L.-S. Wang & Stoner, 2008). Besides, the yellow coffee cherry is attributed to carotenoids (Esquivel et al., 2020).

The processing of coffee can create many by-products. Finished coffee products generally contain coffee beans but not outer layers like the husk, which is comprised of the silver skin, parchment, pectin layer, pulp, and outer skin (Figure 2). The by-products of coffee processing contain various phytochemicals with potential health benefits. The coffee husk contains phytochemical compounds like sucrose, chlorogenic acid, and anthocyanins, contributing to their sweetness, acidity, and bioactive effect (Král et al., 2024). The husk includes around 20% polysaccharides, 1809.9 mg GAE/gm phenolic compounds, and some lipids (Cangussu et al., 2021; Geremu et al., 2016).

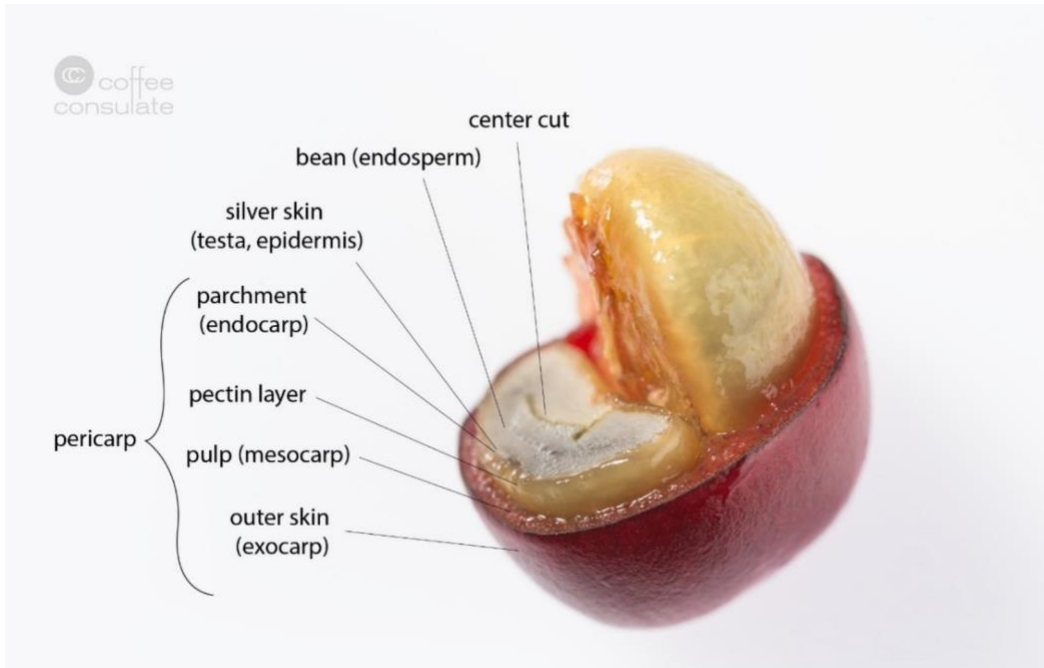


Figure 2 The structure of the coffee in different layers (Klingel et al., 2020).

2.1.3 Polyphenol Extraction Methods

Coffee is a rich source of various bioactive compounds, including polyphenols, organic acids, and sugars. A commonly used method for extracting polyphenols is liquid-liquid extraction, which involves using organic solvents or a mixture of organic solvents and water. Organic solvents can be categorized into polar and less polar solvents, each suited for extracting different types of phenolic compounds.

For polar phenolic compounds, polar organic solvents such as acetone, methanol, and ethanol are frequently used. Methanol, in particular, is considered one of the most effective solvents for the extraction of polyphenols due to its high polarity and ability to solubilize a wide range of phenolic compounds. Acetone, while also effective, is often preferred for its ability to extract specific phenolic compounds and other polar constituents (Galanakis et al., 2013).

However, certain less polar phenolic compounds require the use of less polar organic solvents for efficient extraction. For instance, weak polar organic solvents like ethyl acetate are commonly employed to extract these compounds due to their intermediate polarity, which can selectively dissolve less polar constituents. In a Sarang banua leave extraction study, the polar solvent ethanol and semipolar solvent ethyl acetate were used for antioxidation and antibacterial tests. The results showed that the ethanol-extracted sample had better antioxidation, but the ethyl acetate-extracted sample had a better antibacterial result (Simorangkir et al., 2019).

The ratio of organic solvent to water is a critical factor in the extraction process. Commonly used ratios include 80/20 (v/v) and 50/50 (v/v) mixtures of organic solvent to water. These mixtures not only enhance the extraction efficiency of polyphenols but also facilitate the extraction of other compounds, such as proteins, sugars, and other macromolecules, depending on the extraction conditions (Nielsen, 2024).

Temperature control is another essential aspect of the extraction process. The extraction temperature can range from room temperature to 90°C, significantly influencing the stability and yield of polyphenols. For instance, a low-temperature extraction at room temperature for 24 hours can preserve sensitive polyphenols in the extracting agent with or without the help of an assisted method like ultrasound, whereas a high-temperature extraction at 80°C for a shorter duration, such as 30 minutes, can enhance the extraction rate. In contrast, a high-temperature extraction at 80 °C for a shorter duration, such as 30 minutes, may risk the degradation of some phenolic compounds (Antony & Farid, 2022).

Acidification is also a crucial factor to consider. The addition of a weak acid, such as acetic acid, can improve the stability of polyphenols like anthocyanins during the extraction process (Chethan & Malleshi, 2007). Acidifying the extraction solvent helps maintain an acidic environment, which is beneficial for stabilizing these compounds and preventing their degradation (Biesaga & Pyrzyńska, 2013). For example, anthocyanins, phenolic acids, tannins, and flavonoids are more stable in an acidic environment (Fronde et al., 2019).

However, liquid-liquid extraction with organic solvents can only extract soluble polyphenols. To extract insoluble phenolics with ester linkages, alkaline hydrolysis using agents such as NaOH is required. During this process, the insoluble phenolics are hydrolyzed into the solvent, and subsequent acidification is performed using an acid solvent. This step allows the phenolics to be extracted by less polar organic solvents such as ethyl acetate or diethyl ether (Nielsen, 2024). For example, Chai et al. (2013) used an alkaline-acid hydrolysis method to extract phenolic acid from peanut and soybean. In this alkaline hydrolysis process, the alkaline solvent can denature phenolic acids. To mitigate this, antioxidants such as nitrogen prevent oxidative degradation of the phenolic compounds caused by alkaline hydrolysis (Ross et al., 2009). This

approach ensures that the phenolics maintain their structural integrity and bioactivity throughout the extraction process (Madhujith & Shahidi, 2009).

2.1.4 Methods for Analyzing Polyphenolic Contents

The methods for quantifying polyphenolics include colorimetric and chromatographic techniques. Historically, oxidation reagents such as permanganate or ferric ions were used, as polyphenolics have the ability to reduce these reagents, leading to a color change that can be measured (Tatyana et al., 2018). This qualitative approach provided a basic estimation of polyphenolic content. Over time, more advanced methods like the Folin-Ciocalteu and Folin-Denis assays were developed, employing these reagents due to their increased sensitivity and reliability in detecting various polyphenolic compounds (Nikolaeva et al., 2022). The Folin-Ciocalteu method, in particular, became popular because it can detect a broader range of polyphenolics, providing a more comprehensive measure of total phenolic content.

With technological advancements, high-performance liquid chromatography (HPLC) and gas chromatography (GC) have become more prevalent (Odeh & Allaf, 2017). These chromatographic methods offer the ability to detect a much wider range of polyphenolics with higher precision and specificity compared to the colorimetric methods. HPLC and GC separate individual polyphenolic compounds based on their interactions with the stationary and mobile phases, allowing for detailed analysis and identification. However, these methods require more sophisticated equipment and trained personnel, making them more resource-intensive compared to colorimetric methods like the Folin-Ciocalteu assay. Despite the increased complexity and cost, HPLC and GC are preferred for their superior accuracy and capability to analyze complex mixtures of polyphenolics.

2.1.4.1 Folin-Ciocalteu Method

The Folin-Ciocalteu method is a widely used colorimetric assay for the quantification of total polyphenols in various samples. The principle of this method involves the reduction of the Folin-Ciocalteu reagent, a mixture of phosphomolybdate and phosphotungstate, by polyphenolic compounds under alkaline conditions. This reduction process leads to the formation of a blue complex that can be measured spectrophotometrically at 765 nm.

One of the main advantages of the Folin-Ciocalteu method is its simplicity and cost-effectiveness, making it accessible for routine analysis in many laboratories. It is also relatively fast and requires minimal sample preparation. However, the method has limitations, including its non-specificity. The Folin-Ciocalteu reagent reacts not only with polyphenols but also with other reducing substances such as ascorbic acid, reducing sugars, and some amino acids (Lawag et al., 2023; Zugazua-Ganado et al., 2024). This can lead to overestimation of the polyphenol content. Additionally, the method cannot quantify individual polyphenolic compounds.

2.1.4.2 pH Differential Method

The pH differential method is specifically designed for the evaluation of anthocyanins, a class of polyphenolic compounds that exhibit color changes in response to different pH environments. The principle behind this method involves the structural transformation of anthocyanins, which alters their absorbance properties at different pH levels. At a low pH of 1.0, anthocyanins are in their highly colored flavylum cation form, whereas at a higher pH of 4.5, they convert to a colorless or pale-colored hemiketal form (Lee et al., 2005). In practice, the sample is divided and treated with two buffer solutions, one at pH 1.0 and the other at pH 4.5. The absorbance of each solution is then measured using a spectrophotometer at specific wavelengths, typically 510

nm and 700 nm. The difference in absorbance between the two pH conditions correlates with the concentration of anthocyanins in the sample.

The pH differential method has several advantages. It is relatively simple and quick to perform, requires minimal sample preparation, and provides a direct measurement of anthocyanin content. The method is also highly specific for anthocyanins, as it exploits their unique pH-dependent color changes. However, the accuracy of this method can be affected by the presence of other colored compounds in the sample that may also respond to pH changes, like betacyanins in beetroot, potentially leading to interference in absorbance readings (Rifat et al., 2021).

2.2 Antimicrobial Activities of Polyphenols

2.2.1 Introduction

Phytochemicals, particularly polyphenols, exhibit significant antimicrobial properties, making them a focus of interest in food science and medical research. These natural compounds, found in a variety of plants, have demonstrated the ability to inhibit or even kill a wide range of bacteria through various mechanisms that disrupt bacterial cell functions and structures.

The antimicrobial activity of polyphenols is concentration-dependent. Studies have shown that different polyphenols require specific concentrations to achieve either bacteriostatic (inhibitory) or bactericidal (killing) effects. For instance, research indicates that the inhibitory concentration (IC) of mango seed extract rich in polyphenolic against *Staphylococcus aureus* is 7.81 µg/mL. Meanwhile, the bactericidal concentration (BC) needed to kill this bacteria typically is 23.4 µg/mL (Torres-León et al., 2021).

Polyphenols exert their antimicrobial effects through diverse mechanisms. One primary mechanism is the disruption of bacterial cell membranes, which leads to leakage of cellular content

and ultimately cell death. Additionally, polyphenols can interfere with bacterial energy production, alter cell morphology, and inhibit essential enzymes critical for bacterial survival and proliferation (Piekarska-Radzik & Klewicka, 2021).

Ongoing research continues to explore the specific concentrations and conditions under which these phytochemicals exert their most potent antimicrobial effects. This research aims to harness the benefits of polyphenols in coffee cherries for food safety and nutraceutical applications, highlighting their potential as natural and effective agents for controlling pathogenic bacteria in food.

2.2.2 Foodborne Pathogens

Foodborne pathogens are microorganisms, such as bacteria, viruses, and parasites, which contaminate food and cause illnesses in humans. These pathogens are a significant public health concern due to their ability to cause widespread outbreaks and severe health outcomes. Common foodborne pathogens include *Salmonella*, *L. monocytogenes*, enterohemorrhagic *Escherichia coli*, *Campylobacter jejuni*, and norovirus. It is worth noting that *Salmonella* and *L. monocytogenes* are also among the top five pathogens contributing to domestically acquired foodborne illnesses resulting in death (CDC, 2023).

Typical symptoms caused by foodborne pathogens include diarrhea, abdominal cramps, nausea, vomiting, fever, and muscle aches. Foodborne illnesses have considerable economic implications. According to the CDC, there were 6 multistate foodborne illness outbreaks with more than 100 illnesses and more than 30 hospitalizations in 2023 that resulted in substantial economic loss (*FSIS Foodborne Illness Outbreak Investigations, Fiscal Year 2023*, n.d.). In 2021, the USDA Economic Research Service (ERS) estimated that these illnesses cost the U.S. economy approximately \$17.6 billion (*USDA ERS*, 2021).

2.2.2.1 *Salmonella* Typhimurium

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a prominent pathogen responsible for numerous cases of gastroenteritis worldwide. This bacterium is a Gram-negative, rod-shaped organism and is a significant public health concern due to its strong ability to cause infections in humans (Pink et al., 2014). The primary reservoir of *Salmonella* is farm animals. The transmission route commonly involves the ingestion of water or food contaminated with the feces of carriers (Eng et al., 2015). *S. Typhimurium* infections are commonly associated with the consumption of contaminated poultry, eggs, meat, and dairy products. A *Salmonella* outbreak linked to cucumbers recently occurred and has caused 449 illnesses and 125 hospitalizations (CDC, 2024b).

S. Typhimurium could invade the intestinal epithelial cells, leading to symptoms such as diarrhea, fever, abdominal cramps, and vomiting, typically manifesting within 6 to 72 hours after ingestion (Ngogo et al., 2020). This pathogen is introduced into the intestine by food, and it can survive in the stomach because of its acidic tolerance. This pathogen employs a type III secretion system to inject effector proteins into host cells, facilitating bacterial uptake and survival within host tissues (A. M. P. dos Santos et al., 2020).

One of the major concerns regarding *S. Typhimurium* is its increasing antibiotic resistance. Multidrug-resistant (MDR) strains have emerged, complicating treatment and control measures. For example, *S. Typhimurium* phage type or definitive type (DT) 104, a virulent and drug-resistant pathogen, can resist ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Pope et al., 1998). Resistance genes are often carried on plasmids, facilitating horizontal gene transfer among bacteria.

2.2.2.2 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, facultatively anaerobic bacterium responsible for listeriosis, a serious infection with high mortality rates, particularly among vulnerable populations such as pregnant women, newborns, the elderly, and immunocompromised individuals. *L. monocytogenes* is ubiquitous, and its common food vehicles include ready-to-eat foods, dairy products, produce, deli meats, and seafood. In 2023, a *Listeria* outbreak linked to ‘soft serve on the go’ ice cream cups. The strain was detected in one victim’s freezer (*CDC Newsroom*, 2023). This pathogen is notable for its ability to grow at refrigeration temperatures, making it a significant concern in the food industry (Wiktorczyk-Kapischke et al., 2021). It also has a high salt tolerance and has been linked to cured meat products (Liu et al., 2005).

L. monocytogenes invades host cells, evades the immune system, and spreads intracellularly by hijacking the host's actin cytoskeleton. Symptoms of listeriosis range from mild flu-like symptoms to severe manifestations, including septicemia, meningitis, miscarriage, stillbirth, or neonatal infection in pregnant women (Bucharest Romania et al., 2021; Shi et al., 2021; Zhang & Yi, 2022).

The persistence of pathogenic bacteria in food processing environments and their resistance to various stress conditions underscore the need for stringent hygiene and monitoring practices. *L. monocytogenes* and *S. Typhimurium* cause millions of infections, thousands of hospitalizations, and hundreds of deaths annually in the U.S (CDC, 2023). Effective control of these pathogens involves a combination of good manufacturing practices (GMP), hazard analysis and critical control points (HACCP), and rigorous environmental monitoring. Developing more sensitive detection methods and a better understanding of these pathogens’ ecology are crucial for

improving food safety. Moreover, the development of new and natural antimicrobial agents is urgent because of the rise of antibiotic resistance.

2.2.3 Methods for Determining Antimicrobial Activity

2.2.3.1 Agar Diffusion Method

Agar diffusion methods include the disk diffusion method and well diffusion method. The disk diffusion method, commonly known as the Kirby-Bauer method, involves placing paper disks impregnated with the test antimicrobial agent onto an agar plate inoculated with the target bacteria. After incubation, the zone of inhibition around each disk is measured, with larger zones indicating greater antimicrobial activity. In the well diffusion method, wells are created in the agar, and the antimicrobial agent is introduced into these wells. Similar to the disk diffusion method, the inhibition zones are measured after incubation to determine the agent's efficacy (Erhonyota et al., 2023).

2.2.3.2 Minimum Inhibitory Concentration (MIC)

The MIC is usually determined by preparing serial dilutions of the antimicrobial agent with liquid media in a 96-well plate, followed by inoculation with the test organism. The MIC is determined as the lowest concentration of the agent that inhibits visible bacterial growth (turbidity) after incubation. The agar dilution method is another common method for MIC determination. In this method, serial 2-fold dilutions of the antimicrobial agent will be mixed with microbiological media and agar. The test organism is spotted on each agar plate, and the MIC is the lowest concentration that prevents visible colony after incubation (Wiegand et al., 2008).

2.2.3.3 Minimum Bactericidal Concentration (MBC)

Following MIC determination, samples from wells or tubes that show no visible growth are sub-cultured onto non-selective agar plates without the antimicrobial agent. The MBC is defined

as the lowest concentration that kills 99.9% of the initial bacterial population (X. Liu et al., 2022). After incubation, the number of colonies on the plate will be counted. The MBC is determined by comparing the bacterial concentration treated with the antimicrobial agent to the initial bacterial concentration, identifying the concentration that results in a 99.9% decrease.

2.2.3.4 Biofilm formation Assays

Crystal violet staining is widely used to quantify biofilm formation (Chandrasekharan et al., 2022). The ability of an antimicrobial agent to prevent biofilm formation is evaluated by comparing the biomass of treated and untreated biofilms. Initially, the antimicrobial agent is mixed with a bacterial solution, and the mixture is cultured on a flat surface to allow biofilm formation. Once the biofilm has formed, the culture solution is removed, and the biofilm is washed. Subsequently, the biofilm is stained with crystal violet dye, and its absorbance is measured and quantified using a spectrophotometer.

Another common technique is the plate counting method, which quantifies viable cells within the biofilm to assess the effect of an antimicrobial agent on biofilm formation and the sensitivity of biofilm to the antimicrobial agent after it is formed (Sun et al., 2021). The biofilm is washed, detached, diluted, plated, and counted to determine the number of viable cells.

2.2.3.5 Agar overlay method

The agar overlay method involves pouring the second agar layer on the first layer in the plate. If the secondary selective agar is directly poured into a non-selective agar containing injured but viable cells, the injured cells will not recover (V. c. h. Wu & Fung, 2001). Since overlay agar is poured onto a nonselective agar on which injured cells are recovered first, the overlay method can be used to count both healthy and injured cells. Due to this characteristic, this method has been

used to determine the effect of antimicrobial agents and processes, which may cause injury in target bacteria.

The nonselective medium for the first layer can be Plate Count Agar (PCA) or Tryptic Soy Agar (TSA). The medium for the second layer should be selective for targets of interest. Xylose Lysine Deoxycholate (XLD) agar and Modified Oxford (MOX) Agar are commonly used for detection of *Salmonella* and *L. monocytogenes*, respectively (Kang & Fung, 1999).

2.2.4 Antimicrobial Mechanism of Polyphenols

Polyphenols, naturally occurring compounds in plants, have garnered significant attention for their antimicrobial properties. The primary classes of polyphenols, including flavan-3-ols, flavonols, tannins, and phenolic acids, exhibit various mechanisms to inhibit microbial growth (Daglia, 2012). Flavan-3-ols, such as catechins, cause bacterial cell aggregation, impeding their ability to infect and multiply. They also disrupt the bacterial cytoplasmic membrane, increasing its permeability and leading to cell death. Additionally, these compounds inhibit extracellular microbial enzymes, interfering with essential bacterial metabolic processes (Yi et al., 2010). Another critical mechanism is nutrient deprivation. Flavonols, including quercetin and kaempferol, primarily exert their antimicrobial effects through lipid membrane aggregation and rigidification (Phan et al., 2014). This inhibition results in bacterial cell death. Furthermore, flavonols can modulate protein expression within bacterial cells, either downregulating or upregulating various proteins, thereby disrupting normal bacterial functions. Tannins, such as proanthocyanidins, demonstrate their antimicrobial efficacy by permeabilizing the bacterial cell membrane, causing cellular contents to leak and leading to cell death. They also inhibit crucial bacterial enzymes and chelate metal ions, which are essential for bacterial growth, thereby preventing bacterial proliferation. Phenolic acids, including gallic acid, caffeic acid, and ferulic

acid, directly damage the bacterial cell wall and membrane, exhibiting strong antibacterial properties (Daglia, 2012).

2.2.5 Antimicrobial activity of coffee

Brewing coffee extracts contain significant amounts of polyphenols, particularly caffeic and chlorogenic acids. The antimicrobial activity of brewed coffee varies with the brewing method. For instance, espresso Colombia coffee exhibits higher antimicrobial activity than filter and Italian coffee. At concentrations ranging from 30-300 μL , coffee demonstrated inhibition zones against *Staphylococcus aureus*, *L. monocytogenes*, and *Escherichia coli*, with espresso showing the most substantial effects (Martínez-Tomé et al., 2011).

Extracts from the coffee bean and husk, especially *Coffea arabica*, contain bioactive compounds such as chlorogenic acid and caffeine. These extracts have shown potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. For instance, chlorogenic acid exhibited inhibition zones of 8-11 mm against *E. coli* and *S. aureus* at 8 to 300 μL concentrations. Caffeic acid demonstrated even more vigorous activity, with inhibition zones reaching up to 17 mm at similar concentrations (Monteiro et al., 2017).

Coffee pulp, a by-product of coffee processing, is rich in polyphenols, including chlorogenic acid and caffeine. Studies have indicated that coffee pulp extracts, at concentrations of 150-300 mg/mL, effectively inhibit *Staphylococcus aureus* and *Escherichia coli*, with inhibition zones measuring 10-12 mm. Moreover, coffee pulp containing 0.50 to 79.53 mg GAE/g exhibits a MIC ranging from 3 to 25mg/mL against *Staphylococcus aureus*, 25 mg/mL against *Streptococcus oralis* and *Escherichia coli*, and 12.5 mg/mL against *Pseudomonas aeruginosa*. Notably, the antimicrobial activity of the pulp extract remains effective even after gastrointestinal digestion, suggesting its potential as a nutraceutical agent (É. M. dos Santos et al., 2024; Duangjai et al.,

2016; Khochapong et al., 2021). However, different species of coffee pulp produce varying results. For instance, Sarawak Liberica sp. coffee pulp, with 24.24 mg GAE/g of total phenolic content, shows no significant antibacterial activity against *Staphylococcus aureus* and *S. Typhimurium* (N. S. Ismail et al., 2020).

2.2.6 Conclusion

The antimicrobial properties of various plant species have been widely studied, yet significant gaps remain in understanding the specific mechanisms and efficacy of these properties across different species. For instance, as discussed, different species of coffee pulps exhibit varying efficiencies against *Staphylococcus aureus*. This variability underscores the need for different species to identify those species with the most promising antimicrobial potential. Specifically, red and yellow coffee husks have been chosen for comparison in the following study due to preliminary evidence suggesting differential antimicrobial activities among different coffee husks.

The mechanisms by which polyphenols in coffee pulp exert their antimicrobial activity are not fully understood. It is known that polyphenols in plant extracts can disrupt microbial cell membranes and cause protein leakage (Daglia, 2012). However, the specific effects caused by polyphenols in coffee pulp remain unknown. This research aims to elucidate these mechanisms, providing a clearer understanding of how coffee pulp extracts exert their antimicrobial effects.

Furthermore, the efficacy of antimicrobial agents can be significantly influenced by food constituents. Previous studies have indicated that milk, vegetables, or meat can interfere with the activity of antimicrobials, potentially reducing their effectiveness (Gutierrez et al., 2009). However, the extent of these interactions remains underexplored. Conducting tests in milk, a

common food matrix, allows for a realistic assessment of antimicrobial efficacy, providing valuable insights directly applicable to food safety and preservation.

The exploration of coffee husk polyphenols as natural antimicrobial agents presents exciting future prospects. With rising concerns over antibiotic resistance and the demand for natural preservatives, coffee husk extracts may offer a sustainable and effective solution. Additionally, the valorization of coffee by-products can contribute to waste reduction, save disposal costs for coffee processing waste streams, and create new economic opportunities for coffee-producing regions.

Chapter 3

MATERIALS AND METHODS

3.1 Preparation of Coffee husk Extracts

Red (*Coffea liberica*) and yellow (*Coffea arabica*) coffee husks were harvested from the Island of Hawai'i and O'ahu, respectively. The coffee bean was removed from the fruit using a cherry pitter. Coffee husk was stored at -80°C and dehydrated using a freeze dryer (Labconco) at -40°C and 0.133 mbar for 48 hours. After freeze-drying, the coffee husk was pulverized using a coffee grinder (Black + Decker).

To extract polyphenols, five grams of coffee husk powder were mixed with 100 mL of methanol/water/acetic acid (80:20:0.5, v/v). The mixture was covered with aluminium foil to avoid light exposure and stirred at room temperature for 24 hours. The mixture was then filtered through a Buchner funnel with No. 3 Whatman filter paper under vacuum. The collected liquid was evaporated using a rotary evaporator (IKA) under vacuum at 40°C and a speed of 100 rpm (X. Liu et al., 2022).

After the evaporation, the extract was transferred into tubes and freeze-dried at -40°C and 0.133 mbar for 48 hours. The powder of coffee husk extracts was dissolved in distilled water to achieve a concentration of 100 mg/mL. The extract was filter-sterilized using a sterile syringe filter with 0.2 µm polyethersulfone membrane (VWR) for the subsequent antimicrobial tests. After sterilization, the extract was stored at -20°C and protected from light.

3.2 Chemical Analysis of Coffee Husk Extracts

3.2.1 Quantification of Sugar and Organic Acids

The sugar content of coffee husk extracts was evaluated with the ratio of °Brix using a refractometer (Bio-Rad, iMark). The pH value of each extract was determined with a pH meter (Oaklon). According to Nielsen (2024)'s food analysis, organic acids were measured by titration with 0.1 N NaOH, and this measurement was primarily based on citric acid as the equivalent acid. A precise volume of coffee husk extract was added to a beaker, and 10 µL of phenolphthalein was used as the pH indicator. The titrant was dropped into the analyte little by little until the color of the analyte changed, which meant the endpoint of the titration reflection. The equation for %acid is as follows:

$$\%acid \left(\frac{wt}{vol} \right) = \frac{N * V_1 * Eq \ wt}{V_2 * 1000} * 100$$

N = normality of titrant, usually NaOH (mEq/mL);

V₁ = volume of titrant (mL);

Eq. wt. = equivalent weight of predominant acid (mg/mEq);

Citric acid molecular weight = 192, equivalent weight = 64

V₂ = volume of sample (mL);

1000 = factor relating mg to grams (mg/g);

3.2.2 Total Phenolic Content

The total phenolic content (TPC) of coffee husk extracts was measured using the Folin–Ciocalteu reagent (EMD Millipore Corporation). Gallic acid standard solutions were prepared at concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/mL by diluting gallic acid in sterile

distilled water. Solid phase extraction (ADD CITATION HERE) was conducted on samples prior to measuring total phenolic content to reduce the presence of contaminants. The solid phase extraction was developed based on Georgé et al., (2005). The coffee husk extract was diluted 200-fold with distilled water and filtered using an Oasis HLB cartridge connected to vacuum equipment. To activate the cartridges, 3 ml of methanol followed by 3 mL of distilled water were pipetted into the cartridges and filtered by vacuum. After activation, 4 mL of the diluted sample were added to cartridges. To rinse the samples, 2 mL of 2% formic acid in water was added to cartridges. Subsequently, to collect the filtered extract, 2 mL of 2% formic acid in methanol was added to each cartridge. The extract, mixed with formic acid in methanol, was collected in a tube. The tube was then removed from the vacuum manifold to dry under a stream of nitrogen using a N-EVAP nitrogen evaporator (Organomation, Berlin, MA, USA). Finally, the dried extract was redissolved in 4 mL distilled water to achieve the equivalent dilution. A volume of 30 μ L of distilled water (control), gallic acid standards, or diluted coffee husk extract was added into the wells of a 96-well plate (Costar). Subsequently, 210 μ L of distilled water, 15 μ L of Folin–Ciocalteu reagent, and 45 μ L of 20% sodium carbonate solution were added to each well. The plate was then incubated at room temperature for 2 hours.

After incubation, the absorbance of each well was measured at 765 nm using a spectrophotometer (Bio-Rad, iMark). The absorbance values obtained were used to construct a standard curve based on the gallic acid standards. This standard curve was then used to calculate the total polyphenol content (TPC) of the coffee husk extract samples, expressed as milligrams of gallic acid equivalents (GAE) per liter.

3.2.3 Anthocyanin Concentration

The pH differential method, which relies on the pH-dependent color changes of anthocyanins, was employed to determine the total anthocyanin content in the samples. The results were stated in the equivalent of cyanidin-3-glucoside (cyd-3-glu).

According to the method described by Lee et al. (2005), two aliquots were prepared for each sample and analyzed in a microplate. The first aliquot was mixed with an equal volume of pH 1.0 buffer solution (0.025 M potassium chloride), and the second aliquot was mixed with an equal volume of pH 4.5 buffer solution (0.4 M sodium acetate). Both mixtures were allowed to equilibrate for 20 minutes at room temperature.

Following the equilibration period, the absorbance of each mixture was measured at both 520 nm and 700 nm using a spectrophotometer (Bio-Rad, iMark). The anthocyanin content was calculated with the following equation:

$$\text{Total Anthocyanins} = \frac{A * MW * DF * 10^3}{\epsilon * L}$$

A (absorbance) = (A_{520nm} – A_{700nm})_{pH1.0} – (A_{520nm} – A_{700nm})_{pH4.5};

MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu);

DF = dilution factor established in D;

L = pass length (cm); (0.56 cm in this study)

ϵ = 26900 molar extinction coefficient, in $L * mol^{-1} * cm^{-1}$, for cyd-3-glu;

10^3 = factor for conversion from g to mg;

3.3 Bacterial Strains and Growth Conditions

Two foodborne pathogens, Gram-positive bacteria *L. monocytogenes* F2365 and Gram-negative bacteria *S. Typhimurium* ATCC 14028, and two probiotics, *Lactococcus lactis* (*L. lactis*), and *Lacticaseibacillus rhamnosus GG* (LGG), were used in the antimicrobial tests of coffee cherry extracts. All strains were frozen at -80 °C. Before use, they were transferred twice with Tryptic soy broth (TSB, BBLTM) at 37°C for 24 hours. The confirmation of *L. monocytogenes* and *S. Typhimurium* were done by culturing on Oxford Medium Agar (MOX, Difco™) and Xylose Lysine Deoxycholate Agar (XLD, Difco™), respectively. *L. lactis* and LGG were grown in De Man Rogosa and Sharpe (MRS) broth.

3.4 Determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Coffee Husk Extracts

The minimum inhibitory concentration (MIC) was determined against *S. Typhimurium*, *L. monocytogenes*, *L. lactis*, and LGG. The minimum bactericidal concentration (MBC) was determined against *S. Typhimurium* and *L. monocytogenes*.

According to a method developed by Allkja et al. (2020), the bacterial cultures were ten-fold diluted using peptone water to reach a final concentration of approximately 10⁸ colony-forming units (CFU)/mL. Mueller Hinton Agar (MHA) was prepared by combining Mueller Hinton Broth (MHB, BD) with 1.7% agar. The mixture was autoclaved and allowed to cool to 50°C. Coffee husk extracts were diluted with distilled water to obtain a concentration gradient of 50%, 25%, 12.5%, 6.25%, and 3.125%, and the same volume of sterilized distilled water was prepared as the control. The cooled MHA was mixed with either the extract dilutions or distilled water in petri dishes. The dishes were then left in a biosafety cabinet for 30 minutes to ensure the agar was solidified and agar surface was dry. Then bacterial strains were inoculated on the agar at a volume

of 1 μL per plate. The plates were incubated at 37°C for 24-48 hours. The MIC was determined to be the lowest concentration of the extract, at which no colony was observed on the inoculated agar.

After determining the MIC, the extract was diluted into a two-fold gradient. The diluted extract or sterilized distilled water was mixed in an equal volume of bacterial solution of 10^6 CFU/mL in peptone water. The mixture of distilled water and bacterial solution served as control. Tested extract concentrations included 1 MIC, 2 MIC, and 4 MIC. At 0 hour of incubation, the control was diluted and spread on Plate Count Agar (PCA, BD, USA) for colony counting. All samples were incubated at 37°C for 24 hours and enumerated on PCA. The plates were incubated at 37°C for 24 hours, and then colonies were counted. When the level of tested bacteria was reduced by 99.9% compared to the control at 0 hour, the extract concentration was identified as the MBC.

3.5 Biofilm Formation Capability of *L. monocytogenes* and *S. Typhimurium* Treated with Coffee Husk Extracts

The bacterial culture was diluted with $2\times$ Tryptic Soy Broth (TSB) to achieve a concentration of approximately 10^5 CFU/mL. A volume of $100\ \mu\text{L}$ of the bacterial culture was inoculated into wells of polystyrene, flat-bottom, untreated 96-well plates (Falcon, Corning, USA). Subsequently, $100\ \mu\text{L}$ of coffee husk extracts at concentrations of 1 MIC and $1/2$ MIC were separately added into the wells. The plate was incubated for 24 hours at 37°C . After incubation, the sample was carefully removed, and the wells were gently washed three times with sterile Phosphate-Buffered Saline (PBS, $\text{pH}=7.2$). Finally, $200\ \mu\text{L}$ of PBS was added into each well, and the wells were swabbed with cotton swabs. The detached cell suspension was serially diluted using peptone water and plated on PCA. The plates were incubated for 24 hours at 37°C . The colonies were counted to determine the bacterial level of biofilm.

3.6 Cell membrane integrity of *L. monocytogenes* and *S. Typhimurium* Treated with Coffee Husk Extracts

The bacterial cell membrane integrity was assessed with the LIVE/DEAD BacLight kit (Life Technologies Inc.) following the manufacturer's instructions. The bacterial culture was centrifuged at 13,200 gravitational acceleration (g) for 2 minutes, and cells were washed twice with sterilized saline solution (0.85%, w/v) and resuspended in fresh Muller Hinton Broth-2 (MHB-2) broth. The bacterial suspension was 10-fold diluted with MHB-2 broth and mixed separately with an equal volume of coffee husk extracts at concentrations of 2 MIC and 4 MIC, 70% ethanol (positive control), or water (negative control). The mixture was incubated at 37°C for two hours while rotating on a tube rotator. After incubation, 100 µL of the treated bacterial solution was transferred to a black 96-well plate (Thermo Scientific), and 100 µL of the BacLight reagent was added into each well. The mixture was incubated for 15 minutes at room temperature in the dark. The fluorescence intensity was measured using a microplate reader (Tecan Infinite 200 PRO) with an excitation wavelength of 485 nm and emission wavelengths centered at 530 nm for green fluorescence and 630 nm for red fluorescence. The cell membrane integrity was calculated as the ratio of absorbance at 530 nm vs absorbance at 630 nm.

3.7 Leakage of intracellular proteins in *L. monocytogenes* and *S. Typhimurium* Treated with Coffee Husk Extracts

The leakage of intracellular protein was determined using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). The bacterial culture was centrifuged and cell pellet was washed twice with PBS. The cells were then resuspended in Phosphate Buffered Saline (PBS). The bacterial suspension was ten-fold diluted with PBS to achieve a concentration of approximately 10⁸ CFU/mL. For the assay, the bacterial solution was treated with the same volume

of coffee husk extracts at concentrations of 2 MIC and 4 MIC, 70% ethanol (positive control), or water (negative control). These mixtures were incubated at 37°C with rotation at 40 rpm for 2 hours. Meanwhile, the Coomassie reagent was pre-warmed. After the 2-hour incubation, the mixtures were centrifuged at 12,000 g for 10 minutes. Then, 250 µL of the pre-warmed Coomassie reagents were added to each well of a 96-well plate, followed by the addition of 10 µL of the supernatant from each sample to the wells. Diluted bovine serum albumin (BSA, Thermo Fisher) was used as the standard solution. The plates were left at room temperature for 10 minutes, and the absorbance was measured at 595 nm to determine protein concentration by a microplate reader (Biotek Synergy LX, BioTek Instruments).

3.8 Effect of Coffee husk Extracts on the Growth of *L. monocytogenes* and *S. Typhimurium* in Milk

The bacterial cultures were diluted with milk to achieve a concentration of approximately 6 log CFU/mL. The inoculated milk was mixed at a 1:3 v/v ratio with sterile water (control) or coffee husk extracts at concentrations of 1 MIC and 2 MIC. The mixtures were incubated at room temperature (21°C) and refrigerator temperature (7°C). At 0, 24, 48, and 72 hours, the mixtures were serially diluted and plated on PCA. The plates were incubated at 37°C for 2 hours to allow injured but viable bacterial cells to recover. A layer of melted selective agar was added to each plate, with modified Oxford (MOX) Agar supplemented with Moxalactam Supplement for *L. monocytogenes* and Xylose Lysine Deoxycholate (XLD) agar for *S. Typhimurium*. The plates were further incubated at 37°C for 48 hours. Brown colonies with a black halo on MOX agar were counted as *L. monocytogenes*, whereas red colonies with a black center on XLD agar were counted as *S. Typhimurium*.

3.9 Statistical Analysis

All experiments were performed three times. The bacterial cells counts were converted to Log CFU/ml. Measurement means and standard deviations of different treatments were calculated and compared. The data were analyzed by analysis of variance (ANOVA) and Tukey's multiple comparison test to determine significant differences between treatments at a significance level of 0.05 utilizing Statistical Package for the Social Sciences (SPSS).

Chapter 4

Results

4.1 Chemical Analysis of Coffee husk Extracts

Red and yellow coffee husk extracts were measured for pH, sugar, organic acids, total phenolic content, and anthocyanin content (Table 1). The total phenolic content was determined by using a standard curve generated with gallic acid solutions of known concentrations (Figure 3). Thus, the total phenolic content is presented as the gallic acid equivalents.

The results show that the pH of red coffee husk extract was 0.08 lower than that of yellow coffee husk extract, which was 4.18. Red and yellow coffee husk extracts had similar organic acid and total phenolic contents, which were 1.55% and 5058.27 mg/L for red coffee husk, and 1.41% and 4600.27 mg/L for yellow coffee husk. However, red coffee husk extract had significantly higher sugar and anthocyanin contents than yellow coffee husk extract. The sugar content in red and yellow coffee husk extracts was 9.60 and 8.87 °Brix, respectively. Notably, the anthocyanin content of red coffee husk extract was 22.03 mg/L, compared to 0.06 mg/L in yellow coffee husk extract (Table 1).

Table 1 Chemical analysis of coffee husk extracts¹

Chemical analysis	Red coffee cherry	Yellow coffee cherry
pH	4.10±0.02 ^a	4.18±0.05 ^b
Sugar and organic acids (°Brix/acid)	9.60±0.20 ^a /1.55±0.14 ^a	8.87±0.12 ^b /1.41±0.13 ^a
Total phenolics (Gallic acid equivalents, mg/L)	5058.27±319.60 ^a	4600.27±570.81 ^a
Anthocyanins (cyanidin-3-glucoside equivalent, mg/L)	22.03±2.57 ^a	0.06±0.10 ^b

¹Coffee husk extracts were analyzed at 100 mg/mL. Results represent the mean of three replicates. Means in the same row with different superscript letters (a and b) are significantly different ($p < 0.05$).

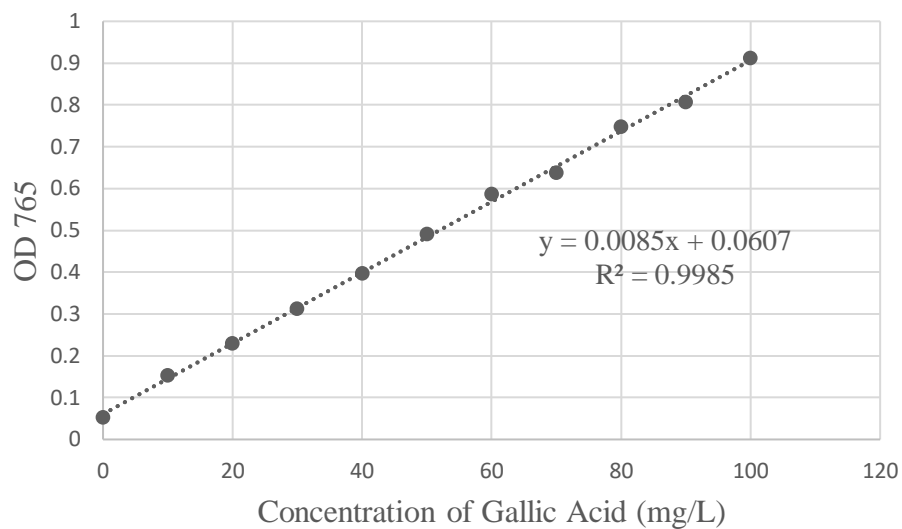


Figure 3 The standard curve for total phenolic content calculation. The standard curve was generated with the absorbance of different concentrations of gallic acid at the wavelength of 765 nm.

4.2 Determination of MIC and MBC

The antimicrobial properties of red and yellow coffee husk extracts were evaluated against multiple bacterial strains, including *S. Typhimurium*, *L. monocytogenes*, *L. lactis*, and *L. rhamnosus GG*. Representative images of bacterial growth on agar plates with varying concentrations of coffee husk extracts are presented in Figure 4 and Figure 5. The results demonstrated a concentration-dependent inhibition of bacterial growth, with higher concentrations of coffee husk extracts resulting in more significant inhibition.

Specifically, inoculated agar plates with 12.5 mg/mL red coffee husk extract showed no colony of *S. Typhimurium* or *L. monocytogenes* (Figure 4B) while these bacteria could grow in presence of 6.25 mg/mL red coffee husk extract (Figure 4A). Yellow coffee husk extract also had MIC of 12.5 mg/mL against these two pathogenic bacteria (Figure 4C and 4D).

Figure 5 shows that *L. lactis* and *L. rhamnosus GG* were much more tolerant to coffee husk extracts than *S. Typhimurium* and *L. monocytogenes*. These probiotic bacteria formed colonies even on agar plates with 50 mg/mL of red coffee husk extract.

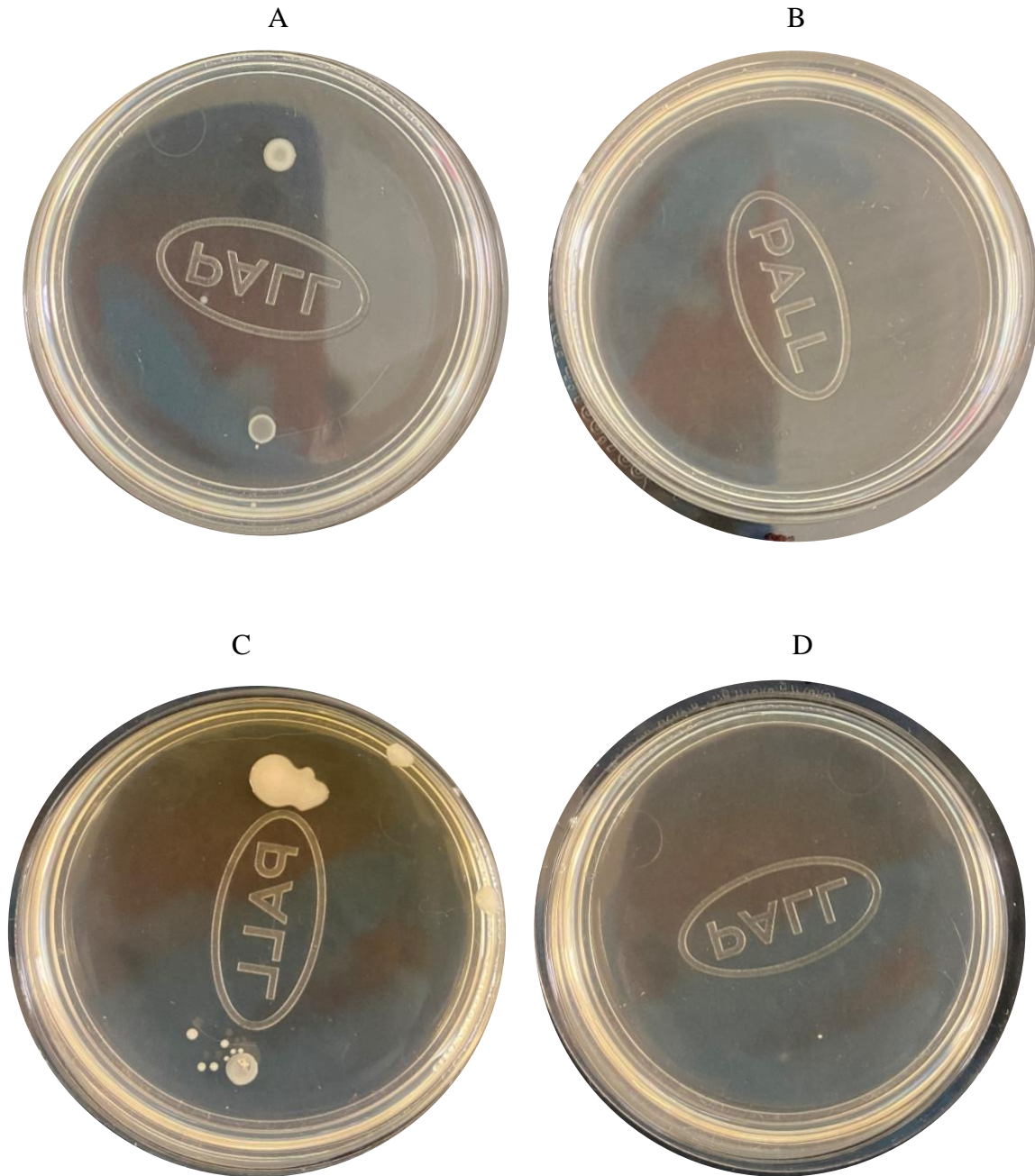


Figure 4 Representative images of bacteria growth on Muller Hinton agar with different concentrations of coffee husk extracts. The bacteria inoculated on the plate include *S. Typhimurium* on the upper side and *L. monocytogenes* on the lower side. (A) shows the plate with 6.25 mg/mL red coffee husk extract; (B) shows the plate with 12.5 mg/mL red coffee husk; (C) shows the plate with 6.25 mg/mL yellow coffee husk extract; and (D) shows the plate with 12.5 mg/mL yellow coffee husk extract.

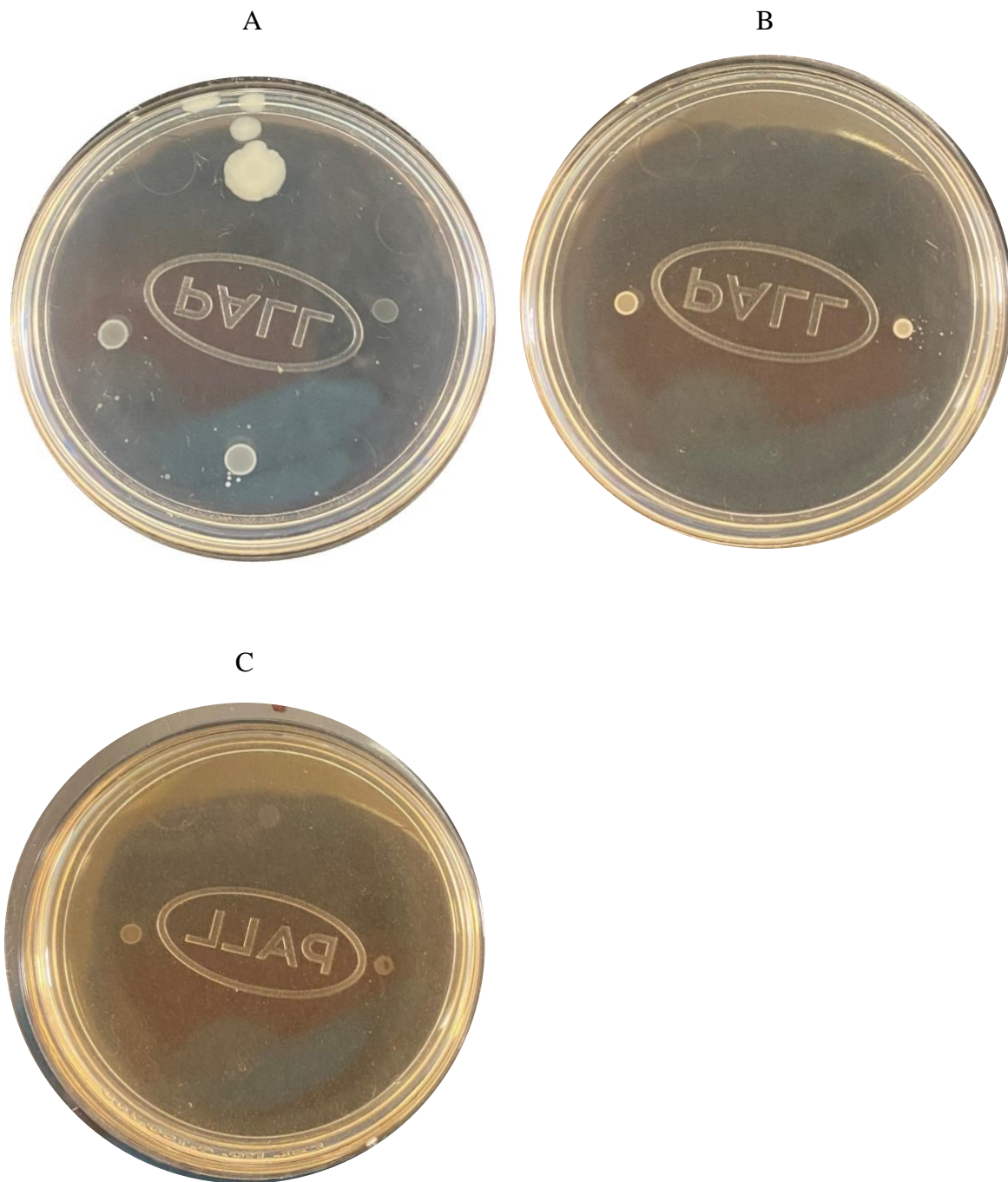


Figure 5 Representative images of bacteria growth on Muller Hinton agar with different concentrations of coffee husk extracts. The bacteria inoculated on the plate include *S. Typhimurium* on the upper side, *L. monocytogenes* on the lower side, *L. rhamnosus GG* on the left side, and *L. lactis* on the right side. (A) shows the plate with water (control); (B) shows the plate with 12.5 mg/mL red coffee husk extract; and (C) shows the plate with 50 mg/mL red coffee husk extract.

Plate counting was used to enumerate viable bacterial cells of *S. Typhimurium* and *L. monocytogenes* in Muller Hinton broth with different concentrations of red and yellow coffee husk extracts. In control, *L. monocytogenes* and *S. Typhimurium* increased from about 6 log CFU/mL at 0 hours to 8.41 and 9.55 log CFU/mL, respectively, at 24 hours (Figure 6). Both coffee husk extracts at 12.5 mg/mL suppressed bacterial growth but did not cause much reduction in bacterial counts. 25 mg/mL of red and yellow coffee husk extracts reduced the inoculated *L. monocytogenes* by 2.34 and 1.78 log, respectively. However, coffee husk extracts at this concentration only reduced the inoculated *S. Typhimurium* by approximately 1 log. When the red and yellow coffee husk extract concentration was 50 mg/mL, they could reduce the number of viable *L. monocytogenes* and *S. Typhimurium* cells by more than 99.9% (3 log). This indicates that 50 mg/mL is the MBC of red and yellow coffee husk extracts against both pathogenic bacteria. At this concentration, *L. monocytogenes* counts decreased to 0.85 and 2.79 log CFU/mL in the presence of red and yellow coffee husk extract, respectively. It is worth noticing that all *S. Typhimurium* cells were killed in the presence of either coffee husk extract (Figure 6).

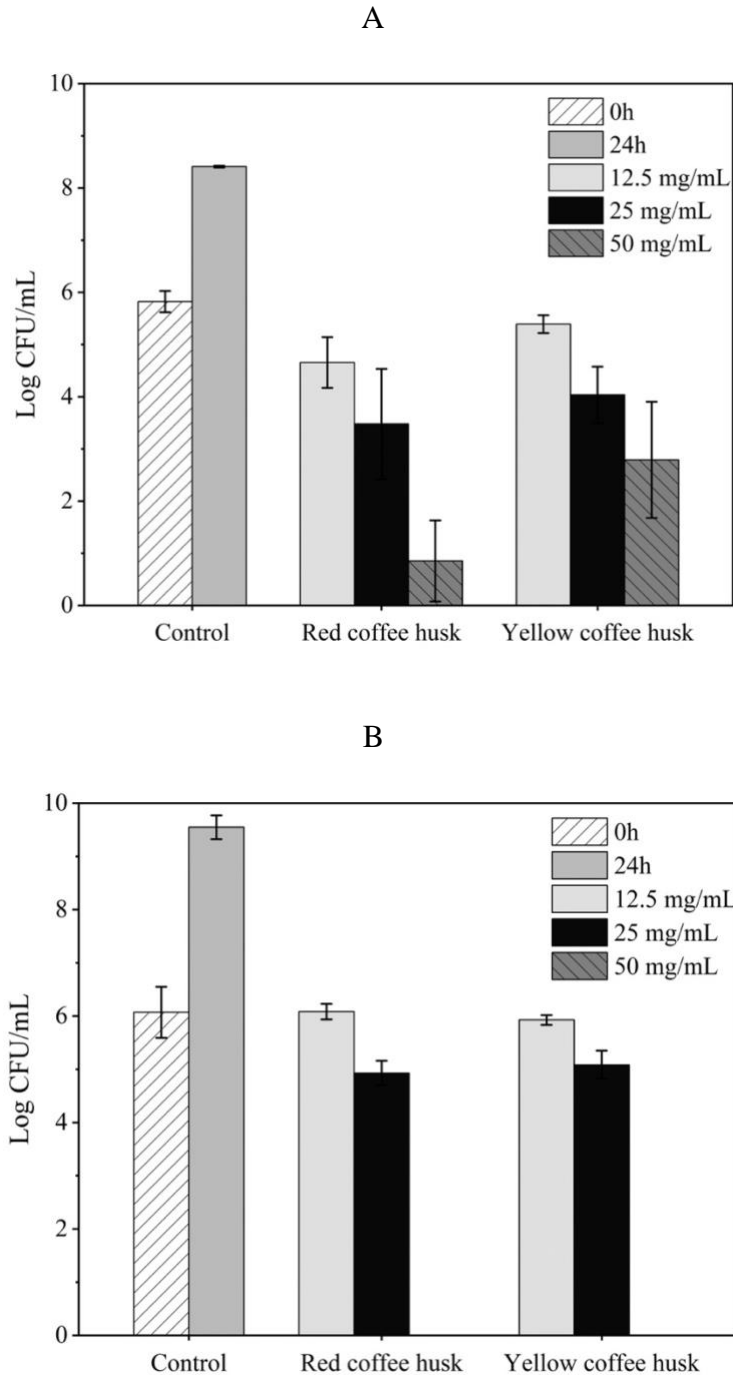


Figure 6 Concentration-dependent antimicrobial evaluation of red and yellow coffee husk extracts against (A) *L. monocytogenes* and (B) *S. Typhimurium*. The viable cell counts of all treatments were performed at an incubation time of 24 hours. The viable cell count of control was also performed at 0 hours. The experiments were repeated three times, and the results are expressed as mean \pm standard deviation.

4.3 Evaluation of Biofilm Formation by *L. monocytogenes* and *S. Typhimurium*

The effect of coffee husk extracts on the biofilm formation capability of *S. Typhimurium* and *L. monocytogenes* on polystyrene plates was determined. This was assessed at two sub-inhibitory concentrations 6.25 mg/mL (1/2 MIC) and 3.125 mg/mL (1/4 MIC).

Figure 7 illustrates the bacterial counts of formed biofilms. Neither red nor yellow coffee husk extract showed a significant effect on biofilm formation by *L. monocytogenes* (Figure 7A). For biofilms formed by *S. Typhimurium*, 3.125 mg/mL (1/4 MIC) coffee husk extracts did not reduce the bacterial count. At 6.25 mg/mL (1/2 MIC), red and yellow coffee husk extracts showed a significant effect ($p < 0.05$). The bacterial counts were 7.7 and 7.81 log CFU/ml, respectively, compared to 8.23 log CFU/ml for control (Figure 7).

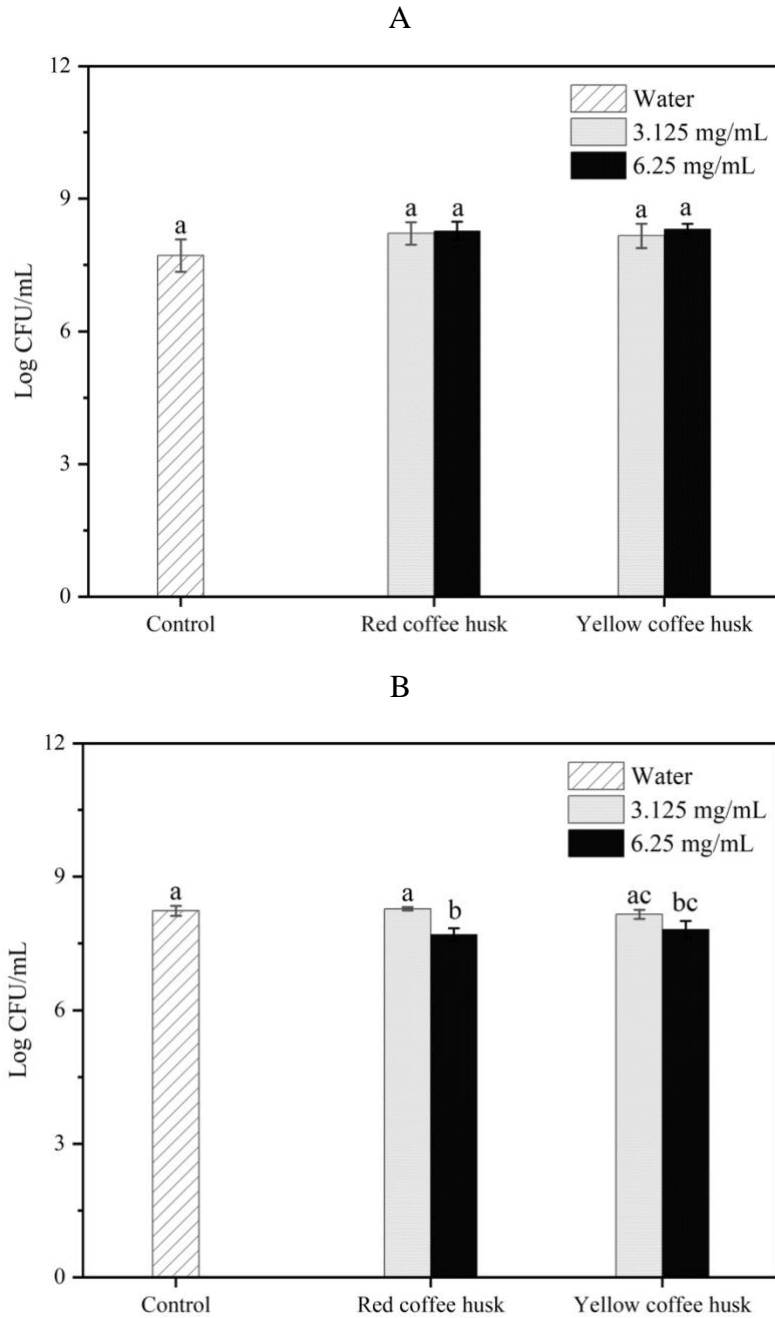


Figure 7 Effects of red and yellow coffee husk extracts at sub-inhibitory concentrations on biofilm formation by (A) *L. monocytogenes* and (B) *S. Typhimurium*. The viable cell counts of biofilms were performed at an incubation time of 24 hours. The experiments were repeated three times, and the results are expressed as mean \pm standard deviation. Means in the same figure with different letters are significantly different ($p < 0.05$).

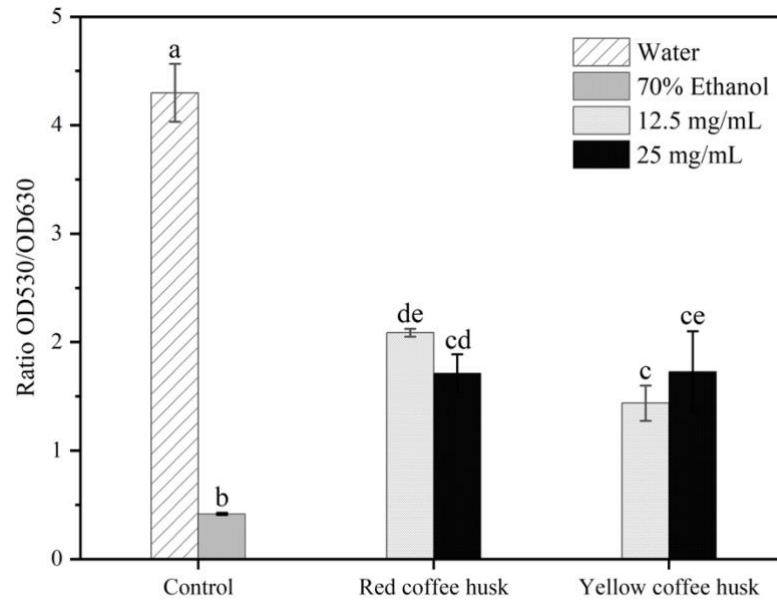
4.4 Membrane integrity of *L. monocytogenes* and *S. Typhimurium*

After bacterial cells were stained by the Live/Dead BacLight reagent, OD530 represented cells with a complete cell membrane that would show a green fluorescence, whereas OD630 represented cells with an incomplete cell membrane that would show a red fluorescence. The ratio of OD530/OD630 reflected the relative amounts of cells with intact membranes to those with compromised membranes. This method was used to determine the effect of coffee husk extracts on bacteria cell membrane integrity and understand the mode of related antimicrobial activity. The concentrations of coffee husk extracts chosen for this analysis were 12.5 mg/mL (MIC) and 25 mg/mL (2 MIC).

As shown in Figure 8A, both coffee husk extracts at both concentrations caused significant damage to the cell membrane of *L. monocytogenes*, compared to the negative control treated with water. Nevertheless, all coffee husk treatments exhibited less effect than the positive control treated with 70% ethanol. *L. monocytogenes* cells treated with 12.5 mg/mL yellow coffee husk extract had more compromised membranes than those treated with 12.5 mg/mL red coffee husk extract.

Regarding the impact of coffee cherry extracts on the cell membrane of *S. Typhimurium* (Figure 8B), the damage appeared to be concentration-dependent. However, there was no significant difference between red and yellow coffee husk extracts at both concentrations. The OD530/OD630 values of all coffee husk treatments were significantly lower than that of the negative control. Notably, the cell membrane damage on *S. Typhimurium* caused by 25 mg/mL red and yellow coffee husk extracts was comparable to the positive control treated with 70% ethanol.

A



B

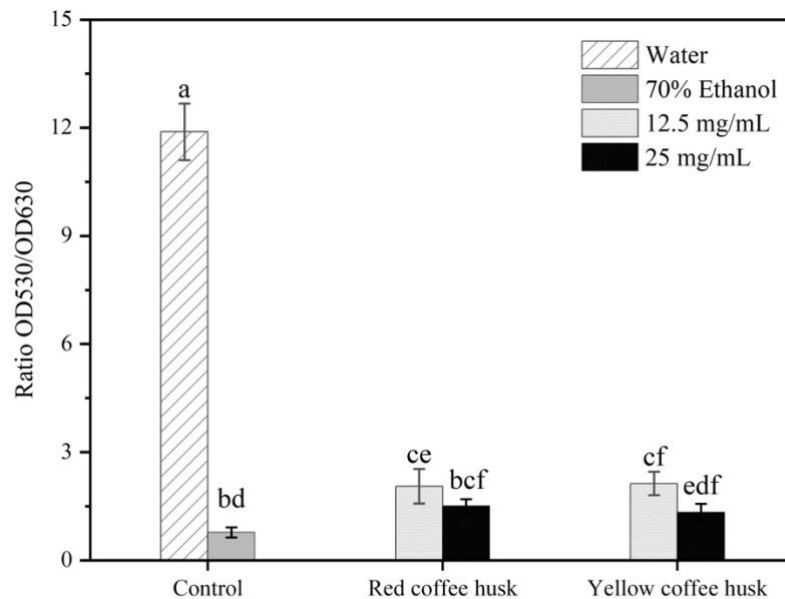


Figure 8 Effects of red and yellow coffee husk extracts on cell membrane integrity of (A) *L. monocytogenes* and (B) *S. Typhimurium*. Cells with intact membranes and cells with damaged membranes were analyzed using the Live/Dead BacLight Kit and fluorescence spectrophotometry at 530 nm and 630 nm, respectively. The experiments were repeated three times, and the results are expressed as mean \pm standard deviation. ($p < 0.05$)

4.5 Leakage of proteins in *L. monocytogenes* and *S. Typhimurium*

Loss of membrane integrity may lead to the leakage of cellular contents, which ultimately results in cell death. This was investigated by measuring proteins released from *L. monocytogenes* and *S. Typhimurium* cells treated by coffee cherry extracts. In the Coomassie protein assay, bovine serum albumin (BSA) solutions of known concentrations were used as standards. Therefore, the protein content is presented as BSA equivalent (Figure 9).

For *L. monocytogenes*, all coffee cherry treatments caused significantly higher protein leakage than the negative control. 25 mg/mL red coffee husk extract resulted in a protein concentration of 58.09 $\mu\text{g/mL}$ in the cell suspension, which was significantly higher than those caused by 12.5 mg/mL red coffee husk extract and yellow coffee husk extract at two tested concentrations. Moreover, both coffee husk extracts at 25 mg/mL led to significantly higher protein leakage than 70% ethanol ($p < 0.05$). It is worth noticing that the protein concentration of 25 mg/mL red coffee husk treatment was about three times that of the positive control (Figure 10A).

As shown in Figure 10B, all coffee husk treatments also caused significantly higher protein leakage in *S. Typhimurium* than the negative control. The protein concentrations of 12.5 mg/mL red coffee husk and both yellow coffee husk treatments were not significantly different from the positive control treated with 70% ethanol. 25 mg/mL red coffee cherry extract resulted in a protein concentration of 59.78 $\mu\text{g/mL}$, significantly higher than any other coffee husk treatments and controls ($p < 0.05$).

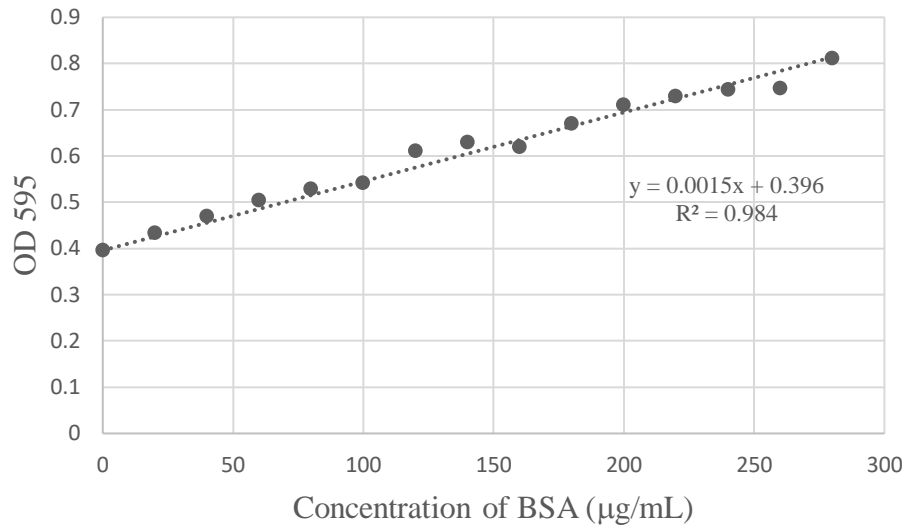


Figure 9 The standard curve for protein concentration calculation. The standard curve was generated with the absorbance of bovine serum albumin (BSA) solutions of different concentrations at the wavelength of 595 nm.

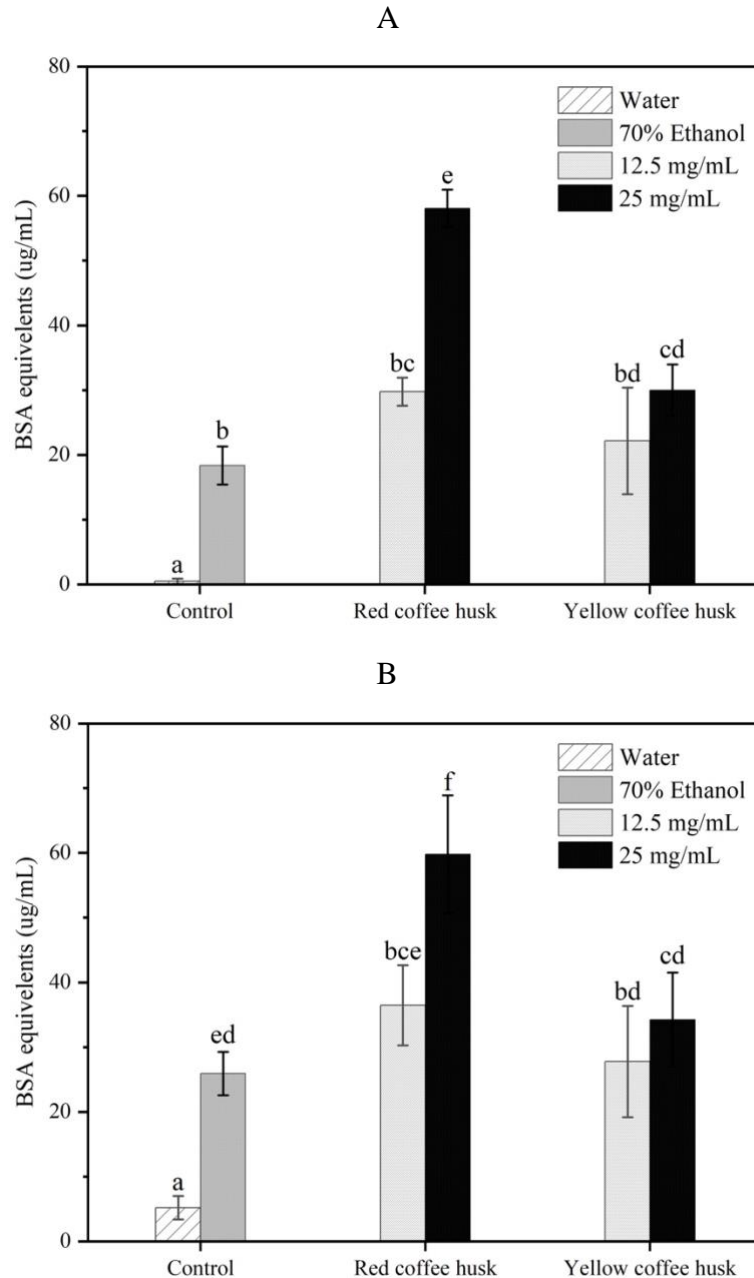


Figure 10 Effects of red and yellow coffee husk extracts on the leakage of proteins in (A) *L. monocytogenes* and (B) *S. Typhimurium*. Protein concentrations were quantified using the Coomassie Protein Assay Kit and spectrophotometry at 595 nm. The experiments were repeated three times, and the results are expressed as BSA equivalent mean \pm standard deviation. Means in the same figure with different letters are significantly different ($p < 0.05$).

4.6 Effect of Coffee husk Extracts on *L. monocytogenes* and *S. Typhimurium* in Milk

In inoculated milk stored at 7°C, neither *L. monocytogenes* nor *S. Typhimurium* gained significant growth within 72 hours. Compared to the controls, none of the coffee husk treatments showed significant antimicrobial effects on the bacteria (Figure 11).

The initial concentration of *S. Typhimurium* and *L. monocytogenes* were 6.26 and 6.28 log CFU/mL, respectively. At room temperature (21°C), the antibacterial effect of coffee husk extracts in milk appeared to be concentration-dependent (Figure 12). Against *L. monocytogenes*, 25 mg/mL coffee husk extracts showed significantly stronger inhibition than 12.5 mg/mL coffee husk extracts over 72 hours of incubation. Both red and yellow coffee husk extracts at 12.5 mg/mL and 25 mg/mL showed significant antibacterial effects at 24 hours. However, 12.5 mg/mL coffee husk extracts lost their effectiveness against *L. monocytogenes* at 48 and 72 hours. Compared to the control, 25 mg/mL red and yellow coffee husk extracts completely inhibited *L. monocytogenes* growth during the course and the bacterial level did not increase in milk (Figure 12A).

For *S. Typhimurium* inoculated in milk stored at 21°C, both coffee husk extracts at 12.5 mg/mL and 25 mg/mL showed significant antimicrobial effects over 72 hours, compared to the control. The only exception was 12.5 mg/mL yellow coffee husk extract, which resulted in a bacterial count lower than but not significantly different from the control at 48 hours. The antimicrobial effect of 25 mg/mL coffee husk extracts was significantly stronger than 12.5 mg/mL coffee husk extracts. Again, both coffee husk extracts at 25 mg/mL suppressed *S. Typhimurium* growth in milk during the storage (Figure 12B).

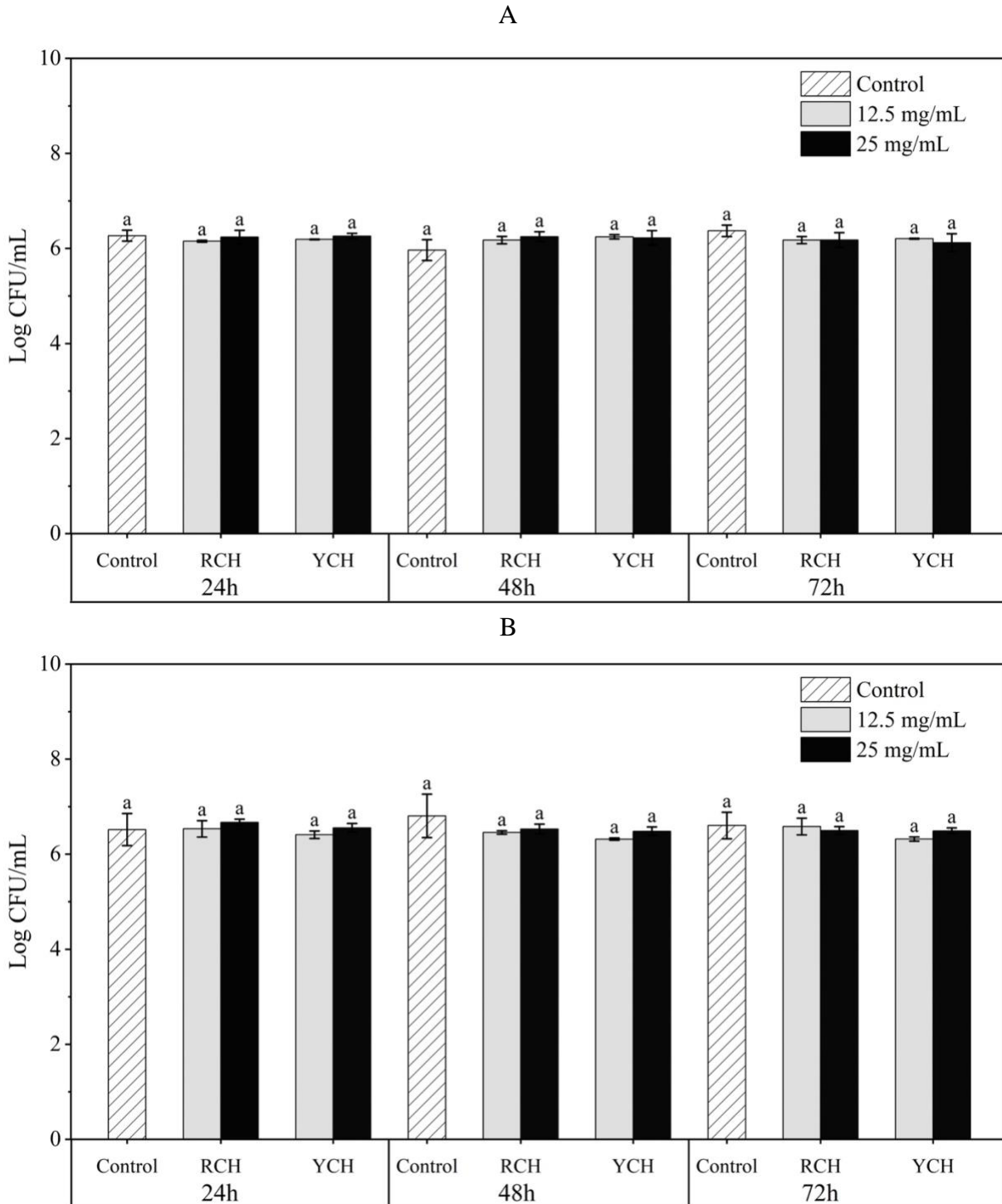


Figure 11 Effects of red coffee husk (RCH) and yellow coffee husk (YCH) extracts at concentrations of 12.5 mg/mL and 25 mg/mL on the growth of (A) *L. monocytogenes* and (B) *S. Typhimurium* in milk at 7°C for 72 hours. The experiments were repeated three times, and the results are expressed as mean \pm standard deviation.

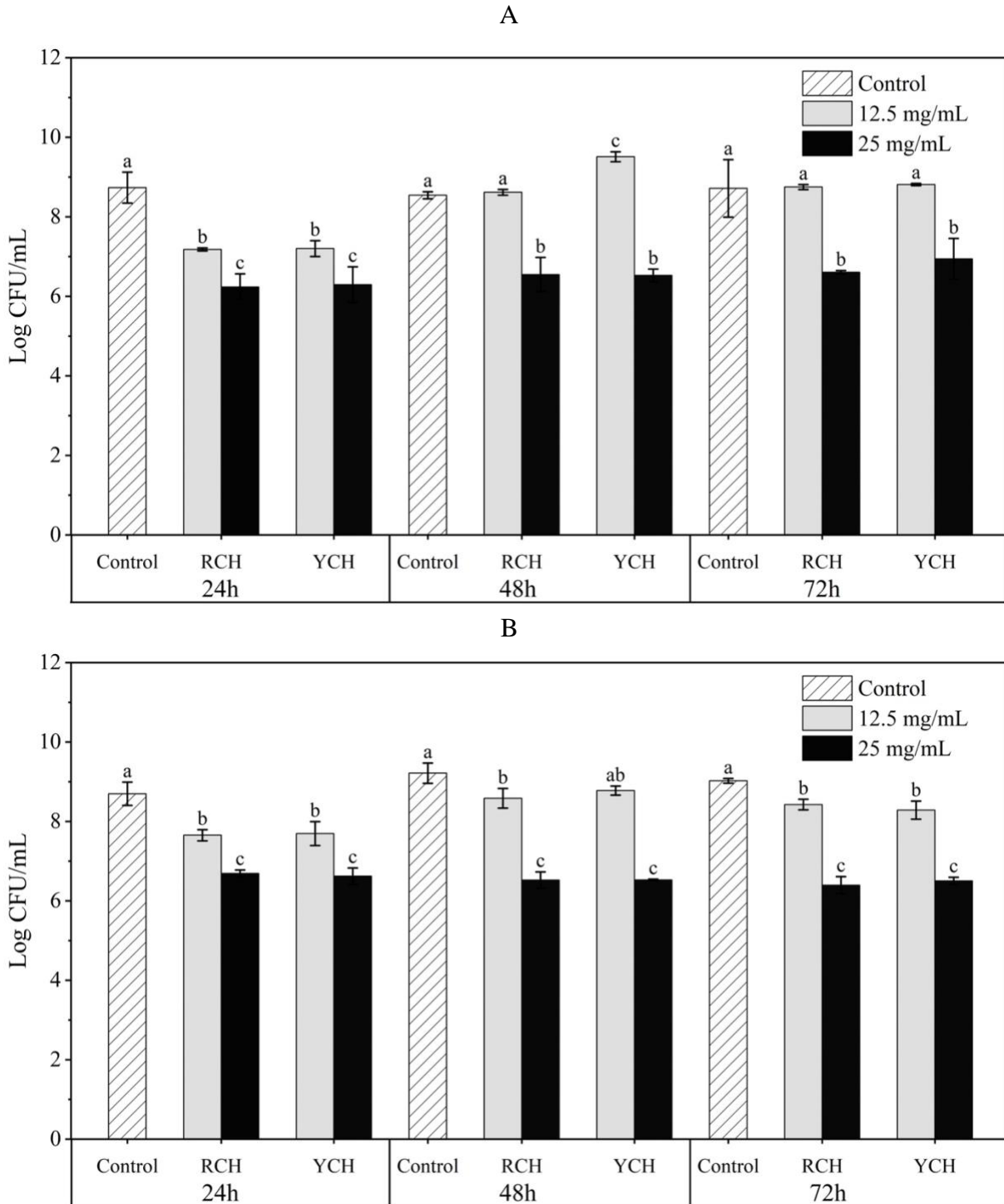


Figure 12 Effects of red coffee husk (RCH) and yellow coffee husk (YCH) extracts at concentrations of 12.5 mg/mL and 25 mg/mL on the growth of (A) *L. monocytogenes* and (B) *S. Typhimurium* in milk at 21°C for 72 hours. The experiments were repeated three times, and the results are expressed as mean \pm standard deviation. Within each time point, means with different letters are significantly different ($p < 0.05$).

Chapter 5

DISCUSSION

The evaluation of pH and organic acid content can help study the flavor, quality, and nutritional value of food. In a previous study, coffee husk had a pH of 4.3 (Kumar et al., 2018). This study found the pH of red and yellow coffee husk extracts to be 4.10 and 4.18, respectively, with organic acid content measured at 1.55% and 1.41%, respectively. To decrease the influence of pigment and other large organic molecules, including lipids and other hydrophobic impurities for the Folin-Ciocalteu method, the solid phase extraction method was used. The phenolic content was determined to be 5058.27 mg GAE/L and 4600.27 mg GAE/L for red and yellow coffee husk extracts, respectively. The discrepancy between these findings and other coffee cherry pulp of Heeger et al., (2017) may reflect differences in raw materials, extraction agents, temperature, and time (Antony & Farid, 2022). Further studies should aim to optimize the extraction process to enhance polyphenol extraction efficacy. In this study, the yellow coffee husk was shown to contain minimal anthocyanins, while the anthocyanin content of the red coffee husk extract was 22.03 mg/L when extracted by methanol. According to Esquivel et al. (2012), yellow coffee husks contained pigments like carotenoids of chloroplasts, violaxanthin, neoxanthin, lutein, β -carotene, α -carotene, and various lutein esters were also identified.

The antimicrobial tests showed that the MIC and MBC of red and yellow coffee husk extracts against *S. Typhimurium* and *L. monocytogenes* were both 12.5 mg/mL and 50 mg/mL, respectively. This indicates that red and yellow coffee husks have similar antimicrobial properties against these pathogenic bacteria. Notably, tested probiotic strains, *L. lactis*, and *L. rhamnosus* GG, grew even in the presence of 50 mg/mL coffee husk extracts, suggesting that the polyphenols in coffee husks have different effects on beneficial and pathogenic bacteria.

Polyphenols generally have adverse effects on most bacteria, but some lactic acid bacteria (LAB) can survive in the presence of polyphenols. High concentrations of polyphenols can negatively impact the membrane integrity of LAB, reduce the pH gradient, and delay carbohydrate metabolism within the cell (Filannino et al., 2014). However, when polyphenols are partially degraded, LAB can metabolize them. For example, strains of *Lactobacillus plantarum* can convert polyphenols into phenolic acids and aromatic compounds. This metabolic process breaks down polyphenols, thereby reducing their antimicrobial activity and partially mitigating their harmful effects on LAB (Filannino et al., 2018). In experiments involving the fermentation of polyphenol-rich elderberry juice by LAB, the levels of dihydrocaffeic acid and catechol increased while the concentrations of caffeic and protocatechuic acids decreased during fermentation. This change in chemical composition reflects the metabolism characteristics of LAB (Ricci et al., 2019).

This study was the first to explore the effects of coffee husk extracts on biofilm formation by pathogenic bacteria. The results demonstrate that at 6.25 mg/mL (1/2 MIC), red and yellow coffee husk extracts could reduce biofilm formation capability of *S. Typhimurium*. But coffee husk extracts at this concentration did not affect biofilm formation by *L. monocytogenes*. This difference may reflect the distinct biofilm formation capabilities of *S. Typhimurium* and *L. monocytogenes*, suggesting that a higher concentration of coffee husk extracts is needed to inhibit the latter.

This finding is particularly significant because biofilms confer increased resistance to antimicrobial agents and are a major concern in food safety and public health. Biofilms are communities of microorganisms attached to a surface and composed of exopolysaccharides and proteins (O'Toole et al., 2000). Polysaccharides composed of D-glucuronic acid can protect bacteria from some antimicrobial agents (Sutherland, 2001). If biofilms cannot be eliminated,

surviving bacteria within the biofilm can quickly reform the biofilm. Previous studies on sweet cherry stems with 30 mg GAE/g total phenolic content demonstrated 62.12% - 84.78% biofilm inhibitory effects on *Escherichia coli* (Afonso et al., 2020; Mahmutović-Dizdarević et al., 2022). In the present study, 6.25 mg/mL coffee husk extracts showed similar potential in reducing biofilm formation by *S. Typhimurium*. Compared to the control, red and yellow coffee husk extracts significantly reduced the bacterial levels in formed biofilms by 0.53 log and 0.42 log, respectively (Figure 7). Higher concentrations of coffee husk extracts may offer a promising natural alternative for controlling biofilm-associated infections and contamination in food processing environments.

Intracellular protein leakage is a clear indicator of cell membrane damage. This study demonstrated significant protein leakage in both *L. monocytogenes* and *S. Typhimurium* when treated with red and yellow coffee husk extracts. Notably, the intracellular protein leakage caused by 25 mg/mL (2 MIC) red coffee husk extract in *L. monocytogenes* and *S. Typhimurium* was significantly higher than that caused by 70% ethanol. Additionally, the intracellular protein leakage caused by 25 mg/mL (2 MIC) yellow coffee husk extract in *L. monocytogenes* was also higher than that induced by 70% ethanol. This leakage implies that the cell membranes could be compromised, leading to loss of essential intracellular contents. The loss of intracellular proteins has profound effects on bacterial viability. Proteins are critical for various cellular functions, including enzyme activity, structural integrity, and metabolic processes (Beynon & Bond, 1986). Leakage of proteins may disrupt these functions, ultimately leading to cell death. Therefore, the observed protein leakage indicates that coffee husk extracts exert bactericidal effects by compromising cell membrane integrity and inducing the loss of vital cellular components.

The evaluation of cell membrane integrity further supported the findings on intracellular protein leakage. This study showed that coffee husk extracts significantly disrupted the membrane

of both *L. monocytogenes* and *S. Typhimurium*, as evidenced by lower fluorescence ratios of OD530/OD630. This correlation between loss of membrane integrity and leakage of intracellular proteins confirmed the detrimental effects of coffee husk extracts on pathogenic bacterial cells.

Previous research has shown that polyphenols can damage cell membranes and alter cell morphology. Using electron microscopy, Yi et al. (2010) demonstrated that tea polyphenols could change the shape of *Pseudomonas aeruginosa*. The authors also compared the extracellular protein profiles of tea polyphenols-treated cells and untreated cells. They found that for the treated cells, there was a higher membrane protein content outside of the cell, including dihydrodipicolinate dehydrogenase, succinyl Co-A synthetase beta subunit, biotin carboxyl carrier protein, elongation factor Ts, 50S ribosomal protein, single-stranded DNA-binding protein, glycine cleavage system protein T2, and polyamine transport protein (Yi et al., 2010). These proteins control the metabolism, transportation, and DNA replication of bacteria. This finding suggests the potential for future studies to explore DNA binding and cell membrane potential to further understand the mode of antimicrobial activity of coffee husk extracts.

The applicability of coffee husk extracts as a natural food preservative was assessed in artificially contaminated milk. The results showed that 12.5 mg/mL red and yellow coffee husk extracts had significant effects on the growth of *L. monocytogenes* and *S. Typhimurium* in milk at room temperature (21°C). Both coffee husk extracts at 25 mg/mL completely suppressed the bacterial growth in milk during storage for up to 72 hours. However, except for the yellow coffee husk extract at 48 hours, none of the 12.5 mg/mL treatments grew. The control did not significantly change after growing to around 9 log CFU/mL at 24 hours.

In comparison, the antimicrobial properties of coffee husk extracts were less effective in milk than in broth media. In Muller Hinton broth, 12.5 mg/mL (MIC) red and yellow coffee husk

extracts inhibited the growth of *L. monocytogenes* and *S. Typhimurium* at 37°C (Figure 6). In milk, a concentration of 25 mg/mL was required to achieve a similar bacteriostatic effect at 21°C. This difference might reflect the effects of both incubation temperature and the food matrix. A previous study found that the MIC of vanillin against *L. monocytogenes* in broth medium and milk at 35°C for 24 hours was 3000 ppm and 4000 ppm, respectively. Interestingly, the MIC in milk at 7°C for 24 hours was 2500 ppm (Cava-Roda et al., 2012). This reduction in MIC at a lower temperature was not observed in the present study.

Additionally, milk constituents, such as fat, can reduce the antimicrobial properties of glycolipids against *L. monocytogenes* (Sun et al., 2021). β -lactoglobulin, a protein present in milk, can interact with tea catechins through hydrogen bonds, hydrophobic interactions, or van der Waals forces (Yildirim-Elikoglu & Erdem, 2018). The interactions between milk components and polyphenols may influence the antibacterial activity of coffee husk extracts. Moreover, the reduced effectiveness of preservatives at low temperatures might be due to the fact that microbial growth and metabolic activity slow down significantly. Preservatives, like other antimicrobial agents, need actively growing and metabolizing microorganisms to exert their antimicrobial effects. At room temperature or higher, microbes are more active and thus more susceptible to these agents. However, in refrigeration, the reduced metabolic activity of microbes means they are not effectively targeted by preservatives, leading to prolonged bacterial survival (Bongaerts & Jansen, 2003).

Chapter 6

CONCLUSION AND FUTURE WORKS

The increasing concerns regarding pathogenic bacteria, particularly the significant number of annual foodborne illness infections, hospitalizations, and deaths, underscore the urgent need for developing new antimicrobial agents. The rise of antibiotic-resistant bacterial strains exacerbates these concerns. Infections caused by *S. Typhimurium* and *L. monocytogenes* are particularly alarming due to their high rates of hospitalization and mortality among foodborne pathogens.

In response to these challenges, the development of new antimicrobial agents is not only crucial but also promising. Additionally, the adverse effects of traditional chemical antimicrobial preservatives on human health, coupled with a growing preference for natural foods and an interest in finding value in by-products from food processing, have fuelled interest in the antimicrobial potential of natural extracts. Previous studies have demonstrated that plant extracts possess significant antimicrobial properties, indicating their potential as natural preservatives, which could reduce the reliance on antibiotics and synthetic chemical preservatives.

Coffee cherry, a crop widely cultivated in Hawaii, is rich in polyphenolic compounds and has shown some antimicrobial properties. Coffee husk is the by-product of coffee cherry during the processing of coffee beans. This study aimed first to analyse the biochemical composition and antimicrobial activity of red and yellow coffee husks. Secondly, it sought to explore their antimicrobial mechanisms. Lastly, it examined the antibacterial efficacy of coffee husk extracts in milk. The study included both Gram-positive and Gram-negative pathogenic bacteria, as well as probiotic lactic acid bacteria, to assess their susceptibility to coffee husk extracts.

The investigation into the characteristics of coffee husks, particularly their polyphenol content, formed the foundation of this research, as polyphenols are known for their antimicrobial

properties and concentration dependency. The total phenolic content in red and yellow coffee husk extracts was found to be 5058.27 mg/L and 4600.27 mg/L, respectively, confirming the feasibility of proceeding with the experiments.

Subsequent testing determined that the MIC and MBC against *S. Typhimurium* and *L. monocytogenes* were 12.5 mg/mL and 50 mg/mL, respectively, for red and yellow coffee husk extracts. Additionally, both coffee husk extracts inhibited biofilm formation by *S. Typhimurium* even at a low concentration of 6.25 mg/mL. Notably, tested probiotic strains demonstrated remarkable resilience to high concentrations of the extracts, which can resist the extracts at 50 mg/mL.

Having confirmed the antimicrobial potential of coffee husk extracts, this study investigated their antimicrobial mechanisms. Research on cell membrane integrity and intracellular protein leakage indicated that polyphenol extracts from coffee husks damage the cell membrane, causing the efflux of intracellular substances, including proteins. This is likely one of the reasons for cell death.

Finally, this study explored the antimicrobial efficacy of coffee husk extracts in milk. Results showed significant antibacterial effects on *S. Typhimurium* and *L. monocytogenes* inoculated in milk that was stored at room temperature (21°C), with inhibition lasting up to 72 hours at a concentration of 25 mg/mL. However, the extracts were not effective at refrigerator temperature (7°C) in the concentration range used in this study.

In conclusion, this study has enhanced our understanding of the antimicrobial properties of various compounds present in coffee husks, highlighting their potential applicability in food preservation and providing a foundation for future research. This study primarily focused on the correlation between total phenolic content and antimicrobial effects. Future research could

investigate the specific polyphenolic components in coffee husks and optimize extraction methods for enhanced antimicrobial efficacy. Additionally, exploring the detailed antimicrobial mechanisms could include examining the impact on intracellular components or sites, such as determining whether leaked proteins include enzymes or transport proteins or assessing changes in cell membrane potential. Understanding why cells lose viability - whether due to disrupted metabolism or the inability to replicate DNA - could provide deeper insights. Furthermore, research on the effects of the extracts on cell motility and biofilm dispersal can also be conducted. This would provide valuable insights into the impact of coffee husk extracts on bacterial populations and their behavior. Lastly, based on the finding that high-concentration extracts did not inhibit probiotics, future research could explore coffee husk extracts' antimicrobial and anti-inflammatory effects on the human gut using an in vitro human cell model.

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