Efficacy of cleaning and sanitation methods in reducing foodborne pathogens on common food contact surfaces in Cambodian fresh food markets

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Abstract

Fresh food markets are common shopping destinations in many parts of the world, especially in low- and middle-income countries (LMICs). However, these markets are also known for being potential sources of microbial contamination. Foodborne pathogens such as *Salmonella enterica* (*Salmonella*) and *Escherichia coli* (*E. coli*) are the two most common bacteria associated with fresh produce and animal source foods (ASF). These pathogens can contaminate food at any stage of the food value chain, from farm to table. In LMICs, these pathogens are often found in food items sold in fresh food markets. In fresh food markets in Cambodia, natural food packaging materials such as banana leaves and bamboo baskets are commonly used as food contact surfaces to store, transport, and display food items. These materials are typical used following traditional practices with the lack of hygiene and sanitation measures. Without proper cleaning and sanitation, *Salmonella* and *E. coli* can attach and persist on these surfaces and cross-contaminate other food products, presenting health risk to both market vendors and consumers. Therefore, there is a need to understand how to effectively control both *Salmonella* and *E. coli* on food contact surfaces typically used in fresh food markets.

The objectives of this study were as follows: 1). demonstrate the attachment of *Salmonella* and *E. coli* on banana leaf and bamboo basket surfaces; 2). evaluate the efficacy of cleaning and sanitation methods in reducing *Salmonella* and *E. coli* experimentally inoculated on banana leaf and bamboo basket surfaces; 3). suggest possible food safety interventions for fresh food markets in Cambodia.

To determine the antimicrobial effectiveness of cleaning and sanitation methods in reducing *Salmonella* on banana leaf and bamboo basket surfaces, 15 different treatments combining cleaning and sanitation methods were tested. Each treatment was carried over for 3 or

5 min. The cleaning methods include wiping, scrubbing, and no cleaning (i.e., control), whereas the sanitation methods comprise of 200 ppm bleach, 400 ppm peracetic acid (PAA), and no sanitation (i.e., control). Banana leaf and bamboo basket surfaces were inoculated with an overnight culture of a *Salmonella* cocktail. Surfaces were then cleaned and sanitized in accordance with each defined treatment. Survival populations of *Salmonella* were neutralized and enumerated. Data suggested that PAA was the most effective sanitizer in reducing *Salmonella* (4.31 log CFU/cm^2 reduction, 95% confidence interval [CI; 4.14, 4.46]) on banana leaf surfaces regardless of cleaning method or contact time applied. Statistical analysis indicated a significant interaction between cleaning and sanitation method on banana leaf surfaces, suggesting that efficacy of sanitation was dependent on the cleaning method used. Wiping followed by bleach for 5 min significantly reduced *Salmonella* by 2.90 log CFU/cm^2 (95% CI [2.72, 3.09]) on banana leaf surfaces as compared to the control (i.e., no cleaning and no sanitation). On the other hand, there is no evidence of any significant interaction between cleaning and sanitation method in reducing *Salmonella* on bamboo baskets. The most effective treatment on bamboo baskets was the use of PAA for 5 min achieving a 2.26 log CFU/cm^2 reduction of *Salmonella* as compared to the control.

A similar experimental design was followed to examine the effect of cleaning and sanitation method in reducing *E. coli* on banana leaf and bamboo basket surfaces. Data showed that a significant reduction of *E. coli* on bamboo baskets were dependent of both cleaning and sanitation applied, highlighting their interactions or combined effects. On bamboo baskets, the most effective treatment was the use of PAA for 5 min following no cleaning, reducing *E. coli* by 4.03 log CFU/cm^2 as compared to the control. On banana leaf surfaces, PAA reduced *E. coli* counts to below the limit of quantification (1.44 log CFU/cm^2) reaching a 3.70 log CFU/cm^2 reduction regardless of the cleaning method or contact time examined.

The results of this study highlight the importance of cleaning and sanitation practices in fresh food markets. The implementation of proper cleaning and sanitation for food contact surfaces is crucial in reducing the risk of contracting foodborne illnesses associated with the consumption of food originated from fresh food markets. Stakeholders may utilize this finding to select an appropriate food contact surface and develop effective cleaning and sanitation procedures that may contribute to ensuring the safety of consumer.

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Chapter 1 - Literature Review

1.1 The global burden of foodborne diseases

Foodborne diseases (FBD) are the result of consuming contaminated food. Common food contaminants include bacteria, viruses, parasites, toxins, or noninfectious chemicals (WHO, 2022). Contamination can occur naturally or during production, processing, and preparation of food products. It has been estimated that over 200 diseases are caused by contaminated food with health consequences ranging from common diarrhea to cancer (WHO, 2015). Globally, FBD are considered both a public health and economic threat. While most common foodborne illnesses cause self-limiting symptoms such as nausea, vomiting, diarrhea, and dehydration; some illnesses can be severe. Children, elderly, and immunocompromised populations can develop moderate to severe infections that may requires hospitalization and/or lead to death (Buzby, 2001). Additionally, research has suggested that FBD is associated with long-term public health impacts among children in the early years of childhood. For example, recent data correlated FBD with stunting, increased risk of non-communicable disease, and poor cognitive development (Guerrant et al., 2013). In fact, a recent study published by a Joint Child Malnutrition Estimate (JME) group - a collaboration between United Nations International Children's Emergency Fund (UNICEF), World Health Organization (WHO) and World Bank (WB) - showed that 22% of children under the age of 5 in 2020 were affected by stunting and a majority of these children were located in low and middle-income countries (LMICs) (WHO, 2021). Recent data has begun to illuminate the relationship between LMICs and the burden of FBD, suggesting that said burden is substantial (Jaffee et al., 2018).

Despite a persistent threat to socio-economic development due to financial losses from medical expenses and productivity loss, the full extent and burden of FBD remain uncertain. The first estimates of the global burden of FBD were launched by WHO in 2006 and led to the establishment of Foodborne Diseases Burden Epidemiology Reference Group (FERG). FERG conducted a study to quantify the burden of FBD based on 31 selected foodborne hazards including bacteria, viruses, parasites, chemicals, and toxins. Following a hazard and incidence-based approach, FERG translated baseline epidemiological data into a mathematically time-based measure called Disability Adjusted Life Years (DALY) which represents the number of life years lost due to illness, disability, or death (Devleesschauwer et al., 2014; Havelaar et al., 2015). These findings were published in 2015 and estimated that the consumption of unsafe food causes 600 million cases of FBD, 420,000 deaths, and 33 millions DALYs globally each year (WHO, 2015). FBD affects both developed and developing nations, with the burden intensifying in countries with developing economies (Hoffmann & Scallan, 2017).

In the United States (U.S.), according the U.S. Centers for Disease Control and Prevention (US-CDC) one in six people get sick due to unsafe food and 3,000 people die annually (Scallan et al., 2011). In Canada, it is estimated that one in eight people contracts FBD each year (Thomas et al., 2013). In Australia, a study in 2010 estimated that in average Australians experience an episode of FBD once every 5 years (Kirk et al., 2014). Beside public health impacts, Jaffee et al., (2018) published the first ever estimates of the financial burden of FBD. These publications stated that FBD contributes to an estimate of financial loss of 110 billion USD annually in LMICs in which 95.2 billion USD are attributed to productivity loss and 15 billion USD loss to the medical treatment. In the Netherlands, cost associated with FBD accounts for 486 million Euros annually while in New Zealand six major pathogens impose 86 million NZD in cost of disease each year

(Lake et al., 2010; Mangen et al., 2015). In addition to these costs, foodborne outbreaks cause significant damage to the food industry, impact a country's reputation, and disturb global food supply chain (Adley & Ryan, 2016). Foodborne outbreaks result in economics loss for food companies, including the cost of recalling and destroying contaminated products, legal action, and the loss of consumer trust. In addition to these financial burdens, outbreaks can have a long-term reputational damage to food companies (Hussain & Dawson, 2013). Furthermore, foodborne outbreaks can also impact a country's international reputation and trade relations. The detection of contaminated food products can lead to increased restrictions on imports and ultimately disrupt the global food supply chains (Buzby & Unnevehr, 2004). These data highlight the significance of prioritizing food safety research and developing effective food safety interventions to reduce foodborne outbreaks and ensure the global food supply chain.

1.2 Foodborne pathogens of concerns – *Salmonella* and *Escherichia coli*

1.2.1 Salmonella

The genus *Salmonella* belongs to the family Enterobacteriaceae and is a medically important pathogen for both animals and humans. *Salmonella* is the leading cause of foodborne bacteria illnesses and the second most common foodborne pathogen in the U.S. with an estimate of 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually (CDC, 2022). In addition, *Salmonella* ranks the highest in the estimates of the economic cost of FBD followed by *Toxoplasma gondii,* and *Listeria monocytogenes* (ERS, 2018). Infection with *Salmonella* causes two major types of diseases in humans including typhoidal salmonellosis (*Salmonella typhi* and *Salmonella paratyphi*) and non-typhoidal salmonellosis. Non-typhoidal salmonellosis is often caused by the consumption of animal-source foods (ASF) such as poultry, eggs, seafood, beef and

pork as well as fresh fruits and vegetables (Brands et al., 2005; Nguyen et al., 2021; Rortana et al., 2021; Trongjit et al., 2017).

Salmonella is a gram-negative, rod-shaped, non-sporulating, facultatively anaerobic bacterium that colonizes the intestinal tracts of animals and humans. The *Salmonella* genus comprises of two main species: *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* contains six subspecies according to the antigenic specificity including *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. According to the WHO Collaborating Center for Reference and Research on *Salmonella*, the genus of *Salmonella* consists of over 2,579 serovars, with 22 of those belonging to *Salmonella bongori* (Grimont & Weill, 2007). Serology is an essential method in identifying the complete antigenic formulas of *Salmonella* isolates. Determination of antigenicity depends on somatic (O) lipopolysaccharides (LPS) on the external surfaces of the bacterial outer membrane, flagellar (H) antigens on peritrichous flagella, and virulent capsular (Vi) antigens. Of all serovars discovered, 100 of them are found to be important in public health causing illnesses in animals and humans.

Although *Salmonella enterica* (henceforth referred to as *Salmonella*) serotype Typhimurium and Enteritidis are the most frequent serotypes causing nontyphoidal salmonellosis worldwide, it is worthy to note that other serotypes are emerging and circulating in certain places (Biswas et al., 2019; Jajere, 2019). For example, *Salmonella* Rissen was reported as one of the top three serotypes associated with swine production in Southeast Asia and Europe (J. W. Schmidt et al., 2012), while *Salmonella* Corvallis was associated with international travelers who visited Southeast Asian countries (Thailand, Cambodia, and Vietnam) and contracted diarrheal diseases such as salmonellosis from contaminated food or water sources (McDowall, 2010). *Salmonella* 4,[5],12,:i:- is a monophasic variant of *Salmonella* Typhimurium and is an emerging serotype

commonly isolated from pig, pork, and humans worldwide. This serotype has been reported to be resistant to multiple antibiotics, heavy metals, and sanitizers (Clark et al., 2020; de Quadros et al., 2020; Elnekave et al., 2018). A study investigating *Salmonella* in environmental samples in Cambodian fresh food markets, (Schwan et al., 2021) identified 16 *Salmonella* serotypes across multiple food contact surfaces (FCS) and non-food contact surfaces (NFCS). Rissen (18 isolates), Hvittingfoss (11), Corvallis (10) were among the three most isolated serotypes circulated on FCS and NFCS in Cambodian fresh food markets (Schwan et al., 2021). In addition, one isolate collected from cardboard surfaces was identified as *Salmonella* 4,[5],12,:i:, indicating that this serovar is prevalent in Cambodian fresh food markets (Schwan et al., 2021). The circulation of various *Salmonella* serotypes in fresh food markets in Cambodia presents public health risk to the country's food system.

1.2.2 Escherichia coli

Escherichia coli (*E. coli*) is a gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacterium belonging to the genus of *Escherichia* which inhabits the intestinal tracts of warm-blooded animals, including humans (Nataro & Kaper, 1998). *E. coli* cells are typically small rods, ranging from 1.1 to 1.5 μm in width and 2 to 6 μm in length. This bacterium can either be motile and nonmotile. The motility of this bacteria depends on the ability to produce flagella which are typical polar. In addition to flagella, many strains of *E. coli* possess the ability to produce pili (a polymer of pilin) that are surface organelles anchored to the outer membrane and extend outwards from bacterial surfaces (Desmarchelier & Fegan, 2002). This particular property plays an important role in bacteria-host responses by facilitating adherence to host cells which are critical in the first step of colonization and biofilm formation (Sauer et al., 2000).

Although most *E. coli* strains are non-pathogenic commensals, several groups of *E. coli* are pathogenic that can cause a variety of diseases ranging from common gastrointestinal diseases to sepsis, meningitis, and urinary tract infections (Percival & Williams, 2014). Groups that cause diarrheal diseases are commonly spread through contaminated food, water, direct contact with animal or people carrying the diseases. Infections with *E. coli* are characterized by bloody diarrhea, stomach cramps, vomiting, and usually symptom persist from 5 to 7 days (Dennehy, 2005). Children under the age of 5, adult aged 65 and older, pregnant women, and individual with immunocompromised immune system tend to develop more severe illnesses compared with the general population (Smith & Fratamico, 2017).

The characterization of *E.coli* is mainly based on serotyping the somatic (O), flagella (H), and capsular (K) antigens present on the surfaces of bacteria. Somatic O antigens consists of the outer lipopolysaccharide chain connected to the core lipopolysaccharide (LPS) of gram-negative bacterial cell wall. Flagella (H) antigens are fine long tubular structure composed of a polymer of flagellin. Capsular (K) antigens are composed of high molecular weight of polysaccharide that forms coat outside the bacteria. Based on clinical infections and virulence factors, *E. coli* causing human disease are classified into six groups: Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Shiga Toxin-Producing *E. coli* (STEC), and Diffusely Adherent *E. coli* (DAEC) (Meng et al., 2012).

In the United States, the economic burden of *E. coli* in 2018 was about 342 million USD resulting from 175,905 reported cases. Despite resulting in twice lower cases, Shiga toxin-producing *E. coli* (STEC O157) costs ten times higher compared to (STEC non-O157) (ERS, 2021). In addition, CDC estimates that 3,600 hospitalization and 30 deaths are attributed to STEC each year in the United States. One of the deadliest *E. coli* outbreaks in United States occurred in

2006 linked to spinach originating from California. This muti-state outbreak led the death of four people and was attributed to 199 cases across 26 states, and 31 of the total patients developed Hemolytic Uremic Syndrome (HUS) caused by *E. coli* O157:H7 that was isolated from cattle fields near the growing site (CDC, 2019).

In Cambodia, the public health and economic burden attributed by *E. coli* is unknown. However, previous studies have shown *E. coli* contamination on various vegetables sold in fresh food markets and distribution centers in Cambodia (Desiree et al., 2021; Phoeurk et al., 2019; Schwan et al., 2022). A study investigating prevalence of *E. coli* in fresh vegetables collected from different types of markets in Phnom Penh reported that the prevalence of *E. coli* on lettuce, herb, and cucumber was estimated at 31%, 36%, and 6%, respectively (Phoeurk et al., 2019). Desiree et al., (2021) studied the prevalence of *E. coli* on vegetables samples and found that 29% of all 312 vegetables collected from fresh food markets in Cambodia were contaminated with *E. coli*.

1.3 Overview of food safety in Cambodia

Cambodia is a developing nation in Southeast Asia with a land area of 181,035 square kilometer. The country shares its borders with Thailand to the northwest, Laos to the north, Vietnam to the east, and the Gulf of Thailand to the southwest. In the last two decades, Cambodia experienced a rapid development that transitioned the nation from a low-income to a lower-middle income country in 2015 (Ehrentraut, 2011). With an average annual economic growth of 7.7% between 1998 to 2018 the country aims to become upper middle-income nation by 2030 (World Bank, 2022). In 2021, the population reached 16.9 million, of which nearly 61% live in rural areas (NIS, 2020), and 77% of the rural households rely heavily on agriculture, fisheries and forestry (USAID, 2022). It is estimated that 18% of Cambodians live below the national poverty line (2.70$) and 45% have no access to a sufficient quantity of affordable and nutritious food. Poverty

and food insecurity are two major drivers for diarrheal diseases among the population (USAID, 2022). Globally, up to 9% of deaths in children under the age of 5 are due to diarrheal diseases contracted from contaminated food or water (UNICEF, 2022). Between 2014 to 2019, the Cambodian Ministry of Health (MOH) reported 134 FBD outbreaks that resulted in 5,825 cases, 5,598 hospitalizations and 81 deaths (Mekong Institute, 2019). However, it is worth noting that estimating the burden of FBD poses a challenge for LMICs, such as Cambodia. This is due to the fact that patients rarely seek medical attention and even when they do, cases are mostly not reported to public health authorities (Hoffmann, 2022). In Cambodia, large-scale foodborne outbreaks are investigated by an event-based surveillance and most of the time the etiological agent causing the outbreaks is not identified (Thompson et al., 2021; Vandy et al., 2012). In addition, most foodborne diseases occurring at home are under-diagnosed and/or under-reported (Redmond & Griffith, 2003). A study conducted by the Cambodian Communicable Diseases Control Department (CCDC) reported that the FBD hospitalization cost is relatively high ranging from 25 to 185 USD with an average of 63 USD per case (Srey, 2021). Several factors were responsible for these foodborne outbreaks including poor hygiene and sanitation practices, temperature abuse, cross contamination, and the use of water and materials from unsafe sources (Mekong Institute, 2019). In addition, lack of established and enforced food safety infrastructures, along with developing health systems, makes the population more vulnerable to foodborne outbreaks.

Cambodia's first food safety law was signed and promulgated on June 8, 2022, by the King Norodom Sihamoni after passing through years of revision. The law was designed to provide frameworks and mechanisms to manage and ensure the quality, safety, hygiene, and authenticity of food through the food value chain, as well as provide public health and food safety protection for consumers and ensure fair food trade. Despite significant efforts towards improving food

safety, the existing policies are still limited and insufficient (Thompson et al., 2021). Most importantly, food safety is an inter-ministerial and shared responsibility, regulated by six different ministries: Ministry of Agriculture, Forestry, and Fisheries (MAFF), the Ministry of Industry, Science, Technology, and Innovation (MISTI), Ministry of Commerce (MOC), Ministry of Health (MOH), Ministry of Tourism (MOT), and Ministry of Economy and Finance (MEF). Although legally recognized duties and responsibilities have been established, overlapping areas in performance has continuously lowered the effectiveness of the implementation of food safety and quality control systems (ILRI, 2021). To facilitate barriers and achieve improved food safety measure in Cambodia, Thompson et al., 2021 proposed four strategic approaches including: i). enhancing surveillance and identification of etiology of foodborne diseases, ii). identifying effective food safety innovations, iii). facilitating food safety behavior change, and iv). improving access to clean water in rural areas (Thompson et al., 2021). Food safety is a key driver to reduce malnutrition and an important building block to achieve positive public health outcomes. Unless food safety agendas are prioritized and committed, foodborne disease outbreaks will remain a persistent threat to public health and economic opportunity.

1.4 Fresh food market system in Cambodia

Fresh food markets can be found everywhere in the world. Typically, fresh food markets (i.e., informal markets, wet markets, or traditional markets) consist of a large collection of open-air stalls where fresh fruits, vegetables, seafood, and meat from domesticated animals are sold (Figure 1.1). In Europe and America, fresh food markets are broadly comparable to farmer's markets. In Asia, fresh food markets are a staple and the majority of the population relies on these markets for their affordable food sources (Maron, 2020). Nevertheless, most fresh food markets escape effective health and safety regulation and are often untaxed and unlicensed (Grace, 2015).

In Cambodia, fresh food markets play a crucial role in shaping the country's food culture, lifestyle, and economy. They can be found throughout the country and are popular amongst Cambodians since food items are often cheaper and fresher compared to supermarkets. Fresh food markets are also places where locals greet and socialize. Food products sold by market vendors are very diverse and mostly consist of a variety of animal meats and vegetables including chicken, pork, beef, fish, seafood, minimally processed food, home-cook meals, and different types of fruit and vegetables. In most stalls, vendors either sell only animal meat, vegetables, or fruits. However, in some places, animal meat, fruits, and vegetables are displayed in close proximity and sold by a single market vendor, increasing the potential occurrence of cross-contamination between products. Furthermore, food products are stored at room temperature and in open-air stall directly exposed to the environment (Muramastu et al., 2020). In some places, fresh food markets are located on the side of a street with heavy vehicles and people traffic. Food items are typically displayed for sale on plastic mats, bamboo baskets, cardboard, lotus flower leaves, or banana leaves. When food contact surfaces are not properly cleaned and sanitized, cross-contamination can occur, resulting in a potential risk of a foodborne hazard to the consumers. Insufficient food safety infrastructure, lack of hygiene and sanitation practices, and unregulated fresh food market systems present high risk of microbial contamination. Therefore, there is an urgent need to examine potential interventions to enhance hygiene and sanitation practices at fresh food markets to ensure the safety and well-being of the consumers.

1.5 Microbial contamination in fresh food markets in Cambodia

Several studies have reported the contamination of food items sold in Cambodia's fresh food markets (Desiree et al., 2021; Lay et al., 2011; Phoeurk et al., 2019; Rortana et al., 2021; Schwan et al., 2021; Trongjit et al., 2017). Lay et al., (2011) reported that raw poultry sold in fresh

food markets in Phnom Penh were highly contaminated with *Salmonella* spp. and *Campylobacter* spp. with an estimated prevalence of 88% and 81%, respectively. In a study published in 2017 investigated bacterial contamination in vegetables sold in various types of fresh food markets in Phnom Penh, the author reported that the prevalence of total coliforms, *E. coli*, and *Salmonella* on lettuce samples were 97.2%, 30.7% and 55.7%, respectively. Similarly, 97.2%, 36.1%, and 55.7% of saw-leaf herb samples were contaminated with total coliform, *E. coli*, and *Salmonella*, respectively. In addition, total coliforms, *E. coli*, and *Salmonella* were detected in 50%, 5.7%, and 11.1% of cucumber samples (Phoeurk et al., 2019). Another study on 68 fermented vegetables samples collected from wet markets in Phnom Penh detected the presence *Enterococcus* spp., *Bacillus* spp. *E. coli* from 34%, 31%, 24% and 10% of samples, respectively (Chrun et al., 2017). Desiree et al., (2021) studied the presence of coliforms, generic *E. coli*, and *Salmonella* in lettuce, cucumbers, and tomatoes in two provinces in northeast part of Cambodia and reported that 28.2% and 21.8% of the total vegetable samples were contaminated with *Salmonella* spp. and *E. coli*, respectively. The author also noticed that the prevalence of both pathogens was significant higher in dry season (October to April; 56.5% *Salmonella* spp., and 34.0% for *E. coli*) as compared to the rainy season (May to September; 15,4% *Salmonella* spp., and 9.1% for *E.coli*) for lettuce samples, indicating seasonal changes on contamination (Desiree et al., 2021). Rortana et al., (2021) showed that the prevalence of *Salmonella* spp. was 43% and 46% in chicken meat and pork, respectively, collected from 52 traditional markets and 6 supermarkets in 25 provinces in Cambodia.

The study of foodborne pathogens in Cambodian fresh food markets expands beyond vegetables and animal-sourced foods. Rortana et al., (2021) also reported that chicken and pork cutting boards were contaminated with *Salmonella* spp. indicating cross-contamination during food handling operation. Of the total of 124 swabs, *Salmonella* spp. was detected in 43% and 31%

on the chicken and pork cutting board swabs, respectively (Rortana et al., 2021). Schwan et al., (2021) demonstrated that *Salmonella* was more prevalent on food contact surfaces (75%) as compared to non-food contact surface (25%). These two studies highlighted the lack of food safety practices in Cambodian fresh food markets and the need to improve public health infrastructures.

1.6 Common food contact surfaces in Cambodian fresh food markets

Food contact surfaces (FCS) are all types of surfaces that directly come into contact with food products during production, processing, and packaging. Food contact surfaces must be easy to clean and sanitize because during use they can become contaminated with harmful microorganisms and potentially transfer to food (Zhao et al., 2017). In the food industry in developed countries (i.e., United States), FCS are typically made of stainless steel or plastics such as polypropylene and high-density polyethylene, and elastomers (Hasting, 2009).

In fresh food markets in Cambodia, FCS are unique surfaces that are used to store, display, and transport food products. These surfaces include bamboo basket, cardboard, mat, styrofoam box, lotus flower leaves, and banana leaves. In fact, a study of *Salmonella* contamination on FCS and NFCS in fresh food markets in Cambodia reported that the most frequently isolated serotype was *Salmonella* Rissen which was prevalent on various types of FCS (Schwan et al., 2021). The author also highlighted that *Salmonella* serotype abundance varied depending upon the surface type and evaluated the diversity of serotypes on floor (13 serotypes) baskets (11), mats (5), scales (5), and lotus flower leaves (4). Baskets, scales, and lotus leaves were the top three surfaces with the most diverse *Salmonella* serotypes (Schwan et al., 2021). Similarly, another study also reported that chicken and pork cutting boards used in Cambodian fresh food markets were contaminated with *Salmonella* (Rortana et al., 2021). These findings suggest that food contact surfaces in fresh food markets in Cambodia might be a vehicle of food contamination, for which food safety

measures and infrastructure need to be improved (Rortana et al., 2021; Schwan et al., 2021). Since FCS may be sources for microbial contamination, finding effective food safety interventions that reduce microbial contamination on FCS may contribute to the reduction of foodborne outbreaks associated with food products originated in fresh food markets in Cambodia.

1.7 Sanitation and food safety

Food products can be contaminated at numerous points though the value chain. The food industry employs various strategies in preventing food product contamination. The most widely used intervention is cleaning and sanitation (Marriott et al., 2018). Cleaning is a prerequisite for an effective sanitization and is defined as "the removal of organic matters such as food residue, oils, grease, and visible soil, using appropriate detergent chemicals" (R. H. Schmidt, 1997). The purpose of cleaning is to physically remove organic matters and visible soils to prevent the persistence of microorganisms and increase the efficacy of chemical sanitizers. There are two types of cleaning, wet and dry cleaning. Wet cleaning involves the use of water and cleaner such as detergents, while dry cleaning does not involve water or detergents but rather the physical removal of visible contaminants. Wet cleaning typically consists of several steps: dry cleaning to remove contaminants from the surfaces, pre-rinsing with potable water, and using cleaning agents such as detergent to remove cleaning agents and remaining contaminants (Chauret, 2014). It is important to note that only surfaces cleaned with cleaning agent and water are eligible for sanitation. Dirty surfaces cannot be sanitized due to the presence of organic matter, which can inactivate or reduce the efficacy of sanitizers, making sanitation ineffective. Cleaning is followed by sanitation, which is defined as "any procedure to lower the population of microorganisms to levels considered safe from a public health perspective" (R. H. Schmidt, 1997). In the food industry, sanitation of surfaces and equipment is focused on ensuring hygienic conditions in food processing environment.

Sanitation can be achieved by different application methods including thermal, stream, radiation, hot water, high hydrostatics pressure (HHP), and chemical sanitizers. Selection of sanitation methods depends upon food and surface types, availability, cost, and equipment design. However, the ideal sanitizer should possess the following characteristics (Marriott et al., 2018):

- Have a wide antimicrobial spectrum against vegetative bacteria, yeasts, and mold.
- Be fast acting in killing microorganisms.
- Be nontoxic and nonirritating to users.
- Be compatible with a broad range of surfaces.
- Have an acceptable level of odor.
- Be stable in concentrated and diluted solution.
- Be resistant to environmental factors such as the presence of organic matters, water hardness and pH variability.

It is important to note these considerations for sanitation are mainly focused on the food industry in developed countries. Such considerations may not be feasible for sanitation application in fresh food markets in LMICs since the nature of factors influencing the efficacy of cleaning and sanitation are different between these two food environments (Table 1.1). For instance, access to clean water is still questionable in most fresh food markets in Cambodia. Hence, the use of maximum allowable concentration of each sanitizer should be recommended so that if the water itself contains microbial contaminants, sanitizers treat the water, and there is still a substantial proportion of sanitizer available in the solutions.

1.8 Chemical sanitizers

Chemical sanitizers are frequently used in the food industry to maintain food quality and safety in processing facilities. There are different types of chemical sanitizers which can be

grouped as oxidizing agents, surface active compounds, and iodophors (Van Houdt & Michiels, 2005). In the United States, chemical sanitizers used in food processing facilities are required by law to register and gain approval from the Environmental Protection Agency (EPA). The Food Code requires that chemical sanitizers used in retail food facilities must reduce bacterial loads to at least 99.999% (5 log reduction) on food contact surfaces and 99.9 (3 log reduction) on non-food contact surfaces (Sanders, 2003). The efficacy of chemical sanitizers is affected by temperature, pH, concentration, exposure time, and organic matters such as food residue and soils (Holah et al., 1990; Mosteller & Bishop, 1993). Characteristics of commonly used chemical sanitizer in food industries are summarized in Table 1.2. Among these chemical sanitizers, chlorine and peracetic acid (PAA) are the two most widely use sanitizers in the food industry (Marriott et al., 2006; Zoellner et al., 2018).

1.8.1 Sodium hypochlorite (Bleach)

Sodium hypochlorite (Bleach, NaOCl) is an inorganic chemical compound that are extensively used as household disinfectant products since it is relatively inexpensive and highly effective against various types of microorganism (Van Houdt & Michiels, 2005). Sodium hypochlorite is chlorine gas dissolved in sodium hydroxide. The addition of sodium hypochlorite to water produces hypochlorous acid (HOCl) and sodium hydroxide (NaOH). The antimicrobial activity of sodium hypochlorite depends upon the availability of hypochlorous acid (free chlorine) present in water that comes into contact with microbial cells (Sapers, 2003). Even though the detailed antimicrobial activity of chlorine is not fully understood, it is generally accepted that hypochlorous acid damages microbial cells through inhibiting glucose oxidation by chlorine-oxidizing sulfhydryl groups of particular enzymes important in metabolism of carbohydrates (Marriott et al., 2018). Other proposed mode of actions are that chlorine disrupts protein synthesis,

reacts with nucleic acids, oxidatively decarboxylates amino acids to nitrites and aldehydes, destructs key enzymes, and inhibits oxygen uptake and oxidative phosphorylation (Marriott et al., 2018).

The maximum allowable concentration of sodium hypochlorite used on food contact surfaces must not exceed 200 ppm (Chauret, 2014). Although 200 ppm is effective for numerous surfaces, 800 ppm is suggested for porous areas (Marriott et al., 2018). A study published in 1989 by Mustapha and Liewen reported that 200 ppm sodium hypochlorite was effective in reducing a 4 log reductions of *Listeria monocytogenes* on stainless steel surfaces (Mustapha & Liewen, 1989). Frank et al., 1997 suggested a similar result in which 200 ppm of choline were effective in destroying 4 log of *Staphylococcus* populations on stainless steel (Frank & Chmielewski, 1997). A study by Kim et al., 2017 reported that sodium hypochlorite in 200 ppm in 5 minutes were effective in lowering 4 log of *Staphylococcus aure*us populations on smooth and non-biofilm stainless steel (Kim et al., 2017). Another newly published study showed that chlorine-based disinfectants (100 ppm of NaOCl) were effective against *S. Enteritidis* planktonic cells in low concentration of organic substances (minimum 5.58 log CFU/cm^2 reduction at clean condition) and biofilms formed on food contact surfaces (minimum 3.21 log CFU/cm^2 reduction at silicone rubber) (Byun et al., 2021). Bang et al., (2014) also suggested that 1 min of NaOCl treatment at a concentration over 50 ppm was effective in reducing *E. coli* O157:H7 biofilm on stainless steel, glass, plastic, and wood surfaces.

1.8.2 Peracetic acid (PAA)

Peracetic acid (PAA) is an organic acid and a strong oxidizing agent with a low pH, and vinegar-like odor. PAA is commercially produced as a quaternary equilibrium mixture of acetic acid and hydrogen peroxide. It is effective in a wide spectrum of temperature (0 to 40°C) and pH

(3.0 to 7.5) (Kitis, 2004). In addition, PAA is compatible with hard water and maintains its efficacy in the presence of organic soil (Rutala & Weber, 2015). It was reported to have a relatively low reaction with organic matter as compared to chlorine (Banach et al., 2015) and produce non-toxic by-products such as acetic acid and oxygen (Gehr et al., 2003; Monarca et al., 2002). PAA was first registered as a pesticide in 1985 in the U.S. to be used as sanitizer, disinfectant, and sterilant. Two main mechanisms of action of PAA are the production of reactive oxygen species which can lead to DNA and lipid damage of microbial cells (Small et al., 2007), and the oxidation of sulfhydryl and disulfide bonds which denatures proteins and enzymes and increases cell wall permeability (Hilgren et al., 2007; Small et al., 2007).

Although PAA is not explicitly categorized as "generally recognized as safe (GRAS)" antimicrobial agents, the degradation of PAA resulted in the production of acetic acid and hydrogen peroxide (Moondra et al., 2018). Both of these agents have been listed as GRAS by the U.S. Food and Drug Administration (FDA). In produce industry, it is approved to be used at 80 ppm as a wash water to reduce microbial load attach to produce surfaces without any further rinse requirement (FDA, 2022). On food contact surfaces, PAA is typically used at concentrations of 150-200 ppm. However, in the presence of organic matter, 200-500 ppm is required (Chauret, 2014).

In the last few decades, PAA has been broadly used in the food industry as a chemical sanitizer to eliminate biofilm, prevent cross-contamination, and improve product shelf-life. In the produce industry, PAA is used to control microbial contamination of minimally processed fruits and vegetables (Banach et al., 2020; Gonzalez et al., 2004; Hellström et al., 2006; Neo et al., 2013). Various studies had assessed the efficacy of this sanitizer against various planktonic cells and biofilm formation of *Listeria monocytogenes*, *Salmonella enterica*, and pathogenic *E. coli* (Aryal

& Muriana, 2019; Hua et al., 2019; Iñiguez-Moreno et al., 2021; Nahar et al., 2022). Aryal and Muriana, 2019 reported that the application of 5 min of PAA reduced the biofilms of both *L. monocytogenes* and *E. coli* O157:H7 to below the detection levels (>7.5 log reduction) while the population of *S.* Montevideo was reduced to less than 3 logs (Aryal & Muriana, 2019). In the study of efficacy of PAA on planktonic cells in suspension without food residues, it was reported that the application of PAA for 30 s reduced the population of *E. coli*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *P. aeruginosa*, *L. monocytogenes*, and *B. cereus* by 99.9999% (>6 log CFU/ml reduction). When assessing the antimicrobial activity of PAA on *Salmonella* attached to FCS, Shi et al., (2016) demonstrated that PAA treatment of 200 ppm for 30 s caused 2.6 log reduction of *Salmonella* Typhimurium on reusable plastic containers.

1.9 Figures and Tables

Figure 1.1. Variety of fresh vegetables sold at a typical fresh food market in Cambodia.

Table 1.1 Comparison of factors affecting the efficacy of cleaning and sanitation in the food industry and fresh food markets.

Factors	Food Industry	Fresh Food Market
Food contact surfaces	FCS are smoother surfaces: stainless steel, plastics, glass, or ceramics	FCS are unique surfaces: bamboo baskets, banana leaves, lotus leaves, or wooden cutting boards,
Presence of contaminants	Protein, fat, sugars, or microorganisms from raw products	Visible dirt, soils, or microorganisms from raw products
Water availability	Water is highly accessible	Water is limited, only available in fresh food market in the city
Sanitizer availability	Various types of sanitizers used	Only chlorine is widely available, other sanitizers are not easily accessible
Temperature	Controlled temperature	Temperature fluctuation depending upon seasonal changes
pH	Plenty of resources to adjust pH of sanitizers	No resources to adjust pH of sanitizers

Table 1.2. Characteristics of commonly used chemical sanitizers in food industries [Adapted from (Gaulin et al., 2011; Wirtanen & Salo, 2003)]

Sanitizer	**Concentration**	**Microorganism**	**pH**	**Temperature**	**Organic matter**
Chlorine	50-500 ppm Although 200 ppm is effective for numerous surfaces, 800 ppm is suggested for porous surfaces Require rinsing above 200 ppm	Broad microbial spectrum against bacteria, fungi, viruses Spores: Yes Biofilm: No	Optimum antimicrobial activity at pH 6.5 to 7.0	Moderately affected	Highly affected
Peracetic acid (PAA)	50 to 500 ppm	Broad microbial spectrum against bacteria, fungi, viruses Spores: Yes Biofilm: Yes	Optimum antimicrobial activity at neutral pH Effective in a wide range of pH (3.0 to 7.5)	Not affected Effective in a wide spectrum of temperature (0-40°C)	Little to no effect
Quaternary ammonium compound (QAC)	200 to 1000 ppm Require rinsing above 200 ppm	Various microorganisms, More effective against vegetable cells Limited effectiveness against most gram-negative bacteria excepts for *Salmonella* and *E. coli*	Little to no effect	Optimum activity at 18 - 37 °C	Moderately affected
Iodophors	6.5 to 75 ppm Require rinsing above 25 ppm	Broad microbial spectrum against bacteria, fungi, and yeasts Spores: No Biofilm: No	Effective in acid to neutral range (pH: 2-7)	Highly affected	Moderately affected

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Chapter 2 - Research Importance

Salmonella and *Escherichia coli* are foodborne pathogens of significant public health interest, particularly for fresh fruits, vegetables, and animal source foods (ASF). Globally, the contamination of fresh fruits, vegetables and ASF has been extensively investigated, especially in fresh food markets in low and middle-income countries (LMICs), where the burden of foodborne illnesses is relatively high. However, these studies have primarily provided information on the presence of microbial contamination within these environments. To date, few studies have provided data on effective interventions to reduce bacterial presence and cross-contamination within fresh food market environments, leading to a significant amount of data defining the problem and scarce data providing solutions.

Natural food packaging materials such as bamboo baskets and banana leaves are commonly used in Cambodian fresh food markets. These materials are a traditional, natural, and sustainable option to store, transport, and display food products. They are increasingly popular due to their local abundance and growing global concerns over environmental impacts of plastic consumption. In fresh food markets where there is often lack of hygiene, sanitation practices and inadequate food safety infrastructures, these traditional surfaces (i.e., banana leaves and bamboo baskets) are commonly used. Foodborne pathogens such as *Salmonella* and *E. coli* can attach and persist on these surfaces, and, if not properly cleaned and/or sanitized, can cross-contaminate other food products presenting risk to public health for both market vendors and consumers.

Hence, there is a need to develop interventions that are specifically designed to reduce bacterial cross-contamination on food products and food contact surfaces in fresh food markets. Since banana leaves and bamboo baskets are widely used in Cambodia, this study aimed to:

- Investigate the attachment of *Salmonella* and *E. coli* on the surfaces of banana leaves and bamboo baskets.
- Determine the efficacy of cleaning and sanitation methods against *Salmonella* and *E. coli* experimentally inoculated on banana leaves and bamboo baskets.
- Suggest effective interventions to clean and sanitize food contact surfaces that can be potentially adopted in Cambodian fresh food market context.

Chapter 3 - Efficacy of cleaning and sanitation methods in reducing *Salmonella enterica* on banana leaves and bamboo baskets: common food contact surfaces used in Cambodian fresh food markets

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Abstract

Banana leaves and bamboo baskets are natural and sustainable food contact surfaces commonly used to display, store, and transport various food products in fresh food markets in Cambodia. These surfaces are normally used following traditional practices with lack of proper cleaning and sanitation measures. The objective of this study was to evaluate the efficacy of cleaning and sanitation methods against *Salmonella enterica* (hereafter referred as *Salmonella*) on banana leaf and bamboo basket surfaces. Banana leaf and bamboo basket surfaces were inoculated with *Salmonella* cocktail and allowed to attach for 60 min. Surfaces were assigned to a treatment defined as a combination of cleaning and sanitation method. Three cleaning methods were evaluated (i.e., wiping, scrubbing, or no cleaning) alone or in combination with 200 ppm bleach, 400 ppm peracetic acid (PAA), applied for 3 or 5 min, or no sanitation. A total of 15 treatment combinations were tested using a factorial design. Surfaces inoculated but not treated were considered as positive controls. Regardless of cleaning method or contact time used, the use of PAA reduced *Salmonella* on banana leaf surfaces to below the Limit of Quantification (1.44 Log CFU/cm^2) achieving 4.31 log CFU/cm^2 reduction (95% CI [4.17, 4.46]). A significant interaction was found between cleaning and sanitation method on banana leaf surfaces (P=0.440), indicating that the efficacy of sanitation depends upon the cleaning method used. Wiping followed by the use of bleach sanitation for 5 min reduced *Salmonella* counts on banana leaves to 2.85 log CFU/cm^2 (95% CI [2.52, 3.18]) as compared to the control (i.e., no cleaning and no sanitation; 5.75 log CFU/cm^2; 95% CI [5.61, 5.90]). On bamboo baskets, the most effective treatment was the use of PAA for 5 min, reducing *Salmonella* counts to 3.11 log CFU/cm^2 (95% CI [2.89, 3.33] . This study provides an initial understanding of the application of cleaning and sanitation strategies on common food contact surfaces utilized in Cambodian fresh food markets.

3.1 Introduction

Foodborne illnesses (FBI) remain a major public health concern worldwide despite remarkable advances in food safety. FBI result from consumption of contaminated food with physical, chemical, or biological hazards (Hoffmann & Scallan, 2017). Biological hazards associated with diarrhea diseases such as bacteria, parasites, and viruses account for 70% of the global burden of FBI (WHO, 2015). FBI have negative impacts on tourism, trade, country reputation, and pose burden on socio-economic development especially for low- and middle-income countries (LMICs) (Crutchfield et al., 2001; Grace, 2015; Lee et al., 2019). In Cambodia, FBI represent a significant cause of mortality among children under 5 accounting for up to 8% of all deaths (Ebner et al., 2020). In addition, 51% (146/288) of reported disease outbreaks in Cambodia from 2010 to 2015 were caused by FBI (Lawpoolsri et al., 2018). Recently, the Cambodian Ministry of Health (MOH) reported 134 foodborne diseases outbreaks between 2014 to 2019, resulting in 5,825 cases, 5,598 hospitalizations, and 81 deaths. Lack of hygiene in food preparation, improper cooking, cross-contamination, and the use of unsafe water and food sources were associated with these outbreaks (Mekong Institute, 2019).

One of the major foodborne pathogens in LMICs is *Salmonella*: this microorganism has often been isolated from various animal source foods (ASF) and fresh produce sold in fresh food markets. *Salmonella* is a gram-negative, facultative anaerobic, foodborne enteric pathogen and a leading cause of gastroenteritis in human (Majowicz et al., 2010). *Salmonella* is predominantly found in ASF such as poultry, eggs, pork, and dairy products (Andino & Hanning, 2015). However, in the last few decades fresh produce outbreaks associated with *Salmonella* highlighted fresh fruits and vegetables as potential sources of *Salmonella* infection (Carstens et al., 2019), especially in

LMICs, where most FBI are associated with the consumption of fresh and perishable foods sold in informal markets (Grace, 2015).

Fresh food markets (or informal markets) are a large collection of open-air stalls where fresh fruits, vegetables, and ASF are sold (Roesel & Grace, 2014). In LMICs, Fresh food markets are the daily destination of millions of consumers since food products are often cheaper and fresher compared to supermarkets (Sary et al., 2019). Fresh food markets are also places where locals greet and socialize. Nevertheless, these environments can represent a health risk to consumers (Jaffee et al., 2018). Most vendors either sell only ASF, fresh fruits, or vegetables. In some places, however, ASF, vegetables, and fruits are sold by a single market vendor and displayed in close proximity favoring cross-contamination. Cross-contamination is further increased due to the extensive food-handling scenario typically observed during market operations. Most importantly, food items are often displayed at ambient temperature and directly exposed to open-air environment increasing the risk of bacterial contamination (Muramatsu et al., 2021). Studies on microbial contamination of food products in fresh food markets have been documented in various parts of the world, especially in LMICs (Anal et al., 2020; Nguyen et al., 2021; Salleh et al., 2003; Sapugahawatte et al., 2022; Sripaurya et al., 2019). In Cambodia, various food products sold in fresh food markets were reported to be contaminated with different foodborne pathogens including *Campylobacter* spp., *Salmonella* spp., *Escherichia coli,* and *Staphylococcus aureus* (Desiree et al., 2021; Lay et al., 2011; Phoeurk et al., 2019; Schwan et al., 2022). Lay et al., (2011) reported that raw poultry sold in fresh food markets in Phnom Penh, Cambodia had a high prevalence of *Salmonella* spp. (88%) and *Campylobacter* spp. (81%). A study in two northeast provinces in Cambodia by Desiree et al., (2021) suggested that 28% and 22% of total vegetable samples collected from fresh food markets were contaminated with *Salmonella* spp. and *E. coli*,

respectively. While most studies focused on fresh produce and ASF, environmental contamination is also a concern in Cambodian fresh food markets (Rortana et al., 2021; Schwan et al., 2021). Schwan et al., (2021) showed that food contact surfaces used in fresh food markets were highly contaminated with *Salmonella* (75%) as compared to non-food contact surfaces (25%). Similarly, *Salmonella* was isolated from 42% and 31% of chicken and pork cutting boards, respectively (Rortana et al., 2021). The presence of *Salmonella* on these food contact surfaces were associated with poor personal hygiene and sanitation practices among vendors (Rortana et al., 2021; Schwan et al., 2021). The lack of hygiene and sanitation practices along with insufficient food safety infrastructures as reported at Cambodian fresh food markets might increase the risk of bacterial contamination and potentially lead to foodborne outbreaks.

Cleaning and sanitation play an important role in preventing FBI and reducing foodborne outbreaks (Marriott et al., 2018). Improved sanitation has positive impacts on human health and economy. According to the WHO study in 2012, every dollar invested in sanitation had a USD 5.50 return in reduced health costs, increased productivity, and lower premature death (WHO, 2022). Hence, maintaining clean and sanitary food contact surfaces is essential in reducing prevalence of foodborne pathogens in fresh food markets since there is a lack of information on the efficacy of cleaning and sanitation measure on food contact surfaces commonly used in fresh food markets in Cambodia.

The objective of this study was to investigate the efficacy of cleaning and sanitation in reducing *Salmonella* experimentally inoculated on banana leaf and bamboo basket surfaces commonly used in fresh food markets in Cambodia.

3.2 Materials and Methods

3.2.1 Salmonella *strains*

Three serotypes of *Salmonella enterica* subspecies *enterica* were used in this study: Rissen, Corvallis, and 4,[5],12,:i:- isolated from food and non-food contact surfaces in fresh food markets in Cambodia. *Salmonella* strains were chosen based upon their prevalence on environmental surfaces (Schwan et al., 2021). *Salmonella* stock cultures were maintained at -80 °C in a CryoCare Organism Preservation System (Key Scientific, Stamford, TX).

3.2.2 Inoculum preparation

A single frozen bead of each *Salmonella* strain was transferred into 10 ml Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) and grown overnight (16-18 h) at 37 °C on an orbital shaker at 70 rpm. A loopful of *Salmonella* culture was then streaked on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD) and incubated at 37 °C for 24 h. A single colony from the streak plate was inoculated into a freshly prepared 10 ml TSB and incubated overnight at 37 °C on an orbital shaker at 70 rpm. Overnight cultures were centrifuged at 4,000 rpm for 10 min. Supernatant was discarded and pellet was resuspended in 1 ml Phosphate Buffer Saline (PBS, VWR Chemicals, Solon, OH). *Salmonella* strains were combined in equal ratio to form a 3 ml multi-strain *Salmonella* cocktail. Initial population was verified by plating on TSA.

3.2.3 Preparation of chemical sanitizers

Peracetic acid (PAA, Shield-Brite PAA 15.0, Pace International Wapato, WA) and bleach (Clorox disinfecting bleach, The Clorox Company, Oakland, CA) were used in this study. 200 ppm of bleach and 400 ppm of PAA solution were diluted with sterile distilled water. Sanitizer solutions were freshly prepared from bulk prior to each experiment. The concentration of each

sanitizer was measured using commercial test kits (Peracetic Acid Test Kit and Chlorine Test Kit, ChemWorld, Kennesaw, GA).

3.2.4 Sample preparation and bacterial inoculation

Experiments were performed on banana leaf surfaces and bamboo basket surfaces. Observations were obtained from 6 different banana leaves or bamboo baskets. For each leaf or basket, 15 leaf pieces (3 x 3 cm^2) or 15 bamboo basket pieces (1.2 x 3 cm^2) were prepared and assignment to treatment. Individual pieces of banana leaves or bamboo baskets were recognized as a blocking structure to account for natural leaf-to-leaf and basket-to-basket. Samples were prepared using a sterile template under aseptic condition. Samples were placed into a sterile petri dish and exposed to UV-C in the Biological Safety Cabinet (BSC, ESCO Life Sciences, South Yorkshire, England) for 15 min. Inoculation was performed by aliquoting 10 drops (each 10 μl) of cocktail on each sample. *Salmonella* was allowed to attach to surfaces up to 60 min.

3.2.5 Treatment design

Each sample was assigned to a treatment defined by a combination of cleaning and sanitation. Three cleaning methods were evaluated 1). wiping with a dry paper towel vertically and horizontally 5 times in each direction, 2). scrubbing with a dry kitchen sponge vertically and horizontally 5 times in each direction, or 3). no cleaning. Upon the completion of the cleaning method, five sanitation methods were subsequently tested 1). 200 ppm of bleach for 3 min, 2). 200 ppm of bleach for 5 min, 3). 400 ppm of PAA for 3 min, 4). 400 ppm of PAA for 5 min, or 3). no sanitation. A total of 15 treatments were assigned into 15 pieces derived from each individual leaf or bamboo basket as previously mentioned. The schematic diagram of the experiment is illustrated in Figure 3.1.

3.2.6 Neutralization and enumeration of Salmonella *cells*

Upon the completion of treatment, samples were transferred into 10 ml Dey and Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA). Controls were submerged into 10 ml Buffered Peptone Water (BPW, BD Difco, Sparks, MD). To recover cells, samples were vortexed for 30 s. Serial dilutions were then performed in 0.1% peptone water (Bacto, Sparks, MD) and plated on TSA in duplicate. Plates were incubated for 24 h in 37 °C. Survival populations after each treatment were counted the next day. Colonies were counted if they were in the countable range (25-250) and reported as log CFU/cm^2. Log reduction was also reported to express the relative number of *Salmonella* cells that are eliminated by treatment. It was calculated based upon the initial *Salmonella* attached to surface minus the remaining *Salmonella* cells after each cleaning and sanitation treatment.

3.2.7 Salmonella *attachment under scanning electron microscopy*

Samples (1 x 1 cm^2) were prepared and inoculated as previously described above for microscopy visualization. Inoculated samples were strained with SYTO9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR) and fixed with 10% formalin as previously described by (Mendez et al., 2020). Samples were visualized by Scanning Electron Microscopy (SEM, Hitachi S-3500N, Tokyo, Japan) located in the Microscopy Facility, Division of Biology at Kansas State University.

3.2.8 Statistical analysis

A general linear mixed model was used to evaluate the effect of cleaning and sanitation methods on *Salmonella* counts obtained as the average of duplicate count plates and expressed as log CFU/cm^2. The linear predictor included the fixed effects of cleaning, sanitation methods, and their 2-way interactions. For banana leaves, the statistical analysis excluded treatments consisting

of PAA as *Salmonella* counts were below the Limit of Quantification (1.44 log CFU/cm^2). The random effect of leaf or basket was incorporated into the model as an overarching blocking structure. The corresponding variance component estimate converged to zero and thus was effectively dropped from the model. In addition, heterogeneous residual variances by sanitizers were also fitted into the model to meet model assumptions. The decision to incorporate heterogeneous residual variances was supported by enhanced model fit to the data, as indicated by Bayesian Information Criterion. Model assumptions were evaluated using externally studentized residuals and were considered to be reasonably met.

Statistical models were fitted using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute, Cary, NC). Restricted maximum likelihood was employed to estimate variance components, and the Kenward-Roger's approach was used to estimate degrees of freedom and correct estimated standard errors. Estimated least square means, corresponding standard errors, and 95% confidence interval were presented at the appropriate level of inference in the factorial treatment structure. Relevant pairwise comparisons between treatment groups were conducted using either a Tukey-Kramer or a Bonferroni adjustment, as appropriate in each case, to avoid inflation of Type I error rate due to multiple comparisons.

3.3 Results

3.3.1 Attachment of Salmonella *on bamboo basket and banana leaf surfaces under SEM*

The attachment of *Salmonella* on banana leaf and bamboo basket surfaces were visualized by Scanning Electron Microscopy. Figure 3.2A shows a typical surface structure of a banana leaf which comprises of stomata and wax platelets distributed randomly on the leaf surface. The high abundance of wax platelets formed peaks and valleys, creating air pockets, and increasing the surface roughness. These structures made it difficult to clearly observe the attachment of

Salmonella cells on banana leaf surfaces (Figure 3.2B). However, *Salmonella* cells were observed with increase magnification as indicated by the green arrow in Figure 3.2C. Figure 3.3A reveals the roughness surface morphology of the uninoculated bamboo basket surface. *Salmonella* cells strongly attached and formed a lump of microorganism on the groove of bamboo epidermal cells as highlighted by the green arrow in Figure 3.3B and Figure 3.3C.

3.3.2 Reduction of Salmonella *on banana leaf surfaces*

Regardless of cleaning method, the use of PAA resulted in a reduction of *Salmonella* counts on banana leaf surfaces below the limit of quantification (LOQ; 1.44 log CFU/cm^2) in all cases. For remaining treatment, results showed that reductions in *Salmonella* counts on banana leaf surfaces were dependent on the combination of cleaning and sanitation method utilized (P=0.0440). It is important to note, that the interaction between cleaning and sanitation was only valid regarding treatments that incorporated bleach.

Estimated *Salmonella* counts for each treatment are shown in Table 3.1. For the control treatment (i.e., no cleaning and no sanitation), estimated *Salmonell*a counts were 5.75 log CFU/cm^2 (95% CI [5.61, 5.90]). The most effective treatment was bleach with a contact time of 5 min combined with wiping as a cleaning method (P=0.0121), reducing *Salmonella* counts to 2.85 log CFU/cm^2 (95% CI [2.52, 3.18]) achieving 2.90 log reduction as compared to the control. The efficacy of bleach improved with increase contact time (i.e., 3 vs 5 min) when banana leaf surfaces were wiped prior to bleach sanitation. For example, bleach sanitation for 3 min preceded by wiping (4.15 log CFU/cm^2; 95% CI [3.82, 4.47]; 1.60 log reduction) appeared to have significant lower antimicrobial potency as compared to bleach sanitation for 5 min followed by wiping (2.85 log CFU/cm^2; 95% CI [2.52, 3.18]; 2.90 log reduction) (P<0.001). There is no evidence for any main

effect of cleaning method (i.e., wiping, scrubbing, or no cleaning) in reducing *Salmonella* on banana leaf surfaces (*P*=0.1531).

3.3.3 Reduction of Salmonella *on bamboo basket surfaces*

No evidence of any interaction between cleaning and sanitation methods was observed on reduction of *Salmonella* counts on bamboo basket surfaces (*P*=0.9691), and neither the main effect of cleaning method (*P*=0.3795). However, a significant effect was observed between sanitation methods in reducing *Salmonella* counts on bamboo baskets, regardless of the cleaning method used (P<0.0001). PAA was the sanitation method that resulted in the largest reduction of *Salmonella* across all sanitation methods (*P*<0.0001).

Table 3.2 presents estimated *Salmonella* counts for each sanitation method on bamboo basket surfaces. The highest reductions of *Salmonell*a counts observed on bamboo basket surfaces were those treated with PAA for 5 min reducing *Salmonella* counts to 3.11 log CFU/cm^2 (95% CI [2.89, 3.33]; 2.26 log reduction), followed by bleach for 5 min (3.91 log CFU/cm^2; 95% CI [3.68, 4.15]; 1.46 log reduction). In addition, longer contact time enhanced the efficacy of PAA in reducing *Salmonella* counts on bamboo basket surfaces. However, there were no significant differences in *Salmonella* counts between bleach (i.e., 3 min and 5 min) and PAA for 3 min (*P*=0.6338).

3.4 Discussion

In the present study we investigate the attachment of *Salmonell*a on banana leaf and bamboo baskets surfaces and evaluated the effect of cleaning and sanitation methods in reducing *Salmonella* counts on food contact surfaces commonly used in Cambodian fresh food markets, specifically, banana leaf and bamboo basket surfaces. Our findings indicate that *Salmonella* reduction on banana leaf surfaces depended upon the cleaning and sanitation method used. A

significantly greater reduction of *Salmonella* was observed on banana leaf surfaces treated with a combination of cleaning and sanitation method as compared to cleaning or sanitation alone. Previous studies have reported similar results in which the sanitation efficacy was enhanced by the cleaning method applied (Masuku et al., 2012; Røssvoll et al., 2015; Ruiz-Llacsahuanga et al., 2022). Rossvoll et al., (2015) confirmed that the efficacy of chlorine-based disinfectant was improved with the combination of a cleaning method (1-2 log increase). Similarly, Masuku et al., (2012) showed that the sanitation effect was significantly improved with the use of wipe clothes combined with silver hydrogen citrate as compared with wiping with cloth by itself. Moreover, in a study evaluating the efficacy of cleaning and sanitation methods against *Listeria innocua* on apple packing equipment surfaces, Ruiz-Llacsahuanga et al., (2022) showed that the use of a degreaser or detergent to clean rollers and conveyor belt followed by sanitizer application caused the greatest reduction. These studies suggested that effective interventions in reducing pathogens on food contact surfaces require incorporating selected combination of cleaning and sanitation methods to ensure maximum pathogen reduction.

Our study demonstrates that bleach treatment could only partially reduce *Salmonella* populations on banana leaf surfaces. As compared to PAA treatment, the lower efficacy of bleach on banana leaf surfaces may be due to the complex microstructure of epidermal cells and the presence of wax platelets on the surface of leaf. In particular, the high abundance of wax platelets created a large surface area of plant organic matters likely to react with free chlorine and cause chlorine depletion, lessening its efficacy (Fransisca & Feng, 2012; Wang et al., 2009; Yi et al., 2022).

For bamboo basket surfaces, we observed a significant reduction effect of sanitation methods. The lower reduction of *Salmonella* observed on bamboo basket surfaces as compared to

banana leaf surface may be explained by the surface topological structure (Figure 3.3). The porous structure of the bamboo basket surface may provide potential physical protection to *Salmonella* cells, therefore limiting penetration of sanitizer and reducing its efficacy (Chiu et al., 2006; Frank, 2001). Consistent with this explanation, in a study assessing the efficacy of electrolyzed water in reducing *Vibrio parahaemolyticus* on kitchen cutting boards, Chiu et al., (2006) showed that *Vibrio* was most persistent on bamboo and wood cutting boards following treatment with electrolyzed oxidizing water as compared to plastics.

Wiping or scrubbing with cloths or kitchen sponge are common practices used to physically remove food residues on surfaces. In our study, we observed that traditional cleaning methods by themselves (with no sanitation incorporated) such as wiping with a dry paper towel and scrubbing with a dry kitchen sponge had no significant effect on *Salmonella* reduction. Conversely, Sun-Young et al., (2011) reported that wiping with a dry paper towel showed a 1.15 log CFU/cm^2 reduction of *E. coli* O157:H7 attached to wood cutting boards. Similarly, Kim et al., (2012) also reported 1.20 log CFU/cm^2 reduction of *Vibrio parahaemolyticus* counts was observed when wood cutting boards were wiped with a dry paper towel. Although studies indicated that cleaning may reduce *Salmonella* prevalence, it is important to note that the surfaces still maintain a substantial number of *Salmonella* highlighting that combining cleaning with sanitation may be necessary.

Although our results are an impactful contribution to the understanding of the use of cleaning and sanitation methods in Cambodian fresh food markets, this study has limitations. To our current knowledge, PAA is not readily available in Cambodian fresh food markets, though it is typically available for large food manufacturing industries. Meanwhile, bleach is widely available and relatively cheap. Therefore, incorporating bleach as a sanitation method is a potential strategy in reducing the prevalence of *Salmonella* associated with fresh and perishable food

products in fresh food markets in Cambodia. In addition, we should acknowledge that these results are based on an experimental study conducted in a controlled laboratory environment. Additional factors should be considered when applying these findings to real-world situations, such as in fresh food markets. These considerations include - but are not limited to – type of food contact surface used, the presence of organic, biological, and chemical contaminants on food contact surfaces, availability of portable water, and availability and pH sanitizer solutions. Specifically, access to clean and potable water is still limited in most fresh food markets in Cambodia. Hence, the use of maximum allowable concentrations of each sanitizer should be recommended so that if the water itself contains some microbial contaminants, sanitizers can first work on treating the water with enough residual sanitizer available in the solutions to sanitize food contact surfaces.

3.5 Conclusion

The presence of *Salmonella* in food environments is a potential contaminant which may lead to foodborne outbreaks. Development of effective cleaning and sanitation methods to decontaminate food contact surfaces has the potential to reduce cross-contamination of *Salmonella*, particularly in fresh food markets. Our findings demonstrate that proper cleaning and sanitation practices can significantly reduce the survival of *Salmonella* on banana leaf and bamboo basket surfaces, highlighting the importance of maintaining a hygienic environment in fresh food markets. Overall, the efficacy of sanitation against *Salmonella* on banana leaf surfaces depended upon the cleaning method used. PAA was the most effective sanitizer on both surfaces. Longer contact time enhanced the efficacy of PAA in reducing *Salmonella* populations on bamboo baskets. This study offers an initial contribution to understanding effective cleaning and sanitation methods that can be introduced in Cambodian fresh food markets. Market vendors may use this

information to make informed decision on how to develop effective practices to clean and sanitize surfaces used at fresh food markets.

3.6 Figures and tables

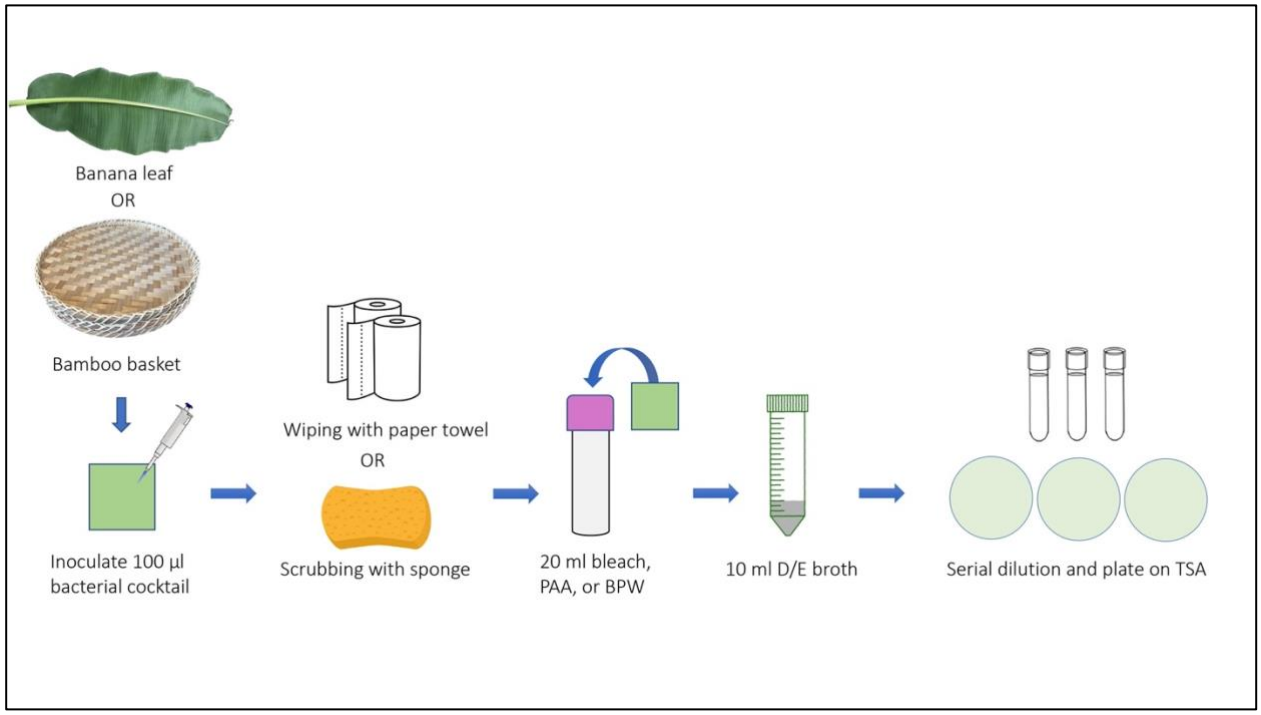

Figure 3.1. Schematic diagram followed in this study for the treatment of inoculated banana leaves and bamboo baskets.

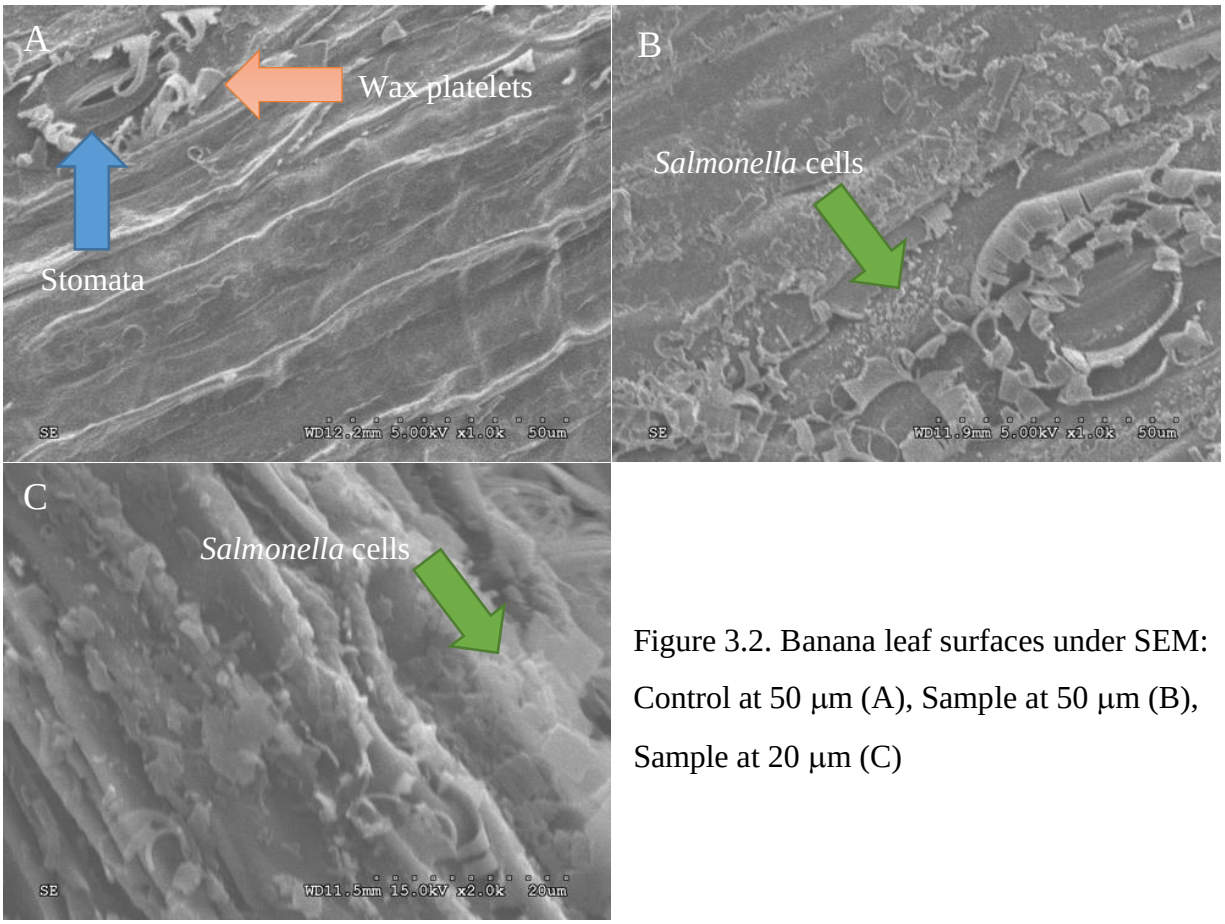

Figure 3.2. Banana leaf surfaces under SEM: Control at 50 μm (A), Sample at 50 μm (B), Sample at 20 μm (C)

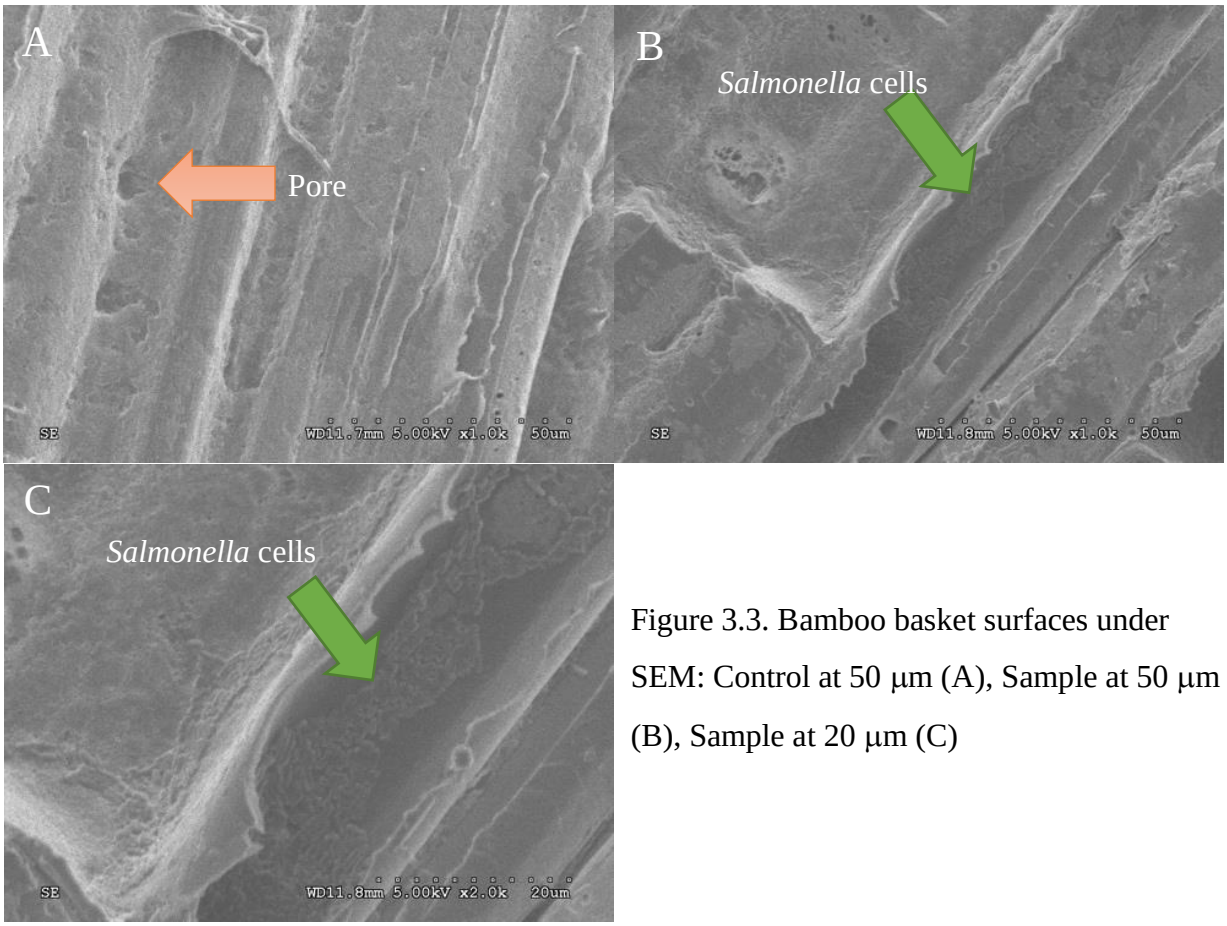

Figure 3.3. Bamboo basket surfaces under SEM: Control at 50 μm (A), Sample at 50 μm (B), Sample at 20 μm (C)

Table 3.1. *Salmonella* counts (Log CFU/cm^2 ± SEM) on banana leaf surfaces after treatments.

Cleaning	Sanitation	Remaining Log CFU/cm^2	Log Reduction	95% CI
Wiping	Bleach 3 min	4.15 ± 0.16^b	1.60	3.82, 4.47
	Bleach 5 min	2.85 ± 0.16^c	2.90	2.52, 3.18
	PAA 3 min	<LOQ	4.31	-
	PAA 5 min	<LOQ	4.31	-
	No sanitation	5.73 ± 0.07^a	0.02	5.59, 5.87
Scrubbing	Bleach 3 min	4.02 ± 0.16^b	1.73	3.69, 4.35
	Bleach 5 min	3.58 ± 0.16^b	2.17	3.25, 3.90
	PAA 3 min	<LOQ	4.31	-
	PAA 5 min	<LOQ	4.31	-
	No sanitation	5.75 ± 0.07^a	0.00	5.61, 5.90
No cleaning	Bleach 3 min	4.01 ± 0.16^b	1.74	3.68, 4.33
	Bleach 5 min	3.45 ± 0.16^b	2.30	3.19, 3.78
	PAA 3 min	<LOQ	4.31	-
	PAA 5 min	<LOQ	4.31	-
	No sanitation	5.75 ± 0.07^a	-	5.61, 5.90

Values are presented as mean ± standard error (n = 6).

CI, confidence interval

LOQ: Limit of Quantification (1.44 log CFU/cm^2).

Letters indicate significant differences between sanitation within each cleaning method (Bonferroni adjusted P<0.05).

Table 3.2. *Salmonella* counts (Log CFU/cm^2 ± SEM) on bamboo basket surfaces after treatments.

Sanitation	Remaining Log CFU/cm^2	Log Reduction	95% CI
Bleach 3 min	4.23 ± 0.12^{b}	1.14	3.99, 4.46
Bleach 5 min	3.91 ± 0.12^{b}	1.46	3.68, 4.15
PAA 3 min	4.01 ± 0.11^{b}	1.36	3.79, 4.23
PAA 5 min	3.11 ± 0.11^{c}	2.26	2.89, 3.33
No sanitation	5.37 ± 0.03^{a}	-	5.29, 5.45

Values are presented as mean ± standard error (n = 6).

CI, confidence interval.

Letters indicate significant differences between sanitation within each cleaning method (Bonferroni adjusted P<0.05).

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Chapter 4 - Efficacy of cleaning and sanitation methods in reducing *Escherichia coli* on banana leaves and bamboo baskets: common food contact surfaces used in Cambodian fresh food markets

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Abstract

At fresh food markets, conditions such as nutrient availability, moisture levels, and the presence of microorganism originated from raw materials, create a favorable environment that promote bacterial attachment on food contact surfaces. In this study we investigated the efficacy of cleaning and sanitation methods in reducing *E. coli* on food contact surfaces commonly used in fresh food markets in Cambodia, specifically bamboo basket and banana leaf surfaces. Surfaces were inoculated with multi-strain pathogenic *E. coli* cocktails. Combination of cleaning method (i.e., wiping, scrubbing, or no cleaning) and sanitation methods (i.e., 200 ppm of bleach applied for 3 or 5 min, 400 ppm of PAA applied for 3 or 5 min, or no sanitation) were evaluated. Findings indicated significant reductions of *E. coli* on bamboo baskets for selected combination of cleaning and sanitation method interaction (P=0.0019), suggesting that the efficacy of sanitation method was affected by the cleaning method utilized. In addition, 400 ppm PAA had a significantly higher antimicrobial potency than 200 ppm bleach in *E. coli* decontamination, reducing *E. coli* counts on bamboo baskets and banana leaves with a maximum estimated reduction of 4.03 log CFU/cm^2 and 3.70 log CFU/cm^2, respectively. These findings highlight the importance of selecting an appropriate combination of cleaning and sanitation method that can effectively reduce *E. coli* on bamboo baskets and banana leaves, particularly in situations where these surfaces are commonly used such as in fresh food markets in Cambodia.

4.1 Introduction

Cambodia is a developing nation in Southeast Asia with an estimated population of 16.9 million in 2023 (CIA, 2023). In the last two decades, Cambodia has experienced a rapid development that transitioned the country from a low-income to a lower-middle income status in 2015 (Ehrentraut, 2011). With an average annual economic growth of 7.7% between 1998 to 2018 the country aims to become the upper middle-income nation by 2030. Poverty and food insecurity are two major drivers for diarrheal diseases among the population. It is estimated that 18% of Cambodian live below the national poverty line (2.70$) and 45% have no access to a sufficient quantity of affordable and nutritious food (USAID, 2022). In fact, between 2014 to 2019, the Cambodian Ministry of Health (MOH) reported 134 foodborne disease (FBD) outbreaks that resulted in 5,825 cases, 5,598 hospitalizations and 81 deaths (Mekong Institute, 2019). Several factors were responsible for these foodborne outbreaks including poor hygiene and sanitation practices, temperature abuse, cross contamination, and the use of water and materials from unsafe sources (Mekong Institute, 2019). In addition, a study conducted by the Cambodian Communicable Diseases Control Department (CCDC) reported that the FBD hospitalization cost is relatively high ranging from 25 to 185 USD with an average of 63 USD per case (Srey, 2021). These alarming statistics highlight the urgent need for effective food safety interventions and a need to improve food safety infrastructures to minimize the risk of FBD in Cambodia.

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, and facultatively anaerobic bacterium that can be found in the environments, foods, humans, and animals. Most strain of *E. coli* are harmless and permanent inhabitants of the gastrointestinal tract of warm-blooded animals (Nataro & Kaper, 1998). However, certain types of *E. coli* are pathogenic causing mild to severe gastrointestinal illness in humans. Infection generally spreads through direct contact with animals,

from person-to-person, or via consumption of contaminated food and water (Banatvala et al., 2001). Symptoms vary and can manifest as bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), and may lead to death. Higher risk populations includes elderly people, pregnant women, children under the age of 5, and immunocompromised people (Smith & Fratamico, 2017). In Cambodia, *E. coli* contamination on various food items sold at fresh food markets have been previously reported. A study by Phoeurk et al., (2019) showed that *E. coli* were detected in 30.7%, 36.1%, and 5.7% of lettuce, saw-leaf herb, and cucumber samples collected from different types of markets in Phnom Penh, Cambodia. Similarly, Chrun et al., (2017) reported that 10% of total fermented vegetable samples from wet markets in Phnom Penh were contaminated with *E. coli*. These studies concluded that *E. coli* contamination in various food item sold at fresh food market were due to the lack of hygiene and sanitation practices among market vendors.

Bamboo baskets and banana leaves are common food contact surfaces (FCS) used in fresh food markets in Cambodia. They are extensively used to wrap, display, and transport different food items. Market vendors display fresh fruits and vegetables on these surfaces to attract consumers, as these materials often represent freshness to Cambodians. Additional benefits include reducing plastic use to address environmental concerns, which is a priority to many Cambodian consumers. However, without appropriate cleaning and sanitation, these unique FCS may potentially serve as routes for bacterial contamination, which has been previously documented (Rortana et al., 2021; Schwan et al., 2021). For instance, in a study investigating *Salmonella* spp. and *Staphylococcus aureus* in fresh food markets in Cambodia, Rortana et al., 2021 showed that 42% and 31% of chicken and pork cutting boards were contaminated with *Salmonella* spp. In addition, *Staphylococcus aureus* was isolated from 19% and 11% from chicken and pork cutting boards, respectively (Rortana et al., 2021). Similarly, Schwan et al., 2020 showed that FCS used

in fresh food markets were frequently contaminated with *Salmonella* (75%) as compared to non-food contact surfaces (25%). Contamination of foodborne pathogens on these food contact surfaces was associated with lack of hygiene and sanitation practices among vendors and inadequate public health infrastructures (Rortana et al., 2021; Schwan et al., 2021). Therefore, there is a need to develop an effective cleaning and sanitation method that can be adopted in fresh food markets in Cambodia to prevent bacterial contamination and reduce foodborne outbreaks.

The presence of *E. coli* on fresh food products and environments found at fresh food markets may serve as source of cross-contamination. *E. coli* abilities to attach to surfaces and form biofilms have also been extensively investigated, given that this natural phenomenon enhances bacterial survival and promotes bacterial persistence in an environment. To tackle these issues, various decontamination strategies have been developed including the use of chemical sanitizers (Marriott et al., 2018). Specifically, chlorine and peracetic acid (PAA) are the two most widely used disinfectants in the food industry (Marriott et al., 2018; Zoellner et al., 2018). The antimicrobial activity of chlorine and PAA have been investigated in *E. coli* decontamination on different food products and food contact surfaces in presence and absence of biofilms (Banach et al., 2020; Bang et al., 2014; Fu et al., 2018; Lee et al., 2016). However, studies mainly focused on the efficacy of sanitation methods on food contact surfaces commonly used in food industry. Little is known about the combination efficacy of cleaning and sanitation on unique FCS such as bamboo baskets and banana leaves commonly used in fresh food markets in Cambodia.

To our knowledge, no study has ever documented the effect of cleaning and sanitation methods on *E. coli* persistence on bamboo basket and banana leaf surfaces. Therefore, the objective of this study was to determine the efficacy of a combination of cleaning and sanitation methods on

reducing *E. coli* on bamboo basket and banana leaf surfaces commonly used in fresh food markets in Cambodia.

4.2 Materials and Methods

4.2.1 E. coli strains and culture preparation

Three pathogenic *E. coli* strains were used in this experiment: H30 (serotype O26:H11) isolated from infants with diarrhea, CDC 97-3068 (serotype O121:H19), and ATCC 35150 (serotype O157:H7) isolated from fecal samples from patients with severe gastrointestinal illness (Markell et al., 2015). Strains were selected in accordance to their prevalence in postharvest produce outbreak in the United States (CDC, 2018). Cultures were preserved in -80 °C using in a CryoCare Organism Preservation System (Key Scientific, Stamford, TX).

E. coli strains were pre-cultured in Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) for 24 h at 37 °C with 70 rpm shaking. Cultures were streaked on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD) and incubated for 24 h at 37 °C. Isolated colonies of each stain were inoculated into 10 ml TSB and grown at 37 °C for 24 h at 70 rpm shaking, to create overnight cultures. The overnight cultures were harvested by centrifugation at 4,000 rpm for 10 min. Pellets were re-suspended in 1 ml Phosphate Buffer Saline (PBS, VWR Chemicals, Solon, OH). Each of the individual *E. coli* strains were mixed at equal volumes (1 ml / suspension) to create a multi-strain cocktail for inoculation. Survival populations were counted utilizing TSA and traditional count plate methods. Colonies were counted if they were in the countable range (25-250) and reported as log CFU/cm^2. Log reduction was also reported to express the relative number of *E.coli* cells that are eliminated by treatment. It was calculated based upon the initial *E. coli* attached to surface minus the remaining *E. coli* cells after each cleaning and sanitation treatment.

4.2.2 Preparation of sample and bacterial inoculation

Surface samples were created by cutting bamboo baskets (2.6 cm^2) and banana leaves (9 cm^2) using a sterile template and surgical blade. Aseptic technique was maintained during this process. Surface samples were then transferred onto a sterile petri dish and placed under UV-C light exposure a Biosafety Cabinet (BSC, ESCO Life Sciences, South Yorkshire, England) for 15 min. For post-UV treatment, 10 drops of 10 µl of the multi-stain cocktails were placed on surface samples and allowed to dry for 60 min to complete inoculation.

4.2.3 Experimental design

For the purpose of this study, cleaning method was defined as wiping, scrubbing, or no cleaning. Sanitation method was defined as chemical sanitizer (i.e., 200 ppm of bleach for the contact time of 3 or 5 min, 400 ppm of peracetic acid for the contact time of 3 or 5 min, or no sanitation). Treatments were defined by the combination of cleaning and sanitation method as shown in Table 4.1. Experiments were carried out on banana leaf surfaces and bamboo basket surfaces. Observations were obtained from 6 different banana leaves or bamboo baskets. For each leaf or basket, 15 leaf pieces (3 x 3 cm^2) or 15 bamboo basket pieces (1.2 x 3 cm^2) were prepared and assignment to treatment. Individual pieces of banana leaves or bamboo baskets were recognized as a blocking structure to account for natural leaf-to-leaf and basket-to-basket. The schematic diagram of the experiment is illustrated in Figure 4.1.

Bleach (Clorox disinfecting bleach, Oakland, CA), and peracetic acid (Shield-Brite PAA 15.0, Pace International Wapato, WA) solution were diluted with sterile distilled water and freshly prepared prior to each experiment. The concentration of each chemical sanitizers was measured for each experiment using commercial test kits (Peracetic Acid Test Kit and Chlorine Test Kit, ChemWorld, Kennesaw, GA).

4.2.4 E. coli *attachment under scanning electron microscopy*

Sample surfaces (1 x 1 cm^2) were prepared and inoculated as previously described above for microscopy visualization. Inoculated sample surfaces were stained with SYTO9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR) and finally fixed with 10% formalin as previously described by Mendez et al., (2020). Sample surfaces were analyzed by Scanning Electron Microscopy (SEM, Hitachi S-3500N, Tokyo, Japan) located in the Microscopy Facility, Division of Biology at Kansas State University.

4.2.5 Statistical analysis

For statistical analysis, *E. coli* counts was expressed as a log CFU/cm^2 and was based upon the average count recorded from duplicate count plates. Comparison of *E. coli* counts for each treatment was completed utilizing a general linear mixed model. For this model, linear predictor included the fixed effects of cleaning method, sanitation method, and their 2-way interaction. For this study, an overarching block structure of leaf was utilized to account for the random effect of leaf in the leaf in the linear predictor. In addition, heterogeneous residual variances were fitted into the model in order to meet model assumptions. Heteroskedasticity seemed to be driven by chemical sanitizer, either none or bleach. The decision to incorporate heterogeneous residual variances was supported by enhanced model fit to the data, as indicated by Bayesian Information Criterion. Model assumptions were evaluated using externally studentized residuals and were considered to be reasonably met.

The statistical model was fitted using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute, Cary, NC). Variance components were estimated using restricted maximum likelihood. A Kenward-Roger's approach was used to estimate degrees of freedom and make corrections to estimated standard errors. Estimated least square means, corresponding standard errors and 95%

confidence interval are presented at the appropriate level of inference in the factorial treatment structure. Relevant pairwise comparisons between treatment groups were conducted using either a Tukey-Kramer or a Bonferroni adjustment, as appropriate in each case, to avoid inflation of Type I error rate due to multiple comparisons. Furthermore, tailored contrasts were built to evaluate the effect of chemical sanitizer averaged across contact times.

4.3 Results

4.3.1 Attachment of E. coli *on bamboo basket and banana leaf surfaces under SEM*

E. coli attachment on bamboo basket and banana leaf surfaces were analyzed by SEM. Figure 4.2A shows the attachment of *E. coli* cells on the grooves of bamboo basket surfaces as compared to control surface (Figure 4.2B). *E. coli* cells were pretty much abundant on the valleys of the bamboo basket surface as compared to the peaks. On banana leaf surfaces, *E. coli* cells were difficult to visualize under SEM due to the sophisticated nanostructure of banana leaf surfaces comprised of stomata, peak, valley, and wax platelets as showed in Figure 4.3B. However, *E. coli* cells were physically visualized in Figure 4.3A as compared to the control banana leaf surface (Figure 4.3B).

4.3.2 Survival of E. coli *on banana leaf surfaces following each treatment*

There was no evidence of any interaction between cleaning and sanitation methods (P=0.0882) neither for any main effect of cleaning methods (P=0.3899) on *E. coli* counts on banana leaf surfaces. However, there was evidence for the main effect of sanitation method (P<0.0001), whereby regardless of the cleaning method used, *E. coli* counts were highest in absence of sanitation method (P<0.001) and significantly reduced with bleach sanitation (P<0.001) and even further with PAA sanitation (P<0.001).

Table 4.2 presents the estimated *E. coli* counts with standard of error (SEM) and 95% confidence interval (CI) after each treatment. Initial *E. coli* counts on banana leaf surfaces were 5.14 log CFU/cm^2 (95% CI [5.06, 5.21]). PAA was the most effective sanitizer on banana leaf surfaces, reducing *E. coli* counts to below the limit of quantification (1.44 log CFU/cm^2) achieving 3.70 log CFU/cm^2 reduction, regardless of the cleaning method or contact time applied. The use of bleach as a sanitation method resulted in lower efficacy as compared to PAA. Bleach reduced *E. coli* counts on banana leaves to 3.29 log CFU/cm^2 (95% CI [3.15, 3.43]) achieving 1.85 log reduction and 1.87 log CFU/cm^2 (95% CI [1.73, 2.01]) achieving 3.27 log CFU/cm^2 for 3 and 5 min, respectively. This result highlights that the efficacy of bleach on banana leaves was improved with an increased contact time.

4.3.3 Survival of E. coli *on bamboo baskets surfaces following each treatment*

The magnitude of the reduction of *E. coli* counts on bamboo baskets was dependent on the combination of cleaning and sanitation methods (P=0.0019), indicating that the efficacy of sanitation method was influenced by the cleaning method used.

Estimated *E. coli* counts with standard of error (SEM) and 95% confidence interval (CI) for each treatment are shown in Table 4.3. Estimated mean of *E. coli* counts for the control treatment (i.e., no cleaning and sanitation method) were 6.37 log CFU/cm^2 (95% CI [6.24, 6.49]). The most effective treatment was the use of PAA for 5 min following no cleaning method, reducing *E. coli* counts on bamboo basket surfaces to 2.34 log CFU/cm^2 (95% CI [2.08, 2.60]) achieving 4.03 log reduction. In addition, when wiping was used as a cleaning method, estimated *E. coli* counts were highest in the absence of sanitation (P<0.0001), significantly decreased by the use of bleach (P<0.0001), and a significantly further reduction was observed with the use of PAA (P<0.0001). For both bleach and PAA sanitation methods combined with wiping as a cleaning

method, reduction of *E. coli* on bamboo basket surfaces were significantly enhanced with increased contact time (i.e., 3 vs 5 min; P=0.0031 and P=0.0051). However, there was no evidence of any differential effect of bleach 3 or 5 min following most of the cleaning method except for wiping.

There is no evidence for any main effect of cleaning method (i.e., wiping, scrubbing, or no cleaning) in reducing *E. coli* on bamboo baskets surfaces (P=0.4321).

4.4 Discussion

The use of bamboo baskets and banana leaves in food environment has been a longstanding tradition in Cambodia. The objective of this study was to evaluate the efficacy of cleaning and/or sanitation methods in reducing *E. coli* contamination on banana leaf and bamboo basket surfaces, through an inoculation study. Our findings indicate that the efficacy of cleaning and/or sanitation methods in reducing *E. coli* on bamboo basket surfaces was dependent on the combination of cleaning and sanitation methods used. The use of PAA chemical sanitizer for 5 minutes following no cleaning was the most effective combined treatment evaluated on bamboo basket surfaces. On banana leaf surfaces, PAA was the most effective sanitizer on banana leaf surface, regardless of the cleaning method or contact time applied.

The use of cleaning and sanitation strategies to control microbial populations in food environments has been previously investigated (Man et al., 2018; Ribeiro et al., 2019). These studies showed that the cleaning method used improved sanitation efficacy, suggesting that the efficacy of sanitation can be enhanced when associated with selected cleaning method.

One of the most advantages of cooperating bleach as a sanitation strategy in fresh food market is that it is relatively cheap and widely available in Cambodia. Despite resulting in a lower efficacy when compared to PAA, bleach still maintains a substantial reduction of *E. coli* on both surfaces. Our study showed that 200 ppm chlorine combined with selected cleaning method

resulted in 1.88 to 2.65 and 1.85 to 3.27 log CFU/cm^2 reduction of *E. coli* counts on bamboo basket and banana leaf surfaces, respectively. Similar log reductions have been previously documented (Beuchat et al., 2001; Nei et al., 2009; Pahariya et al., 2022). A study by Beuchat et al., (2001) reported that 200 ppm chlorine reduced *E. coli* O157:H7 on alfalfa seeds by 2.7 log CFU/g, which is comparable to our findings.

Traditional cleaning method such as wiping and scrubbing are common practices used to physically remove visible dirt and food residues on surfaces. Our findings suggested that there was no evidence of any effect of cleaning method (i.e., wiping or scrubbing) on reduction of *E. coli* on both surfaces evaluated. The effect of mechanically cleaning method (i.e., wiping or scrubbing) shown by other studies revealed different results. Sun-Young et al., (2011) found that wiping contaminated wood cutting boards with a dry paper towel resulted in a 1.15 log CFU/cm^2 reduction of *E. coli* O157:H7. Additionally, Kim et al., (2012) observed a reduction of 1.20 log CFU/cm^2 of *Vibrio parahaemolyticus* counts on wood cutting boards when wiped with a dry paper towel.

Surfaces characteristics of bamboo baskets and banana leaves are likely to play a role in attachment and survival of foodborne pathogens. The SEM images of bamboo baskets (Figure 4.2) clearly revealed a highly rough and porous nature of bamboo basket surfaces that could potentially serve as reservoirs for *E. coli*. In addition, this characteristic may provide the necessary crevices and niches for *E. coli* cells to attach, establish biofilms and resist the penetration of sanitizers. Previous studies reported that surface roughness is a critical factor in bacterial attachment, with higher roughness leading to greater bacterial attachment (Frank, 2001). Similarly, SEM images of banana leaf surfaces depicted a unique physical structure that could potentially affect the attachment and survival of *E. coli*. The images clearly visualize stomata, and layers of wax materials on the surface, forming multiple layers of wax platelets that created peaks and valleys

throughout leaf surface. These characteristics hampered the visualization of *E. coli* cells under SEM (Figure 4.3). These findings may have practical implications for food safety measures in settings that utilize natural materials such as fresh food markets in Cambodia.

These findings highlight the importance of selecting an appropriate sanitation method that can effectively reduce *E. coli* on bamboo baskets and banana leaves, particularly in situations where traditional cleaning methods such as wiping or scrubbing alone may not be effective. It is also important to acknowledge that these results were generated from a laboratory study. When applied at fresh food market environment, several considerations should be assessed such as the type of food contact surfaces used, the presence of physical and biological contaminants, availability of sanitizer and water, and storage temperature. For example, our study was performed in room temperature of about 25 °C while the temperature at fresh food market in Cambodia could go up to 40 °C in a hot day. These factors directly influence the efficacy of sanitizers and should be further investigated when using cleaning and sanitation methods to decontaminate food contact surfaces used at fresh food markets.

4.5 Conclusion

The presence of *E. coli* on food contact surfaces in fresh food markets is a potential health hazard. Bamboo baskets and banana leaves widely used at fresh food markets in Cambodia may serve as vehicles for foodborne pathogens transmission if surfaces are used without proper cleaning and sanitation measures. Overall, we reported that the effectiveness of cleaning and sanitation methods in reducing *E. coli* was reliant on the specific methods employed. Our statistical analysis confirmed a significant interaction between the cleaning and sanitation methods applied to bamboo baskets, suggesting that the success of the sanitation approach is reliant on the specific cleaning method implemented. Our study showed that cleaning and sanitation play an important

role in reducing *E. coli* contamination on both surfaces evaluated. This study provides insight for development of cleaning and sanitation practices that can be applied at fresh food markets in Cambodia to reduce the prevalence of *E. coli* on food items and prevent foodborne outbreaks.

4.6 Figures and Tables

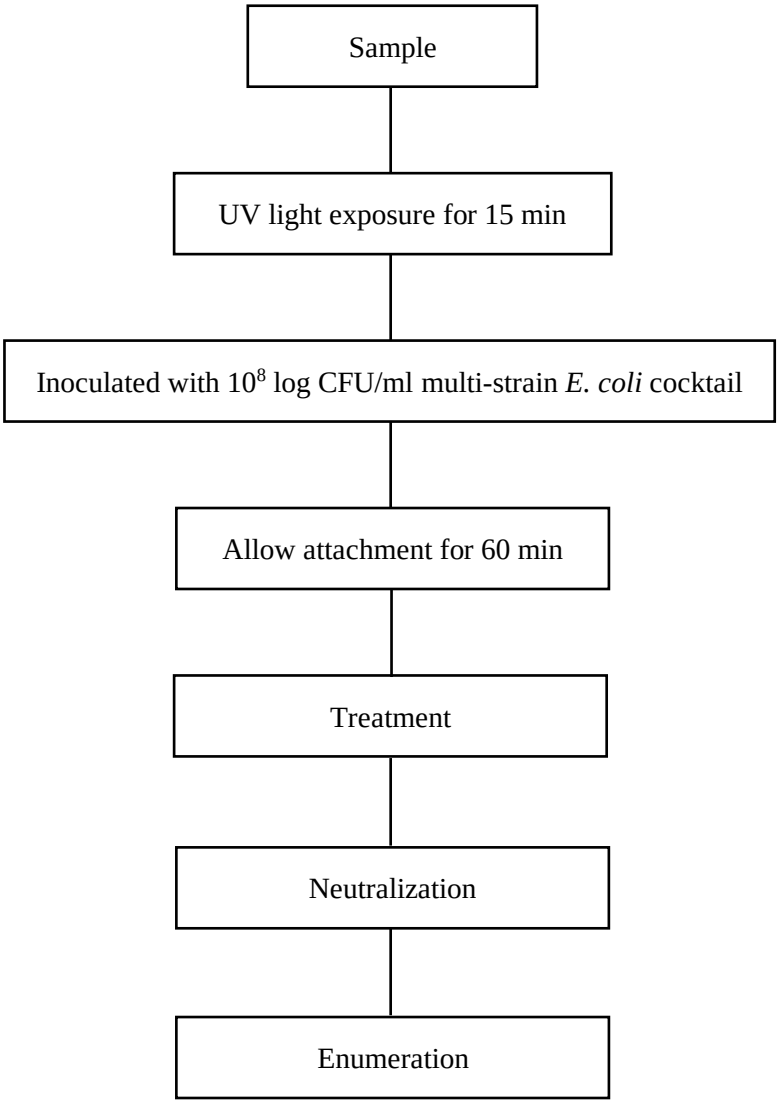

Figure 4.1. Schematic diagram of each treatment

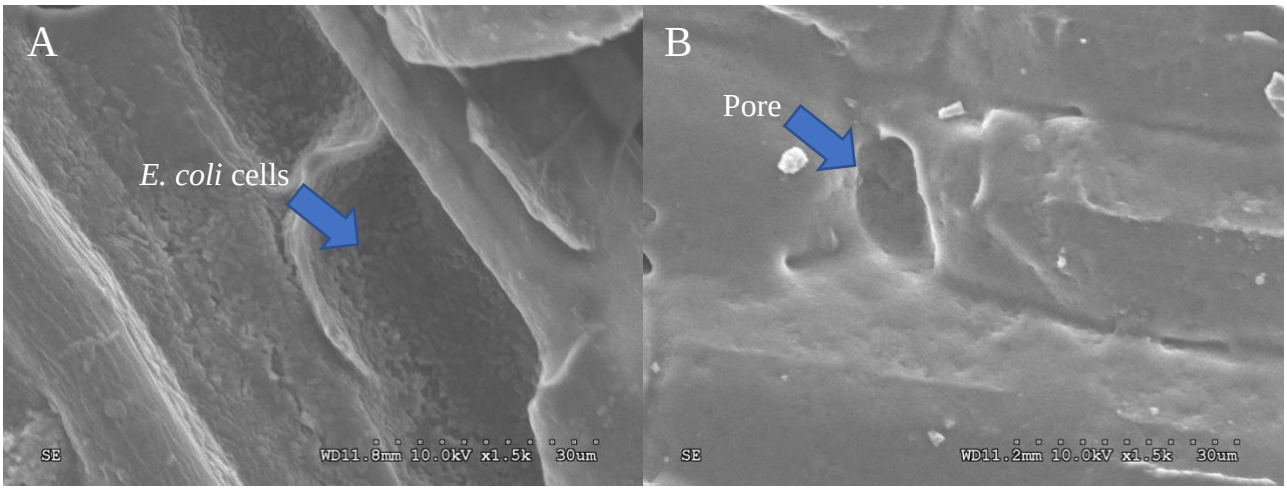

Figure 4.2. Bamboo basket surface under SEM with (A) and without (B) *E.coli* attachment

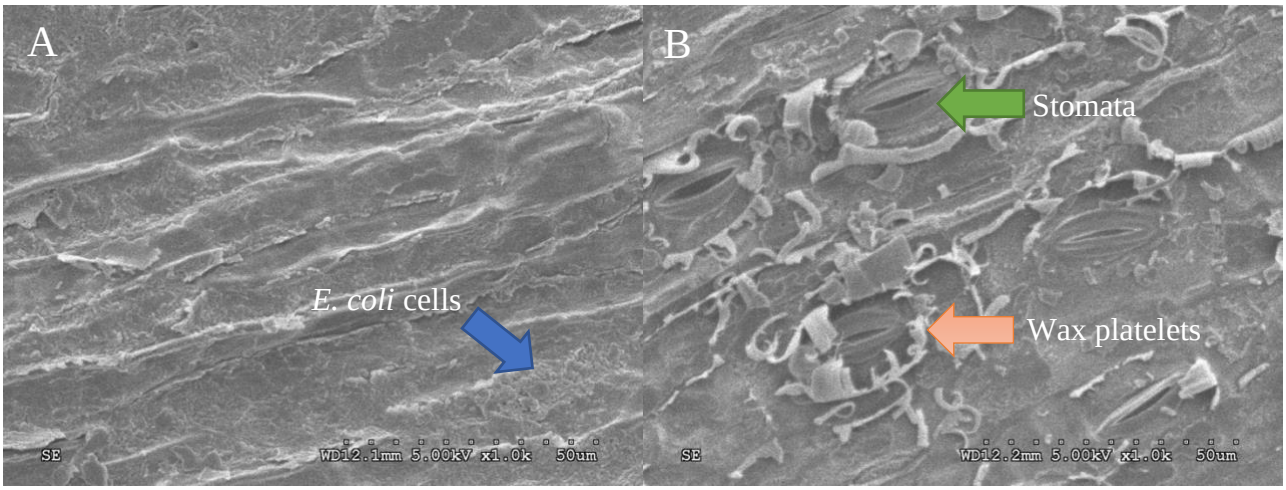

Figure 4.3. Banana leaf surface under SEM with (A) and without (B) *E.coli* attachment

Table 4.1. Summary of experimental design and treatment code

Cleaning methods	Sanitizer and contact time	Abbreviation
Wiping (W)	Bleach 3 min (B3)	WB3
	Bleach 5 min (B5)	WB5
	PAA 3 min (P3)	WP3
	PAA 5 min (P5)	WP5
	No sanitizer (N)	WN
Scrubbing (S)	Bleach 3 min (B3)	SB3
	Bleach 5 min (B5)	SB5
	PAA 3 min (P3)	SP3
	PAA 5 min (P5)	SP5
	No sanitizer (N)	SN
No cleaning (N)	Bleach 3 min (B3)	NB3
	Bleach 5 min (B5)	NB5
	PAA 3 min (P3)	NP3
	PAA 5 min (P5)	NP5
	No sanitizer (N)	Control

Table 4.2. *E. coli* counts (Log CFU/cm^2 ± SEM) on banana leaves by treatment

Sanitation	Remaining Log CFU/cm^2	Log Reduction	95% CI
Bleach 3 min	3.29 ± 0.07^b	1.85	3.15, 3.43
Bleach 5 min	1.87 ± 0.07^c	3.27	1.73, 2.01
PAA 3 min	<LOQ	3.70	-
PAA 5 min	<LOQ	3.70	-
No sanitation	5.14 ± 0.04^a	-	5.06, 5.21

Values are presented as mean ± standard error (n = 6).

LOQ, limit of quantification (1.44 log CFU/cm^2).

CI, confidence interval

Letters indicate significant differences between sanitation within each cleaning method (Bonferroni adjusted P<0.05).

Table 4.3. *E. coli* counts (Log CFU/cm^2 ± SEM) on bamboo baskets by treatment

Cleaning	Sanitation	Remaining Log CFU/cm^2	Log Reduction	95% CI
Wiping	Bleach 3 min	4.49 ± 0.13^{b}	1.88	4.23, 4.76
	Bleach 5 min	3.82 ± 0.13^{c}	2.55	3.55, 4.08
	PAA 3 min	3.35 ± 0.13^{c}	3.02	3.10, 3.61
	PAA 5 min	2.67 ± 0.13^{d}	3.70	2.41, 2.93
	No sanitation	6.24 ± 0.06^{a}	0.13	6.11, 6.36
Scrubbing	Bleach 3 min	4.19 ± 0.13^{b}	2.18	3.93, 4.46
	Bleach 5 min	3.72 ± 0.13^{bc}	2.65	3.46, 3.99
	PAA 3 min	3.27 ± 0.13^{cd}	3.10	3.01, 3.52
	PAA 5 min	3.15 ± 0.13^{d}	3.22	2.90, 3.41
	No sanitation	6.24 ± 0.06^{a}	0.13	6.12, 6.37
No cleaning	Bleach 3 min	4.32 ± 0.13^{b}	2.05	4.05, 4.58
	Bleach 5 min	3.96 ± 0.13^{b}	2.41	3.70, 4.22
	PAA 3 min	3.18 ± 0.13^{c}	3.19	2.92, 3.43
	PAA 5 min	2.34 ± 0.13^{d}	4.03	2.08, 260
	No sanitation	6.37 ± 0.06^{a}	-	6.24, 6.49

Values are presented as mean ± standard error (n = 6).

CI, confidence interval

Letters indicate significant differences between sanitation within each cleaning method (Bonferroni adjusted P<0.05).

4.7 References

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Chapter 5 - Summary and Recommendations

5.1 Summary

The experiments of this research project were conducted to understand the effect of cleaning and sanitation method on unique FCS commonly used in fresh food markets in Cambodia. The findings of these studies could be used as a basis to develop effective cleaning and sanitation interventions to improve product safety in fresh food markets in Cambodia. The main results from this study are summarized below:

1) Reduction of *Salmonella* on banana leaf surfaces and *E. coli* on bamboo basket surfaces depended upon both cleaning and sanitation method applied.
2) Traditional cleaning methods such as wiping or scrubbing did not significantly reduced *Salmonella* and *E. coli* contamination on both surfaces.
3) PAA was the most effective sanitation method in reducing both *Salmonella* and *E. coli* on banana leaf surfaces.
4) Bleach maintained an effective antimicrobial activity against both foodborne pathogens tested.

The data presented in this study contributes to understanding the abilities of *Salmonella* and *E. coli* to attach and resist cleaning and sanitation strategies on different surfaces relevant to food premises.

5.2 Recommendations

Potential recommendations for further studies are listed below:

1) Study the efficacy of other commercially available chemical sanitizers such as silver dihydrogen citrate, lactic acid, acetic acid, and thymol in reducing foodborne pathogens on FCS.

2) Explore the effect of other factors such as surface type, organism type, inoculation protocol, sanitizer delivery method, and the presence of organic matter could be explored.
3) Investigate the effect of hydrophobicity and wetting properties of foodborne pathogen on different FCS.