

1. INTRODUCTION

1.1. Background

Foodborne diseases caused by pathogenic bacteria are still a major threat to public health (Sobel & Watson, 2009). Food borne pathogens bacteria require certain processing to inhibit the growth. However, this process might cause undesirable flavor or texture to food product. One of the solutions is to use antimicrobial agents. Nowadays, there are a lot of trends that use natural products as the source of antimicrobial agents. Natural substances or bioactive compound might be effective to kill the foodborne pathogenic bacteria without decreasing the quality of the food. Naturally antimicrobial compounds are present in plant leaves, stems, bark, roots, flowers, and fruit. Plants have secondary metabolites that can be responsible for the natural resistance. Thus, a lot of researches were done to discover plant's antimicrobial properties so it can treat foodborne diseases (Hashiim *et al.*, 2010).

Zingiber officinale Roscoe or as known as ginger seems to have potential as antimicrobial agent. Ginger contains of secondary metabolites, especially the flavonoid, phenol, terpenoids and essential oils. Those secondary metabolites produced can inhibit the growth of pathogenic microorganisms (Nursal *et al.*, 2006). Essential oils and compounds in ginger are determined by harvesting time. Essential oil of ginger which harvested young, reported to be higher than the old harvested age. In old harvested ginger, the essential oils will decrease, whereas the levels of starch and fiber will increase. Concentration of terpenoids compound is also greater in plants that still young. Thus, young rhizome has higher antimicrobial activity than old rhizome (Gersbenzon & Croteau, 1991).

Many studies have already reported the antimicrobial properties of *Z. officinale* Roscoe against some selected pathogenic bacteria (Akintobi *et al.*, 2011; Indah *et al.*, 2013; Jain *et al.*, 2016) but limited research about antimicrobial activity analysis based on different harvesting time. Therefore, the analysis of antibacterial activity based on different harvesting time might be studied.

According to Burt (2004), secondary metabolites in ginger are affected by the form of ginger (fresh and dried form). Nada *et al.*, (2014) reported that water extract of powdered ginger has higher antibacterial activity than water extract of fresh ginger. According to Widada (1993), grinding process produces heat which causes polymerization of most components in essential oil. Polymerization causes the formation of resin and polymers that have higher molecular weight. Resin consists of phenolic compounds, so the formation of a resin compound will increase the number of phenolic components in oleoresin, which means powdered ginger will have higher antibacterial effect.

Sivasothy *et al.*, (2011) reported that essential oil in ginger are generally more effective against gram positive bacteria compared to gram negative. It is because the outer membrane of Gram-negative bacteria has lipopolysaccharide (LPS) which act as barrier function, but Gram-positive bacteria do not have. The bacteria used in this study are *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes*, *Bacillus cereus*, and *Escherichia coli*. These five pathogenic bacteria can cause many dangerous illnesses and even death if they are too much consumed (Wagner, 2012). *B. cereus* represents Gram positive spore-forming bacteria while *S. aureus* and *L. monocytogenes* represent Gram positive non spore-forming bacteria. *S. Typhimurium* and *E. coli* represent Gram negative non spore-forming bacteria.

1.2. Literature Review

1.2.1. Functional Properties of *Z. officinale* Roscoe

Z. officinale, a member of the Zingiberaceae family, is a household spice used in the daily in many Asian countries. Ginger is usually used as spices in food and drinks, cosmetics, essential oil, flavoring agent, ingredients for some industrial process, and herbal medicine, to treat rheumatic diseases, asthma, stroke, toothache, diabetes, muscle pain, throat, cramps, hypertension, nausea, fever and infection (Wang & Wang, 2005). Oleoresin is one of ginger's products which obtained from extraction using organic solvents. Oleoresin contains of 2-3% essential oil and resin (Uhl, 2000).

Essential oil in ginger or also called the volatile compounds is a component which produces the aroma and flavor in ginger. It is generally soluble in organic solvents and insoluble in water. The main components of essential oil is sesquiterpene hydrocarbon which consist of zingiberene (35%), curcumen (18%), farnescene (10%), α - (7,84%) dan β - bisabolene (3,34%), limonen (1,48 – 5,08%). The other components come from monoterpenoid which are 1,8-cineole, linalool, borneol, neral, dan geraniol (Felipe *et al.*, 2008). Those components are determined by the harvesting time and type of ginger. At the young age, rhizomes have higher essential oils. While in old rhizome, the essential oils decrease due to evaporation, whereas the level of starch and fiber will increase (Gersbenzon & Croteau, 1991).

Resin or known as non-volatile compound is a component which provides a pungent taste in ginger. Resin consists of phenolic compounds, which are gingerol, zingerone, shogaol, and paradol. Gingerol is a compound which is highly sensitive to heat. Heat treatments will make gingerol compound decrease and change into shogaol, which cause fresh ginger is most pungent than the other form of ginger. The volatile compounds reach maximum levels when rhizomes have more than 9 months period after planting, hence the young harvested ginger taste less pungent (Raghavan, 2007).

Nursal *et al.*, (2006) reported that secondary metabolites in ginger which are flavonoids, phenol, terpenoids, and essential oil can inhibit the growth of bacteria *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, fungi *Neurospora sp*, *Rhizopus sp* and *Penicillium sp*. Many studies already conducted that natural preservative and antimicrobial function usually comes from secondary metabolites in plants. Secondary metabolites do not have a direct function in the growth and maintenance of plants, but acts as protective functions against different forms of stress in plants. This secondary metabolites produced by plants besides the primary biosynthetic and metabolic route of compounds for plant daily function used (Veberic, 2010).

Ginger-growth stages consist of several phases, which are germinating stage, seedling stage, growth and development stage, and rhizome dormant stage. Germinating stage

starts when the dormant bud begins to sprout to the opening of the first leaf. The period time of germinating phases is 90 days. The seedling stage is from the first leaf opening to the stage at which small rhizomes with three layers is formed, or usually called three branches stage. The plants grow slowly in seedling stage. This phase begins 90-110 days after planting. Growth and development stage divide into 2 stages, which are growing stage and rhizome-expanding stage. Growing stage is the most crucial phase of growth because the plants show quick growth, many tillers arise and leaf number and area increase sharply, the rhizome branches producing fingers but their growth is slow. This happens during 110-130 days. At rhizome-expanding stage, the growth emphasis is shifted to the rhizome. At this time, the root quantity becomes stabilized, tillering speed decrease, and the leaf area reaches steady state. It begins 130-160 days after planting. The next phase is rhizome dormant stage, a period of temporary suspension of growth activity (Ravindran & Nirmal, 2005). Ginger-growing stages can be seen in Figure 1.

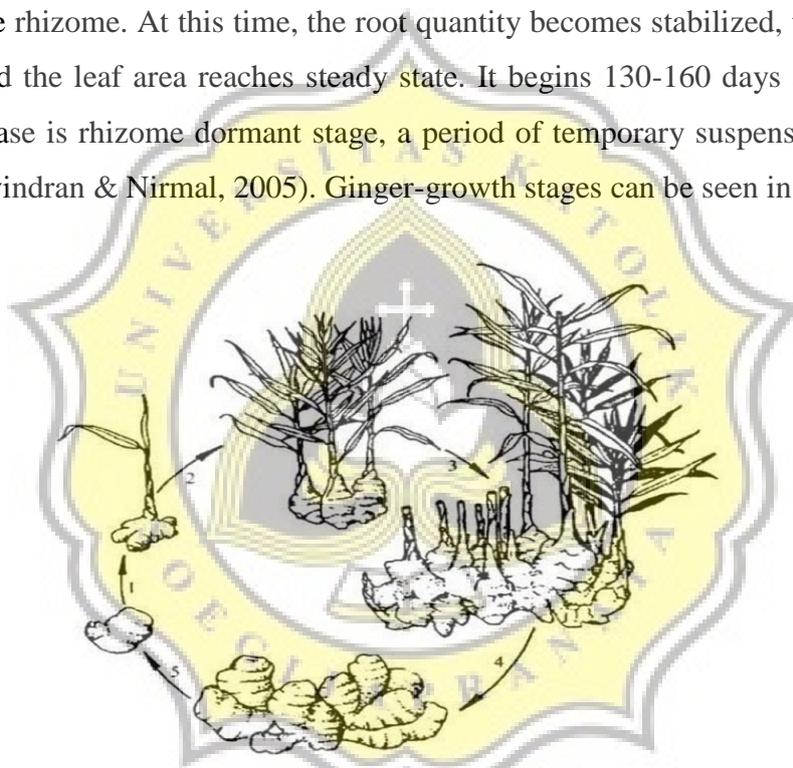


Figure 1. Ginger-growing stages. (1) Germinating stage, (2) Seedling stage, (3,4) Growing and development stage, (5) Rhizome dormant stage (Source: Ravindran & Nirmal, 2005)

1.2.2. Pathogenic Bacteria

Bacillus cereus is a Gram-positive spore-forming bacterium. It is a facultative anaerobe bacterium that grows over a temperature range of 10-48°C and has an optimum growth temperature of 35-45°C. *B. cereus* was responsible to 14 outbreaks and caused 691 reported cases of foodborne illness in U.S. (Keith *et al.*, 2004) *B. cereus* spores are

more resistant to heat and chemical treatments than vegetative pathogens. Milk, meat, spices, and meat additives can be contaminated with the spores (Bhunias, 2008). Priest (1993) added that *B. cereus* is often present as an intrinsic contaminating microorganism in Refrigerated Processed Foods of Extended Durability (REFPED), pasteurized milk, rice dishes and pastas. It produces enterotoxin which is known as diarrheagenic toxin, diarrheal agent, fluid accumulation factor, vascular permeability factor, dermonecrotic toxin and intestinonecrotic toxin (Spira & Goepfert, 1972).

Staphylococcus aureus is a spherical, Gram-positive bacterium. These organisms are facultative anaerobes with an optimum growth temperature around 37°C and capable to survive down to 8 °C or slightly below (Hayes, 1995). The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperatures as high as 50°C, high salt concentrations, and drying process (Tolan, 2012). *S. aureus* grows when the food is left at room temperature for long periods. At temperature 60°C or higher, the organism will not grow. However, below 60°C the organism will grow and produce toxins. It is the major cause of skin and soft tissue infections such as boils, pimples, impetigo, and cellulites. *S. aureus* can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections (Bhunias, 2008).

Salmonella sp. is a Gram-negative bacterium, rod-shaped bacilli that belong to the family Enterobacteriaceae. *Salmonella.sp* is facultative anaerob, with an optimum growth temperature is 35-37°C, unable to form spores, and motile or able to move around spontaneously (Gray *et al.*, 2002). *Salmonella sp.* produces a heat labile enterotoxin which cause diarrhea and some of them also produce cytotoxin which may cause general enteric symptoms and inflammation (Jay *et al.*, 2003). *Salmonella enterica* is widely dispersed in nature. It can colonize the intestinal tracts of vertebrates, including livestock, wildlife, domestic pets, and humans, and may also live in environments such as pond water. The fresh food also has been the source of major outbreaks. A few examples of foods that have been linked to *S. enterica* illness include meats, poultry, eggs, milk and dairy products, fish, shrimp, spices, yeast, coconut, sauces, freshly prepared salad dressings made with unpasteurized eggs, cake mixes, cream-filled desserts and toppings that contain raw egg, dried gelatin, peanut butter,

cocoa, produce (fruits and vegetables, such as tomatoes, peppers, and cantaloupes), and chocolate (FDA, 2012).

Escherichia coli is a Gram-negative, non-sporing bacterium. *E. coli* is a mesophile typical growing at optimum temperature around 37°C. It is not heat resistance, but it can survive in refrigerated condition or frozen storage for extended periods (Adams & Moss, 2008). *E. coli* is most often associate with human illnesses which cause about 73.500 causes of illness and 60 deaths annually in the U.S. (David *et al.*, 2012). It causes acute diarrhea, gastroenteritis, dysentery, hemolytic uremic syndrome (HUS), urinary tract infection (UTI), septicemia, pneumonia, and meningitis (Bhunja, 2008). *E. coli* is not only present in uncooked meat, but also in the environment. It can be found in contaminated water and in people who consume shellfish from the contaminated water (Mallin *et al.*, 2000).

Listeria monocytogenes is a Gram positive, rod-shaped, facultative bacterium, and motile. *L. monocytogenes* can grow in refrigerated temperatures. Raw milk, inadequately pasteurized milk, chocolate milk, cheeses (particularly soft cheeses), ice cream, raw vegetables, raw poultry and meat, fermented raw-meat sausages, hot dogs and deli meats, and raw and smoked fish and other seafood are usually associated with *L. monocytogenes*. Illness that caused by *L. monocytogenes* is generally called listeriosis. Listeriosis is gastrointestinal disease that can cause endocarditis. Endocarditis is an inflammation of heart tissue due to bacterial infection. The other diseases that caused by listeriosis such as brain abscesses, eye infections, hepatitis or liver disease, peritonitis or abdominal infection, lung infection, joint infection, arthritis, heart disease, bone infection, and gall bladder infection. This disease is very dangerous for human especially for pregnant women. In non-pregnant adults, *L. monocytogenes* primarily causes septicemia, meningitis, and meningoencephalitis (FDA, 2012) *L. monocytogenes* is among the major causes of death from foodborne illness. A recent report by the Center for Disease Control and Prevention (CDC) estimated that domestically acquired foodborne *L. monocytogenes* causes 255 deaths in the U.S. annually (FDA, 2012).

1.2.3. Antibacterial Test

The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including bioautographic, diffusion, and dilution methods. The bioautographic and diffusion methods are known as qualitative techniques since these methods will only give an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays (Valgas *et al.*, 2007). Diffusion method can be done in three ways, namely cylinders method, hole-plate method and paper disk method. Hole-plate method or well diffusion is performed by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 μL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. Agar diffusion test is performed by measuring the diameter of clear zone which is indicative of the response of bacterial growth inhibition by an antibacterial compounds in the extract (Balouiri *et al.*, 2016).

Amoxicillin is used as a positive control because it is effective against Gram-negative and Gram-positive bacteria. Amoxicillin has greater efficiency compare to other antibiotics e.g. ampicillin, azithromycin, clarithromycin, cefuroxime, and doxycycline in treatment of various infections. Amoxicillin inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer. Amoxicillin binds the protein which cause deactivation of enzymes involved in the transpeptidation reaction or cross linking of peptidoglycans. Inhibition of this cross linking reaction leads to loss of cell wall integrity, and cause cell lysis (Vincent *et al.*, 2011).

Dimethylsulfoxide (DMSO) is used as a negative control because the ginger extract was diluted with this solvent after being concentrated. DMSO is often used to dissolve extract and rarely applied as an extraction solvent because it has high boiling point of 189⁰ C, making it impossible to be removed at a low temperature (40⁰C) using rotary

evaporator (Thomas, 2010). DMSO polarity is 44.4 which is classified as semi polar solvent where it could dissolve both non-polar or polar molecules (Jan, 1996)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of an organism after overnight incubation. Based on Okeke *et al.*, 2001, MIC test can be done by 2 methods, which are agar well diffusion method and macro-broth dilution method. In agar well diffusion method, plant extracts with different concentration are added in wells in MHA (Muller-Hinton agar) plates pre-inoculated with test bacterial cells. Commercial antibiotic is used as positive control. All test plates are incubated at 37°C for 24 hours. The least concentration of each extract showing a clear zone of inhibition is taken as the MIC. For the macro-broth dilution method, some serial dilutions of the extracts are prepared with MHB (Muller-Hinton broth) as diluent, and tested with organism. The MIC was taken as the last dilution showing no noticeable growth (turbidity).

Evaluation of the inoculation procedure for diffusion method is using 0.5 McFarland standard or equal to 1.5×10^8 cells of approximate cells density. The McFarland equivalence standards are intended to be part of a quality control program for adjusting densities of bacterial suspensions that are used for identification and susceptibility testing. The broth cultures are adjusted using spectrophotometer at a wave length of either 600 or 625 nm (McFarland, 1907).

1.2.4. Total Phenolic Compound Test

Phenolic compounds have the presence of at least one aromatic hydroxyl-substituted. Phenolic compound acts as protective functions against some stress conditions of plants (Veberic, 2010). Jain *et al.*, (2016) stated that phenolic compounds (shogaol, gingerol) has important role as antimicrobial agent in ginger. The roles of phenol on killing microorganism is by precipitated the outer protein membranes, ruptured the cell wall, coagulated and caused loss of the cell contents and energy through the leakages of cell wall. Besides, the essential oils contained in the ginger extract were able to induce the leakage of ions and other cell contents. The extract, therefore, affected the bacterial

cytoplasmic membrane and induced the loss of nucleic acid and ions which cause denaturation of proteins inside the cell (Poeloengan, 2011). If the concentration of phenol is low, the cell constituents (nucleic acid and glutamic acid) are liberated in external media. But, when the concentration of phenol is high, the phenolic compound could inhibit intracellular enzyme thus causing denaturation of bacterial proteins and lysis of the cell membrane (Maris, 1995).

Folin-Ciocalteu method is the most common method used to determine total phenols. Folin-Ciocalteu method is selected because the technique is cheaper and easier. The principle of this reaction is based on the reduction of Folin reagent (phosphomolibdat acid and phosphotungstat acid) with hydroxyl phenolic groups (tyrosine and tryptophan) which produces a blue color (Vermeris, 2006). Phenols in plant extracts react with specific Folin-Ciocalteu reagent (redox reagents) to form a blue chromophore constituted by a phosphotungstic-phosphomolybdenum complex, where the absorption of the chromophores can be quantified by spectrophotometer at OD_{765} (Andressa *et al.*, 2013). As a standard comparison, Gallic acid is used, which is a class of simple phenolic acids. Gallic acid is a compound commonly used as a standard in addition of chlorogenic acid. Gallic acid is chosen because it is more stable, pure, and cheaper (Vermeris, 2006).

1.3. Objectives

The objectives of this research are to observe the antibacterial activity on fresh and powdered of *Zingiber officinale* Roscoe based on different harvesting time (3, 4, and 5 months) against Gram-positive bacteria (*Bacillus cereus* FNCC 0057, *Staphylococcus aureus* FNCC 0047, and *Listeria monocytogenes* FNCC 0156) and Gram-negative bacteria (*Escherichia coli* FNCC 0091, and *Salmonella enterica typhimurium* FNCC 0050), followed by Minimum Inhibitory Concentration (MIC) test, and to determine the total phenolic compounds in ginger.