Antibacterial activities of *Clitocybe nuda* extract on foodborne pathogens

by

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Abstract

The addition of antimicrobial agents to foods is an important approach for controlling foodborne pathogens to improve food safety. Currently, most antimicrobial agents in foods are synthetic compounds. Researchers are looking for natural antimicrobial agents to substitute for synthetic compounds in foods to control microbial growth in foods. *Clitocybe nuda* is an edible macrofungus which produces a large number of biologically active compounds with antibacterial activities. The purpose of this study was to evaluate the antibacterial activities of *Clitocybe nuda* extract on foodborne pathogens and its stability at different temperatures and pHs, and to estimate the molecular weights of some of the antibacterial components of the fungus. The antimicrobial activity was evaluated by testing the minimum inhibitory concentration (MIC) on four foodborne pathogens: *Listeria monocytogenes, Salmonella typhimurium, E. coli* O157:H7, and *Staphylococcus aureus*. The stability of the extract was tested at pH 4-10, and temperatures of 4, 72, 100, and 121 ℃. The growth of pathogens was significantly inhibited by the *Clitocybe nuda* extract. The MIC_{50} for *Listeria monocytogenes, Salmonella typhimurium, E. coli* O157:H7 and *Staphylococcus aureus* are 79.20, 84.51, 105.86, and 143.60 mg/mL, respectively. The antibacterial activities among heat-treated *Clitocybe nuda* extracts were not affected by heat, and there was no significant different among the four temperatures evaluated ($p \geq 0.05$). The pH had no negative impact on the antibacterial activities. The data indicate that the extract can effectively control the growth of foodborne pathogens and is very stable at high temperatures and over a wide range of pH.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

BPB	Butterfield's phosphate buffer
CAP	Controlled-atmosphere Packaging
CFU	Colony Forming Unit
DAEC	Diffusely Adhering *E. coli*
DI	Deionized
EAEC	Enteroaggregative *E. coli*
EPEC	Enteropathogenic *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ETEC	Enterotoxigenic *Escherichia coli*
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
GRAS	Generally Recognized As Safe
HACCP	Hazard Analysis and Critical Control Point
HUS	Hemolytic Uremic Syndrome
LAB	Lactic Acid Bacteria
MAP	Modified-atmosphere Packaging
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MW	Molecular Weight

MWCO	Molecular Weight Cug-off
RH	Relative Humidity
RTE	Ready-to-eat
SE	Staphylococcal Enterotoxin
Stxs	Shiga-like Toxins
STEC	Shiga Toxin-producing *E. coli*
TSB	Tryptic Soy Broth
TTP	Thrombotic Thrombocytopenic Purpura
USDA	US Department of Agriculture
UTI	Urinary Tract Infection
UV	Ultraviolet
VTEC	Verotoxin-producing *E. coli*

CHAPTER 1: INTRODUCTION

This chapter addresses the food safety concerns in the modern food industry. The purpose and significance of the study are also mentioned in this chapter.

1. Background

The consumption of processed foods has been growing rapidly; food safety has become a great concern all over the world. Foodborne illnesses can be induced by foodborne pathogens, chemicals, and toxins contaminated foods. The Center for Disease Control and Prevention (CDC) estimated that there were 48 million foodborne illnesses and 1,000 outbreaks each year in the US, which means one in six Americans got sick due to the consumption of contaminated foods each year. These illnesses caused 128,000 hospitalizations and 3,000 deaths (CDC 2011a). However, not all illnesses were reported, therefore these numbers are underestimated.

Foodborne pathogenic microorganisms can cause diseases in humans. The illnesses can be serious and mortal. For example, *Clostridium botulinum*, *Escherchia coli* O157:H7 and *Salmonella* can cause serious foodborne illnesses that lead to death. Foodborne pathogens also lead to an economic burden every year. According to Scharff (2012), 77.7 billion dollars was lost annually to investigate foodborne illnesses associated with 31 pathogens including *Salmonella, Listeria monocytogenes,* and *E. coli* O157:H7. *Salmonella* nontyphoidal was reported to be the number one pathogen resulting in hospitalizations (CDC 2011a). Foodborne pathogens also lead

to serious economic loss. For example, there are 2-4 million cases of illness associated with *Salmonella*, and these cases caused about 3 billion dollars of economic loss (Bhunia 2008).

In recent years, several multistate outbreaks induced by foodborne pathogens caused a large number of illnesses and deaths. In December 2011, there was a *Listeria monocytogenes* outbreak which involved 28 states, and 146 illnesses. Thirty deaths and one miscarriage were reported (CDC 2011b). In 2009, *Salmonella* contaminated peanut butter resulted in 714 illnesses and 9 deaths (CDC 2009). In September 2000, *Staphylococcus aureus* contaminated barbeque pork resulted in 214 illnesses and 6 hospitalizations (CDC 2012).

Although many antimicrobials are applied to foods to inhibit the growth of foodborne pathogens, there are still several concerns forcing food researchers to search for novel antimicrobials to substitute for the current synthetic compounds. Firstly, "natural" and "minimally processed" food products have become more popular among consumers. Secondly, the transfer of antibiotic resistance to pathogens is also an issue. Thirdly, some antimicrobials may make the sensory properties of the products undesirable to consume.

2. Purpose of Study

Since foodborne pathogens cause serious food safety issues every year, it is important to develop appropriate avenues for controlling foodborne pathogens to improve food safety. Currently, the major antimicrobial agents used in the food industry are synthetic compounds. Due to health concerns, food safety specialists are looking for natural antimicrobial agents to substitute for synthetic compounds for controlling microbial growth. A large number of fungi in the group basidiomycetes have shown antibacterial, antiviral, and antifungal activities against

various human pathogens (Suay and others 2000). Many of these basidiomycetes are edible mushrooms, such as *Clitocybe nuda*.

Clitocybe nuda is an edible macrofungus which produces a large number of biologically active compounds with antimicrobial activities. Most investigations with macrofungi focused on their therapeutics activities of human disorders and few of them focused on their antimicrobial activities against pathogens. Chen and Huang (2009) reported the antimicrobial activity of *Clitocybe nuda* against plant pathogens. The purpose of this study are 1) to evaluate the antibacterial activities of *Clitocybe nuda* extract on foodborne pathogens, 2) to test its stability at different temperatures and pHs, and 3) to estimate the molecular size of the effective compounds.

3. Significance of Study

Currently, the commonly used food preservatives are synthetic or artificial chemicals. There are concerns of using these compounds. Firstly, they might be harmful to human health. For example, nitrite, which is used as a curing agent to inhibit *Clostridium* growth in meat products, can react with amines and ammonium compounds to form nitrosamines which are carcinogenic (Jay and others 2005). Secondly, their effectiveness is highly related to the conditions of the foods, such as moisture content, pH, and oxidation-reduction potential of the food. Thirdly, "natural" is the new trend in food industry. Artificial food preservatives are not preferred by consumers who want natural foods.

Clitocybe nuda is an edible mushroom with bio-activity. Antimicrobial activity is one of the more important functions. This mushroom has shown great antifungal and antibacterial activities against plant pathogens in a previous study (Chen and Huang 2009). However, few studies have reported its antibacterial activities against foodborne pathogens. This study analyzed

the antibacterial activities against four foodborne pathogens and the potential uses of this mushroom extract as a food preservative.

CHAPTER 2: REVIEW OF LITERATURE

This chapter is a review of literature related to food safety concerns, foodborne pathogens, and current food preservation methods. The bio-activity of edible mushrooms are also discussed in this chapter, especially their antimicrobial activity. Since the mushrooms are edible, they are natural and novel sources of food preservatives to substitute for the synthetic chemicals.

1. Food safety concern

1.1 Microorganisms

Microbial growth and metabolism can be influenced by food composition, temperature, relative humidity, and gaseous composition of the environment (Jay and others 2005). According to the role of microorganisms in foods, they can be divided into three major categories: beneficial microorganisms, spoilage microorganisms, and pathogenic microorganisms.

Beneficial microorganisms are used in fermentation to produce enzymes, flavor and odor compounds, acids, and antimicrobial agents in various food products, such as cheese, summer sausage, yogurt, and pickles. These metabolism products can improve the sensory properties of the product, extend the shelf-life of the product and inhibit the growth of pathogenic microorganisms (Bernardeau and others 2006). Probiotics also belong to this group. They are living organisms that have health benefits when consumed. Probiotic is defined as “organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal

microbial balance" (Fuller 1989). Yogurt is a common example of probiotic carrier. When the probiotics are applied to yogurt, it usually refers to the concept bio-yogurt. The microorganisms in the yogurt are beneficial to the human gastrointestinal microecology (Lourens-Hattingh and Viljoen 2001).

Spoilage microorganisms grow in food and result in producing undesirable flavors or odors, changing texture or appearance, and loss of nutritional values of the food products. These undesirable changes make the product not suitable for human consumption. Food spoilage occurs due to inadequate processing and improper handling and storage. Many microorganisms can cause food spoilage, such as *Bacillus*, *Pseudomonas*, *Lactobacillus*, and some molds (Gram and others 2002). Some microorganisms can be beneficial microorganisms or spoilage microorganisms depending on the food products, such as *Lactobacillus* (Bernardeau and others 2006).

Foodborne pathogenic microorganisms may cause diseases in human after consumption. Sometimes the growth of pathogenic organisms may not change the quality and sensory properties of the food. Therefore the contamination of pathogens may not be detected without performing microbiological tests (Flint and others 2005). Foodborne pathogens can induce food intoxication, toxicoinfection, and infection. Food intoxication is caused by the toxin pre-formed by bacteria in foods, such as *Staphylococcus aureus, Clostridium botulinum,* and *Bacillus cereus.* Staphylococcal toxins caused 185,000 cases of foodborne illnesses before the year of 1999 (Mead and others 1999). In toxicoinfection, the toxin is produced inside the host after ingestion of microbial contaminated foods. This type of illness is usually caused by *Clostridium perfringens, Vibrio cholera,* and enterotoxigenic *Escherichia coli* (ETEC). In foodborne infection, the microorganisms are invasive and can be harmful to the tissues and organs of the

host. In 1997, it is estimated that 1,963,000 cases of foodborne infections were caused by *Campylobacter*, and 1,342,000 cases infections were caused by *Salmonella* non-typhooid (Tauxe 2002).

Mycotoxins are secondary metabolites produced by certain fungi and can result in acute or chronic diseases in human. The toxic response caused by mycotoxins is termed mycotoxicosis (Sweeney and Dobson 1998). The primary metabolites are the production of the compounds that are essential for growth. Secondary metabolites occur at the end of exponential phase of growth and the products are usually not relative to growth or metabolism (Jay and others 2005). They may be mutagenic, carcinogenic or hepatotoxic and cause serious diseases. The diseases caused by mycotoxins include cancers, gastrointestinal disturbances, alteration of the immune system, and reproductive problems. Species of *Aspergillus*, *Fusarium*, and *Penicillium* can produce mycotoxins, such as aflatoxin, citrinin, patulin, penicillic acid, ochratoxin, and fumonisins (Sweeney and Dobson 1998). Among those mycotoxins, aflatoxin is the most deadly one and it targets livers (Bhunia 2008).

1.2 Parameters of foods that affect microbial growth

1.2.1 Intrinsic parameters

Most microorganisms grow best in the pH range of pH 6.5-7.5, whereas few of them can grow below pH 4.0. Among all microorganisms, bacteria are more sensitive to low pH than molds and yeasts, whereas pathogenic bacteria are the most sensitive ones. pH effects are usually from the influence of function of enzymes and the transportation of nutrients into cells. Meanwhile, the morphology of some microbes can also be influenced by pH changes (Horner and Anagnostopoulos 1973, Morris 1962). The pH effects against microorganisms depend on the

type of acid used. For example, acetic acid has better inhibitory effect than citric acid, phosphoric acid and tartaric acid at the same concentration (Corlett 1980).

The moisture content of food is very important to microbial growth. The water requirement of microbes is described as water activity, which is the ratio of the water vapor pressure of food to the vapor pressure of pure water at the same temperature. Generally, bacteria require higher water activity than molds and yeasts, whereas Gram-negative bacteria require higher water activity than Gram-positive bacteria. *Staphylococcus aureus* can grow at water activity 0.86. Halophilic bacteria, xerophilic molds, and osmophilic yeasts have been reported to grow at very low water activity 0.75, 0.65, and 0.61, respectively (Christain 1963). In general, the effect of low water activity is to increase lag phase length and decrease the growth rate to reduce the final population (Morris 1962).

Microorganisms also require energy, nitrogen, vitamins, minerals, and other growth factors to grow and function normally. They can utilize sugars, alcohols, and amino acids as a source of energy. Some microbes can utilize complex carbohydrates such as starches and cellulose also. Fat can also be a source of energy for some microorganisms. Amino acids are the primary nitrogen sources for heterotrophic microorganisms. Some microbes can also utilize nucleotides and high-molecular-weight proteins as sources of nitrogen (Jay and others 2005). B vitamins are very important to Gram-positive bacteria, since they cannot synthesize B vitamins themselves, whereas Gram-negative bacteria and molds can synthesize B vitamins and do not require B vitamins from the environment to grow (Ruiz-Barba and Jimenez-Diaz 1995). The naturally occurring antimicrobial constituents in foods can influence the growth of microorganisms, such as the essential oils in some plants, lactoferrin and conglutinin in milk, and

lysozyme in eggs. The natural covering of foods can provide protection against microbial growth such as the testa of seeds, shells of eggs, hide of animals, and shells of nuts (Jay and others 2005).

Microorganisms can also be classified into aerobes, obligate anaerobes, and facultative anaerobes based on their oxygen requirements. Aerobes such as *Bacillus* require oxygen for respiration and energy, whereas obligate anaerobes grow in absence of oxygen. The presence of oxygen in the environment is very important to food quality and safety. Food products are processed and stored in various conditions, thus the oxygen concentration of the environment may vary. Anaerobes can grow in vacuum packaged products, whereas aerobes can grow on the surface of the food (Jay and others 2005).

1.2.2 Extrinsic parameters

Generally, microorganisms can be divided into three categories based on their optimum temperatures: psychrotrphs (20-30 ℃), mesophiles (30-40 ℃), and thermophiles (55-65 ℃) (Jay and others 2005). Both psychrotrophs and mesophiles can cause diseases in humans. Their optimal growing temperature range is 4-37 ℃. Microorganisms can grow over a wide range of temperatures and the considerations of proper storage temperatures are very important to inhibit microbial growth. Basically, molds can grow in a wider range of temperatures than bacteria and yeasts. Yeasts can grow in the psychrotrophic and mesophilic temperature ranges (Horner and Anagnostopoulos 1973).

The relative humidity (RH) of the environment is important to the water activity of the food and the microbial growth on the surface of the food (Horner and Anagnostopoulos 1973). Carbon dioxide and ozone are both important gases used to control microbial growth and extend shelf-life of food products (Byun and others 1998, Clark and Lentz 1973). Additionally, the

presence of other microorganisms may also influence the growth of pathogenic and spoilage microorganisms, since these organisms can produce inhibitory or lethal substances, such as antibiotics, bacteriocins, hydrogen peroxides, and organic acids (Alakomi and others 2000, Cleveland and others 2001, Klebanoff 1968).

1.3 Pathogens and general mechanism of pathogenesis

Pathogens are able to cause cellular damage by establishing in tissues, which will result in morbidity or mortality. Morbidity means general suffering, whereas mortality means death. Pathogens can be classified based on their transmission patterns, movement among hosts and vectors into zoonotic, geonotic, or human origin. Zoonotic diseases are transmitted from animals to humans, such as *E. coli* O157:H7 and *Campylobacter jejuni*. Geonotic diseases are acquired from soil, water, or decaying plants, such as *Listeria monocytogenes*. Human origins are transmitted from human to human, such as Hepatitis A and *Vibrio cholera* (Bhunia 2008).

Pathogens can also be classified into primary pathogens and opportunistic pathogens based on their pathogenicity. Primary pathogens regularly cause diseases in humans, whereas opportunistic pathogens infect immune-compromised populations. To induce disease, pathogens must enter and survive inside a host, avoid the host's defense, be able to replicate to a significant population, be transmitted, and be able to express specific traits within the host. The growth and survival of a pathogen can be affected by the nutrients, pH, mucus composition, natural microflora, oxygen, carbon dioxide, and physiological status such as stress of hormones of the host (Bhunia 2008).

When dealing with pathogenic microorganisms in foods, it is important to know the type and concentration of microorganisms or toxin present in the food. Many foodborne pathogens are

ubiquitous in nature and can be found in soil, water, animals and plants. Pathogens can be introduced into food products in different ways, such as raw materials, packaging materials, humans, and plant equipment. Recontamination of processed food contributes to foodborne outbreaks and illnesses. The packaging, handling, storage, and distribution of food product may also influence the microbial load. The characteristics of the microorganism or toxin are also important to consider, such as their response to heat, pH, salt, and other processing methods (Bhunia 2008).

1.3.1 *Listeria monocytogenes*

Listeria monocytogenes has the highest mortality rate among all foodborne pathogens. There are about 25,000 people that get listeriosis in the US each year (Bhunia 2008). In the US, there is a "zero tolerance" policy for this pathogen in ready-to-eat foods, which means the absence of this pathogen in 25 g food sample (Gilbert 1992). Some European countries and Canada allow 100 CFU/25 g food (Bhunia 2008). The contamination of *Listeria monocytogenes* in ready-to-eat foods led to several large outbreaks in the US in recent years. The estimated cost due to *Listeria monocytogenes* contamination is approximately 2 billion dollars per year (Bhunia 2008). In 1998-1999, the contamination of hot dogs caused a multistate outbreak, which involved 22 states and caused 101 illnesses, 15 deaths, and 6 miscarriages (Graves and others 2005). In 2005, a multistate outbreak involved deli turkey meat, which affected 9 states and led to 12 deaths. After this outbreak, the Food Safety and Inspection Service (FSIS) completed a risk assessment for *Listeria monocytogenes* in deli meats (Gottlieb and others 2006). In December 2011, contamination of *Listeria monocytogenes* in whole cantaloupes led to a multistate outbreak, which involved 28 states and 146 people. Thirty deaths and one miscarriage were also reported (CDC 2011b).

Listeria species are Gram-positive, non-sporeforming, facultatively anaerobic bacteria (Junttila and others 1988). These bacteria are able to survive in extreme environments. *Listeria* can grow in pH range of 4.1-9.6 and temperature range of -0.4-50 ℃ (Buchanan and Phillips 1990, Junttila and others 1988). They are also tolerant to 10% salt and the presence of antimicrobial agents (Buchanan and Phillips 1990). There are six species recognized in *Listeria* genus: *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri,* and *L. grayi* (Rocourt and others 1992). *Listeria denitrificans* has been excluded from this genus and transferred to another genus, *Jonesia* (McLauchlin 1987). Among these species, *L. monocytogenes* is pathogenic to humans and animals. *L. monocytogenes* has 13 distinct O-antigenic patterns. Serotypes of 1/2a, 1/2b, and 4b cause 98% of the outbreaks and serotype 4b is the most virulent one in Europe. In the United States and Canada, serovars 1/2a, 1/2b, and 4b tend to have a even distribution (Gellin and others 1991, Seeliger and Hohne 1979).

Listeria monocytogenes can cause intracellular infections that affect both healthy and immunosuppressed populations. It is able to cause fever and gastroenteritis in healthy humans. In immunosuppressed individuals such as pregnant, older people, alcoholics, and people on hemodialysis, this organism results in serious diseases or death (Nieman and Lorber 1980). The overall mortality rate due to the infection of *Listeria monocytogenes* is about 20-30%. The infection doses may vary from 100 to 10^6 cells depending on the immunological status of the individuals and the types of carriers (Bhunia 2008). The incubation period of the disease varies from 1.5 days to 3 months depending on the immunological status of the host and the population of the organism. This organism causes three forms of disease: gastrointestinal disease, systemic listeriosis, and abortion and neonatal listeriosis (McLauchlin 1990a &1990b).

Serotypes 1/2a, 1/2b, and 4b were involved in the gastrointestinal disease (Seeliger and Hohne 1979). The infection dose associated with these outbreaks was very high, which was about 10^6-10^8 cells (Bhunia 2008). Incubation periods of this form are less than 24 h. The symptoms of gastroenteritis include fever, headache, nausea, vomiting, abdominal pain, and watery diarrhea (McLauchlin 1990b). Systemic listeriosis is a rare but fatal disease, which primarily affects individuals with poor immune systems, such as AIDS patients, organ transplant patients, and cancer patients receiving chemotherapy. The targets of this disease include liver, spleen, gall bladder and the lymph nodes. It may also cause meningitis and encephalitis; and in pregnant women, it may also cross the placental barrier and infect the fetus causing abortion and stillbirth (Bhunia 2008).

Listeria monocytogenes is a ubiquitous pathogen in nature and can be found in soil, water, sewage, plants and the intestinal tract of animals such as sheep, cattle, and goat. Food is one of the major sources of *Listeria monocytogenes*, especially the ready-to-eat or minimally processed foods, such as hot dogs, luncheon meats, smoked fish, and soft cheeses. Additionally, post-processing contamination of food products is another concern of *Listeria monocytogenes* contamination (Roberts and Wiedmann 2003).

Since *Listeria monocytogenes* can cause serious diseases to humans and is commonly found in nature, the United States Food and Drug Administration (FDA) and US Department of Agriculture (USDA) grouped food products into four categories based on their risk levels to educate consumers. High-risk populations such as immunocompromised individuals and pregnant women should avoid high risk products such as deli meats, unheated frankfurters, high-fat dairy products, soft unripened cheese, unpasteurized milk, smoked seafood, ready-to-eat crustaceans, deli salads, and reheated frankfurters (McLauchlin and others 2004). Regulation

also requires the implementation of Hazard Analysis and Critical Control Point (HACCP) plans for all plants that produce RTE products to eliminate or reduce *Listeria monocytogenes* contamination. Products shall be recalled to prevent further incidences once they have been implicated for an outbreak. This organism can be killed by heating at 71 ℃ for 1 min (Bhunia 2008). Thus, food products should be pasteurized or heated before consumption (Junttila and others 1988). Other good practices to reduce *Listeria monocytogenes* in products include implementation of effective sanitation plans, using antimicrobial agents in products, and routine surveillance of the processing plant.

1.3.2 *Salmonella*

Salmonella belongs to the *Enterobacteriaceae* family. It is a Gram-negative, non-sporeforming rod. This organism is motile and facultative anaerobic. The optimum temperature for *Salmonella* is 35-37 ℃ and it can grow in the temperature range of 5-45 ℃ (Matches and Liston 1968). It can also grow in a wide pH range of 4.0-9.0. The pH range for best growth is 6.6-8.2 (Chung and Goepfert 1970). *Salmonella* is widely distributed in nature. It is usually found in the intestinal tract of reptiles, birds, farm animals, humans, and occasionally insects. Human salmonellosis is usually foodborne. Poultry and eggs are the major concern for salmonellosis. Additionally, other meat and dairy products were also implicated in previous outbreaks (Coburn B and others 2007).

Salmonella is one of the most important pathogens affecting public health in both developed and developing countries. It causes 1.4 billion cases of gastroenteritis and 16 million cases of typhoid fever each year, and there were 3 million deaths among those cases in the world (Voetsch and others 2004). In the US, there are 2-4 million cases involving *Salmonella* annually, and the illnesses causes 500-1,000 deaths and about 3 billion dollars in economic loss (Bhunia

2008). Recently, a large multistate outbreak of salmonellosis occurred in the US. This outbreak involved the consumption of contaminated peanut butter. In 1994, a large outbreak involved serotype Enteritidis and caused more than 224,000 illnesses. The vehicle food was ice cream (Hennessy and others 1996). Since 1979, *Samonella* serotype Typhimurium has become the most frequently implicated serotype associated with outbreaks (Glynn and others 1998).

The serotypes of *Salmonella* are grouped based on their somatic (O), flagellar (H), and capsular (Vi) antigens. There are more than 2,400 serotypes of *Salmonella* under two species: *Salmonella enterica* and *Salmonella bongori* (Le Minor and Poppoff 1987). *Salmonella* can cause three forms of diseases: typhoid fever, gastroenteritis, and bacteremia. Typhoid fever is a systemic disease and is usually caused by *Salmonella enterica* serotype Typhi. Other serotypes such as Paratyphi and Enteritidis cause typhoid-like infection in humans. These types of fever are the most severe diseases caused by salmonellae. Typhoid fever has the longest incubation time, causes the highest body temperature, and has the highest mortality rate (Coburn and others 2007). *Salmonella* serotype Typhimurium causes serious gastroenteritis in children and immune-compromised populations. In healthy individuals, it causes self-limiting gastroenteritis with symptoms of this disease include fever, diarrhea, abdominal pain, and vomiting. These symptoms usually appear within 6-24 h and self-resolved within 3-4 days. The infection dose varies from 1 to 10^9 CFU (colony forming unit), and it may decrease if the pathogen is consumed with liquid foods (Zhang and others 2003).

During systemic infection, treatment with ampicillin and amoxicillin could be used to clear *Salmonella.* However, since the gastroenteritis induced by *Salmonella* serotype Typhimurium is self-limiting, it is not recommended to apply antibiotic therapy to gastroenteritis patients (Nelson and others 1980). During the processing of food, several practices can be

applied to prevent the contamination of *Salmonella*, such as the implementation of personal hygiene and application of good sanitation plan in slaughter plants. Proper food handling, avoiding cross-contamination, and education to the public can also help to reduce the contamination of *Salmonella* (Mcllroy and others 1989).

1.3.3 *Escherichia coli* O157:H7

Escherichia coli was established as a foodborne pathogen in 1971 because of a 14-state outbreak happened after the consumption of imported cheese, which involved 400 illnesses (Fantasia and others 1975). It is a Gram-negative facultative anaerobic motile bacillus. It belongs to the *Enterobacteriaceae* family. *E. coli* can contaminate water and soil through warm-blooded animal feces. Fruits and vegetables may also be contaminated if the untreated manures are used as fertilizers. Meats are also a common source of *E. coli*, since the meat may be contaminated by fecal contracts during slaughter (Armstrong and others 1996).

Most *E. coli* strains are non-pathogenic and harmless to humans. The non-pathogenic strains usually exist in the intestinal tract of humans and other warm-blood animals as a common microflora (Bhunia 2008). The pathogenic strains are acid resistant and can cause disease including gastroenteritis, dysentery, hemolytic uremic syndrome (HUS), urinary tract infection (UTI), septicemia, pneumonia, and meningitis. The major concern in recent years about pathogenic *E. coli* is the outbreaks that involved enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7 is a serotype of EHEC, which was emerged as a foodborne pathogen several decades ago. The diseases were usually caused by the consumption of contaminated meat, dairy products, fruits, and vegetables, and especially ground beef (Karmali and others 1985, Leyer and others 1995, Suthienkul and others 1990).

There are two antigens used to determine the serotypes of *E. coli*, "O" and "H" antigens. "O" antigen is the serogroup determinant, and there are 174 O antigens. Additionally, there are 53 serotypes of "H" antigens, which are also called flagellar antigens. Strains that lack flagella are non-motile and are named "NM". In other words, "O" antigens determine the serogroup, whereas "H" antigens determine the serotypes. Virotype is another classification of *E. coli*, which is based on the presence of virulence factors and toxin production. There are 6 virotypes of *E. coli*: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) (Cheasty and Rowe 1983, Merson and others 1979).

E. coli O157:H7 is a serotype in EHEC group. *E. coli* belongs to this group can produce lethal Shiga-like toxins (Stxs) to induce bloody diarrhea and hemolytic uremic syndrome (HUS), which is prevalent in developed countries (O'Brien and others 1992). Therefore, EHEC is also called STEC (Shiga toxin-producing *E. coli*). Since Shiga toxin kills Vero cells, therefore this group also refers to verotoxin-producing *E. coli* (VTEC). This group can be divided into five seropathotypes (A-E). *E. coli* O157:H7 belongs to seropathotype A, which is the principal serotype associated with *E. coli* outbreaks (Armstrong and others 1996). The outbreaks caused by *E. coli* O157:H7 decreased in recent years (Rangel and others 2005).

This organism is sensitive to temperature. It grows rapidly at 30-42 ℃, grows poorly at 44-45 ℃, and does not grow below 10 ℃. The cells can be destroyed by pasteurization temperature (Gonthier and others 2001). This pathogen is usually present in the intestine of animals without causing disease. It also exists in the feces of chickens, sheep, pigs, dogs, and cattle. Ground beef is a major concern of EHEC and has been involved in many outbreaks in the US, Europe, and Canada (Armstrong and others 1996). One of the most important outbreaks

about contamination of EHEC in under-cooked hamburgers occurred in 1993. It was a multistate outbreak and affected over 500 people, and caused 4 deaths (Bell and others 1994). Additionally, raw milk, apple cider, fruits, sprouts, and salad were also implicated in previous outbreaks (Armstrong and others 1996).

Since *E. coli* O157:H7 is acid resistant, it can pass through the stomach and reach the small intestine to cause disease (Conner and Kotrola 1995). The infection dose is 50-100 cells (Bhunia 2008). EHEC can produce two types of Shiga-like toxins, Stx 1 and Stx 2. Stx 2 is highly toxic and is very likely to cause HUS. Stxs also have nephrotoxic, cytotoxic, enterotoxic, and neurotoxic activitites. Nephrotoxic activity may result in massive damage to kidney tubules, bloody urine, and the hemorrhagic uremic syndrome. The neurotoxic activity causes a neurological disorder called thrombotic thrombocytopenic purpura (TTP). Stx may also act as enterotoxin, which results in fluid accumulation and diarrhea. The cytotoxic activity inhibits cellular protein synthesis and causes programmed cell death (Armstrong and others 1996).

The incubation period of EHEC is 3-9 days after ingestion of contaminated food and the symptoms usually last for 4-10 days (Bhunia 2008). The symptoms of colitis include abdominal cramps, watery diarrhea, and vomiting. Bloody diarrhea may occur due to the damage to the blood vessels in the colon. HUS is characterized by acute renal failure, thrombocytopenia, and microangipathic hemolytic anemia. TTP may result in seizures, coma, and death due to blood clots in the brain (Armstrong and others 1996).

1.3.4 *Staphylococcus aureus*

Staphylococcus aureus naturally inhabits human and animal skins and it can cause infections in many organs. Some staphylococci are methicillin-resistant (MRSA) and

vancomycin-resistant. The infection caused by these resistant strains may be fatal due to the lack of antibiotics for treatment. Staphylococci are responsible for both food poisoning and intoxication, which may result in severe vomiting and diarrhea (Carmo and others 2002). In January 1998, contamination of *Staphylococcus aureus* in ham salad caused an outbreak in Texas involved 225 illnesses (CDC 2012). In September 2000, an outbreak occurred in Georgia and induced 214 illnesses with 6 hospitalizations. The source of contamination was barbeque pork (CDC 2012). In 2009, *Staphylococcus aureus* caused a multistate outbreak. More than one hundred illnesses were reported and the contaminated food was chicken (CDC 2012).

Staphylococcus belongs to the family of Micrococcaceae. And the genus *Staphylococcus* is classified into more than 30 species and subspecies. *Staphylococcus aureus* is the primary species in this genus which is responsible for food poisoning. Other species such as *S. intermedius, S. chromongens,* and *S. cohnii* also belong to this genus. Many of the species in *Staphylococcus* genus can produce enterotoxins. *Staphylococcus aureus* is a Gram-positive coccus and it appears as grape-like clusters under microscope. They are non-motile and produce golden yellow colonies. This is a catalase-positive and facultatively anaerobic microorganism. *Staphylococcus aureus* is salt-tolerant (10-15%) and relatively resistant to drying and heating, but it is sensitive to lysostaphin (Betley and others 1992).

The intoxication caused by *Staphylococcus*, staphylococcal food poisoning, is one of the most common foodborne illness reported. The food poisoning caused by staphylococci decreased over the past two decades in the US. However, it is still the major concern of food poisoning in Brazil, Egypt, Japan, and most developing countries (Balaban and Rasooly 2000, Carmo and others 2002). The intoxication occurs due to the ingestion of preformed staphylococcal enterotoxins (SEs). The contamination of *Staphylococcus* usually associate with deli foods,

custard or pudding, salad dressing, meat, hams, fish, and milk products. *S. aureus* grows when the foods are left at room temperature for a long period of time and the enterotoxins are usually produced between the temperature range of 10-46 ℃, with the optimum between 40 ℃ and 45 ℃ (Bhunia 2008, Smith and others 1983).

There are 17 major distinct SEs and many of them can cause food poisoning, acute illness, fever, erythematous lesions, and hypotension (Balaban and Rasooly 2000). SEs are water soluble and heat resistant. They are also resistant to protease. The toxins can remain active after 30 minutes boiling (Anderson and others 1997). The production of toxins depends on water activity, pH, temperature, oxygen and CO_2 levels of the environment and the growth phase of bacteria (Silverman and others 1983). The infection dose of *S. aureus* is 10^5-10^8 CFU and 0.1 μg toxin in adult (Loir and others 2003). The incubation period is 1-6 h and the symptoms include nausea, acute vomiting, abdominal pain, diarrhea, headache, cramping, and anaphylactic shock (Betley and others 1992).

Since staphylococcal intoxication is usually self-limiting. Bed rest and fluid therapy are recommended for patients. The food should not be left at room temperature for long time to prevent bacterial growth. Good hygienic practices and effective sanitation plans should be applied in the processing plant to prevent contamination. The implementation of HACCP plan and rapid microbiological analysis can help to control this pathogen in food processing plants (Coia and others 2006).

2. Current antimicrobial methods in food industry and the challenges of application

2.1. Food protection with chemicals

A large number of chemicals have been reported to have antimicrobial activities and potential use as food preservatives. However, only a relatively small amount of them are allowed to be applied in food products, since FDA has strict regulations of safety about the application of chemicals in food preservation due to their toxicity to humans (Jay and others 2005). Chemicals and probiotic microbes may also change the sensory properties and the quality of the products. The effectiveness of the compounds may also be influenced by the pH, water activity, and composition of the food (Lopez-Malo and others 2005, Praphailong and Fleet 1997).

Benzoic acid and its sodium salt are effective against yeasts and molds. These compounds only have effects in undissociated form. Therefore, they have the greatest activity at low pH, and are applied in high-acid products such as margarine, pickle, apple cider, soft drinks, catsup, and salad dressings. Benzoic acid and its sodium salt were the first chemical preservative approved in foods by FDA. It is still widely used as a food preservative in many food products today. It is generally recognized as safe (GRAS) with the maximum tolerance of 0.1% in foods. Concentration greater than 0.1% of benzoic acid may result in undesirable tastes, which is described as “peppery” or burning (Bosund 1962, Jay and others 2005, Lopez-Malo and others 2005).

Sorbic acid and its calcium, sodium, and potassium salts are used as fungal inhibitors in hard cheeses, figs, salad dressings, syrups, jellies, and cakes with 0.2% maximum tolerance limit (Jay and others 2005). They are also effective in acidic pH range, which is below pH 6.0. These compounds are more effective than sodium benzoate between pH 4.0-6.0. These compounds

have similar effects against molds as benzoic acid at pH below 3.0 (Deuel and others 1954). Sorbic acid and sorbates also showed effects against a wide range of bacteria. Generally, they are more effective against catalase-positive cocci and aerobes. However, the application of their antibacterial activities is not approved by regulations (Ivey and others 1978). Similarly, propionic acid and its salts are permitted in bread, cakes, and cheeses as an antifungal agent. These compounds are highly specific against molds and their mode of action is more fungistatic than fungicidal (Lopez-Malo and others 2005).

Sodium nitrate and sodium nitrite are used as curing ingredient in processed meat products, such as wieners, bacon, smoked fish, and canned cured meats, since they can stabilize the pink meat color, develop flavor, and inhibit the growth of spoilage and food poisoning microorganisms (Gray and Pearson 1984). The antibacterial activity of these compounds increases within the acidic pH range. *Clostridium botulinum* is the major concern related to nitrite inhibition. This organism produces life-threatening neurotoxin. These compounds can inhibit the growth of vegetative cell and prevent the germination of spores which are very heat resistant and can survive during thermal processing and smoking (Reddy and others 1983). It is also effective against *S. aureus* at high concentration, but it generally does not have effect against Enterobacteriaceae (Cassens 1995). Since nitrite is toxic to human, 120 ppm is the maximum tolerance of nitrite in meat products (Jay and others 2005).

NaCl and sugars have been used as food preservatives since ancient times, and they have similar mode of action, which refers to the effect of hypertonic conditions. The hypertonic condition makes water pass out of cells faster than it enters, which results in the plasmolysis of cells. Therefore, the plasmolysis of cells will inhibit the growth of microorganisms and may lead to death. At high concentration of their solutions, there is a drying effect on both food and

microorganisms. However, it requires six times more sucrose than NaCl to get the same degree of inhibition. And the effects of sugars are generally against bacteria rather than yeasts and molds. Since salt and sugars may lead to different sensory and textural effects, their applications in foods are different. Generally, salt is used in meat products, whereas sugars are used in fruit preservation, candies, and condensed milk (Praphailong and Fleet 1997).

Sulfur dioxide and the salts of sulfite, bisulfate, and metabisulfite all act similarly against microorganisms in lemon juice, wines, fruit juices, and molasses (Jay and others 2005, Lloyd 1975). This group of compounds has been used as food preservatives since ancient times. However, they were not used as a meat preservative in the US until 1813. And they are still not permitted in meat now since they are considered as source of thiamine (Banks and Board 1982, Tompkin and others 1980).

Acetic and lactic acids are the two organic acids commonly used as food preservatives. They are produced by lactic acid bacteria in fermented products such as pickles, sauerkraut, and fermented dairy products (Jay and others 2005). The antimicrobial effects are the depression of pH and metabolic inhibition from the undissociated acid molecules. These two organic acids show bactericidal effects against Gram-negative bacteria. The antibacterial activities of these two acids are different at the same concentration. For example, the growth of *E. coli* O157:H7 can be totally inhibited when it was exposed to 10% acetic acid. And the growth of *E. coli* O157:H7 can only be reduced for 6 log cycles when the culture was exposed to the same concentration of lactic acid (Alakomi and others 2000, Entani and others 1998). The sodium and potassium salts of these two acids are also used as preservatives in foods, since they have bacteriostatic effects against psychrotrophic pathogens, such as *Listeria monocytogenes* (Bedie and others 2001, Shelef 1994).

Bacteriocins are toxins produced by bacteria to inhibit the growth of other bacteria. Nisin is a bacteriocin produced by *Lactococcus lactis* and other lactic acid bacteria (LAB), which is widely used as a natural food preservative. It is generally effective against Gram-positive sporeformers, and ineffective against Gram-negative bacteria and fungi. Its antimicrobial activity can be increased when it is combined with other hurdle technologies, such as low temperature, carbon dioxide, sucrose, and modified atmosphere packaging (MAP). About 50 countries permitted its use as a food preservative, and it was approved in the US in 1988 with 1% maximum tolerance. It is desirable as a food preservative because it is nontoxic to human, heat stable, no off-flavor or off-odor, and naturally produced by bacteria (Cleveland and others 2001, Delves-Broughton 1990).

The hurdle concept was applied to some foods for over a century, which is also described as barrier technology, combination preservation, and combined methods. In this concept, several factors or techniques can be applied to control the growth of microorganisms in foods at the same time (McMeekin and others 2000). For example, hurdle concept is demonstrated to prevent the germination of spores of *Clostridium botulinum*. Intrinsic and extrinsic parameters used include pH, water activity, NaCl, $NaNO_2$, temperature, and aerobic bacterial biota (Jay and others 2005).

2.2 Food protection with modified atmosphere packaging

Modified-atmosphere packaging (MAP) is used to alter the gaseous environment around foods to extend shelf-life or inhibit the growth of microorganisms. Carbon dioxide is used as a food preservative in MAP, and about 90% boxed beef products are packaged in MAP in the US (Philips 1996). Carbon dioxide can retard both lag and exponential phases of bacterial growth.

Several factors can influence the antimicrobial activity of carbon dioxide. Firstly, carbon dioxide has greater solubility at lower temperature. Therefore, the inhibitory activity increases as temperature decreases. Secondly, carbon dioxide has the highest inhibitory activity at 20-30%, especially for fresh meats (Gill and Tan 1980). The pH of the environment is another important factor. Generally, the inhibitory activity increases as pH decreased to the acid range. Carbon dioxide has greater antimicrobial activity under pressure. Basically, carbon dioxide has greater effects against Gram-negative than Gram-positive bacteria. The MAP can control the growth of *Yersinia enterocolitica* and *Salmonella*, but cannot control *Staphylococcus aureus* and *Liseria monocytogenes* (Nielsen and Zeuthen 1985).

Vacuum packaging, MAP, and controlled-atmosphere packaging (CAP) are all considered as hypobaric packaging methods, which refer to low pressure, low temperature, and high humidity storage of foods (Gill and Molin 1998). The vacuum packaging can be achieved by evacuating air from gas-impermeable pouches followed by sealing. The composition of gas changes during storage, because the oxygen is consumed due to tissue and microbial respiration and carbon dioxide is released. MAP is achieved by altering the package atmosphere by flushing with the mixture of carbon dioxide, nitrogen, and/or oxygen (Gill and Molin 1998). The difference between CAP and MAP is that the composition of MAP may change during storage, whereas the composition of CAP is constant during storage.

2.3 Food protection with radiation

Radiation can be defined as the emission and propagation of energy through space or other materials. The radiation type primarily used in food preservation is electromagnetic, and the radiations are grouped based on their wavelengths, such as microwaves, ultraviolet rays, X-

rays, and gamma rays. Shorter wavelength makes greater damage to microorganism. Basically, spore-forming microorganisms are more resistant than non-spore-forming microorganisms (Jay and others 2005).

Ultraviolet (UV) is a bactericidal agent, and it is absorbed by proteins and nucleic acids. Since UV has poor penetrative capacity, the application of UV is limited on the surface. However, the UV light may catalyze oxidation and result in rancidity and discoloration, which are undesirable to food quality (Sunada and others 2003). Gamma rays are emitted from the excited nucleus of elements such as ^{137}Cs and ^{60}Co. They have very high penetrative capacities (Byun and others 1998). X-rays are similar to gamma rays except that they are produced by bombardment of heavy-metal targets with high-velocity electrons within an evacuated tube. The power of microwave is from the intermolecular friction due to the oscillate of molecules about their axes (Jay and others 2005).

An international group of microbiologists suggested the terminology for radiation treatments of foods in 1964. Radappertization refers to "commercial sterility" and the typical dose is between 30-40 kGy. Radicidation is equivalent to pasteurization and the typical dose is 2.5-10 kGy. Radicidation can reduce the number of non-spore-forming pathogens. Radurization can be considered as the pasteurization with lower dose, 0.75-2.5 kGy, which can reduce the numbers of specific spoilage microbes (Goresline and others 1964).

The patent of radiation as food preservative was issued in 1929. However, it was not widely used until World War II, and the application of this method is slow in reaching its maximum potential use. One of the most important reasons is that the types of foods approved to be treated with radiation are limited. The CDC has estimated that if half of the pork, poultry,

ground beef, and processed luncheon meats in the US were treated with radiation, it will reduce more than 880,000 cases of foodborne diseases (Jay and others 2005). Another important reason is that radiation can make undesirable changes to the products caused by post irradiation reactions. Water may undergo radiolysis and produce hydrogen and hydrogen peroxide. Free radicals may also be produced to give off-flavors and off-odors. Radiation may also destroy B vitamins in foods and soften fruits and vegetables by degrading pectin and cellulose (Liuzzo and others 1966, Massey and Bourke 1967).

3. Basidiomycetes mushrooms

3.1 General introduction

Basidiomycota is a phyla under Fungi kingdom. Basidiomycota include mushrooms, puffballs, stinkhorns, bracket fungi, and other polypores. There are about 14,000 mushroom species and perhaps as many as 22,000 species. Only 10% are well known. Even among the known species, very low proportion was well investigated (Lindequist and others 2005). The reproduction is primarily sexual via the formation of end cells called basidia and basidia normally produce external meiospores, which are called basidiospores. Some Basidiomycota can also reproduce asexually (Morrow and Fraser 2009).

3.2 Bio-activities

Basidiomycetes produce a large number of secondary metabolites with biological activities, such as antimicrobial, cytotoxic, and hallucinogenic activity. Edible mushrooms such as *Hericium erinaceum, Flammulina velutipes,* and *Lentinus edodes* were reported to be inhibitory against human pathogens (Chen and Huang 2009). The study of Barros and others showed that wild mushrooms, such as *Cantharellus cibarius, Hypholoma fasciculare, Lepista*

nuda, Lycoperdon molle, Lycoperdon perlatum, Ramaria botrytis, and *Tricholoma acerbum*, contain phytochemicals with antioxidant and antimicrobial activities. The antioxidant activities are also related to the control of chronic diseases, such as cancer, cardiovascular diseases, diabetes, and osteoporosis (Barros and others 2008).

The major groups of effective compounds are phenolics and tocopherols. Phenolic compounds play a significant role as an antioxidant. Additionally, they also have antibacterial, anti-inflammatory, antiallergic, antithrombotic, antiviral, anticarcinogenic, and hepatoprotective activities (Barros and others 2008). Flavonoids are the most common phenolics distributed in mushrooms. Tocopherol is the primary form of vitamin E and has health benefits. The rich nutrients and these bioactive compounds give the mushrooms a wide market. According to Barros and others (2008), *Cantharellus cibarius* and *Lepista nuda* has high reducing power, and strong antibacterial against Gram-positive pathogens. However, it has relatively low content of phenols or tocopherols. *Ramaria botrytis* has slightly antibacterial activity against Gram-positive bacteria.

Suay and others screened 204 basidiomycetes species in 17 orders for their antimicrobial activities. Among those species, 109 species showed antimicrobial activity. The species with antibacterial activity were much more than the species with antifungal activity. All species screened in the order of Russulales showed antibacterial activities, but without antifungal activity. Ganodermatales is the order with the second most active species, which is 73%. Screened species have the effects against bacteria, and none of the active species has the effects against fungi. Order Cortinariales has 67% active species, and all of them are effective against fungi. Boletales is the order showed both antibacterial and antifungal activities.

Yamac and Bilgili studied the antimicrobial activities of fruit bodies and mycelial cultures of mushroom isolates by determining their minimum inhibitory concentrations. Their research showed that the effectiveness against microorganisms depends on the species, the extraction solvents used, and the specific microorganisms tested. For example the chloroform extract of *Hygrophorus agathosmus* has strong antimicrobial activity against *Enterobacter aerogenes, Salmonella typhimurium, Staphylococcus epidermidis, Bacillus subtilis,* and *Saccharomyces cerevisiae.* However, its dichloromethane and acetone extracts have little antimicrobial activity. *Armillaria mellea* only has activity against *Staphylococcus aureus* and the activity is not affected by the extraction method. Their study also showed that the extract from fruit bodies has higher antimicrobial activity than the extract from mycelial cultures.

Many wild edible mushroom species showed antioxidant activities, such as *Cantharelle cibarius, Laccaria amethysta, Clitocybe odora, Lepista nuda, Lepista saeva,Macrolepiotata procera,Lactarius deliciousus, Laccaria laccata, Pleurotus ostreatus* and *Hericium erinaceus*. In The antioxidant activity was determined using lipid preoxidation method in tissue homogenates. Among these ten species, *L. nuda,* which is characterized as *Clitocybe nuda* now, and *L. amethysta* showed the strongest antioxidant activity, which is even stronger than the standard, ascorbic acid. The possible bioactive compounds with antioxidant activities are alkaloids, flavonoids, saponins, and tannis. The antioxidant properties of flavonoids may help to protect against diseases induced by oxidative stress (Egwim and others 2011).

The antiviral activities of mushrooms are generally due to the inhibition of viral enzymes and the synthesis of viral nucleic acids (Lindequist and others 2005). Additionally, basidiomycete *Clitocybe nebularis* was reported to have insecticidal activities (Pohleven and others 2011). Chihara and others (1970) also reported that an edible mushroom, *Lentinus edodes*,

contains polysaccharides with antitumor activity. These edible mushrooms also have other potential pharmacological applications, such as inhibition of allergic reactions, antiatherogenic activity, hypoglycemic activity, anti-inflammatory activity, and hepatoprotective activity. The cytostatic activities can also contribute to their antitumor properties (Lindequist and others 2005).

4. *Clitocybe nuda*

4.1 General introduction

Clitocybe nuda is an edible mushroom under Basidiomycota division in Fungi kingdom. However, it is also known to cause allergic reactions in sensitive individuals, particularly when the mushroom is consumed in raw. It has been recognized as *Lepista nuda*, and changed to *Clitocybe* genus in 1871. Wood blewit and blue stalk mushroom are its common names. It is a saprotrophic species and can be found on decayed leaf litter in both coniferous and deciduous woodlands. This mushroom usually has lilac to purple color. However, the strains in North America are commonly tan with purplish tone on stem and gills. This mushroom contains 0.11% fat, 3.70% protein, and 1.55% total carbohydrates. It is also with high unsaturated fatty acids, such as oleic acid and linoleic acid, which have health benefits (Barros and others 2008).

4.2 Bioactivity

The culture filtrates and extraction of *Clitocybe nuda* showed various degrees of antimicrobial activities against pathogenic microorganisms. Different strains of *C. nuda* display different degrees of inhibitory effects against pathogenic fungi and bacteria (Chen and Huang 2009). Their study also showed that the culture filtrate of *C.nuda* can effectively reduce the germination of *Phytophthora capsici* and has antimicrobial activity against human pathogens *Staphylococcus aureus* and *Candida albicans*. The methanol extraction of this mushroom also

showed antibacterial activities against several pathogens, such as *Bacilliu subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* (Dulger and others 2002). The inhibitory substances are stable in wide pH range and resistant to thermal threatments. The effective compounds are hydrophilic and negative charged. The molecular weights of active compounds are between 500 and 1000 Dalton (Chen and Huang 2009). The minimum inhibitory concentration (MIC) of *C. nuda* against some Gram-positive pathogens, such as *Bacillus subtilis* and *Staphylococcus aureus*, is even lower than the positive control, ampicillin, which is a very effective antibiotic used to treat bacterial infections (Barros and others 2008).

According to Yamac and Bilgili (2006), the extraction from fruit bodies has stronger activity against microbial growth than the extraction from mycelial cultures. And the active compounds are in both aqueous and organic fractions. The inhibitory effects against mold germination also depend on the extraction methods. The water, ethanol and methanol extracts of *C. nuda* can totally inhibitory the germination of *Phytophthora capsici*, whereas the acetone and dichloromethane extracts can only inhibitory about 30% germination of the same organism (Chen and Huang 2009).

CHAPTER 3: MATERIALS AND METHODS

1. Bacterial culture preparation

Listeria monocytogenes G3990 4b, *Salmonella typhimurium* ATCC13311, *E. coli* O157:H7 204P, and *Staphylococcus aureus* ATCC 12600 were used in this study. Before use, *Salmonella typhimurium*, *E. coli* O157:H7, and *Staphylococcus aureus* were cultured in tryptic soy broth (TSB) (BD, Franklin, NJ) while *Listeria monocytogenes* was cultured in TSB with 0.6% Yeast Extract (AMRESCO, Solon, OH). The bacterial cultures were incubated on an incubation shaker at 37 ℃ with 200 rpm shaking for 20 h. After incubation, the cultures were washed twice with Butterfield's phosphate buffer (BPB) by centrifuging at 2,800 g for 8 min at 10 ℃. Then, the bacteria were resuspended in BPB. The bacterial populations were estimated from the absorbance at $O.D._{640nm}$ and adjusted to designated populations for use.

2. Mushroom extraction method

Dry mushroom was grounded into powder using a coffee grinder (Mr. Coffee, Los Angeles, CA). Then, 18 g powder was extracted by stirring with 600 mL 95% EtOH solvent for 24 h at 25 ℃, 400 rpm and filtered through Whatman no. 4 filter paper. The residue was extracted two more times with the same procedures described above. All extracts were combined, and the solvent of the combined extract was removed completely by evaporating at 40 ℃. And the dried residue was re-suspended in 15 mL deionized water. Then the suspension was filtered through 10 μm cellulose (Thomas Scientific, Swedesboro, NJ) filter paper and 0.45 μm sterile

filter membrane. The filtrate was stored at 4 ℃ or freeze dried for further use. The weight of dried extract was also measured from freeze dried sample.

3. Minimum inhibitory concentration test of *Clitocybe nuda* extract

The mushroom extract was mixed with cultural media to make a series of concentrations at 0%, 5%, 10%, 20%, 30%, 40%, 50%, and 60%. The negative control was the mushroom extract at 0% concentration. The pH of the samples were adjusted to 7. Bacteria were added to samples to make the final population of 100 CFU/mL. Triplicates were made for each concentration. All samples were incubated at 37 ℃ with 200 rpm shaking for 48 h. Then, 1 mL culture was washed twice with BPB through centrifugation (Thermo Fisher Scientific, Hudson, NH) at 4500 rpm for 4 min. The washed bacteria were re-suspended in 1 mL BPB. And 100 µL of bacterial suspension were transferred in a 96-well microplate. The O.D. values were measured using a microplate reader (UVP, Upland, CA). A linear regression of $O.D._{650nm}$ vs. concentration in mg/mL was made. Minimal inhibitory concentration at 50% inhibition (MIC_{50}) was calculated from the linear regression at 50% $O.D._{650nm}$ values of 0% mushroom extract concentration, which was considered as 100% growth. This experiment was repeated twice for all tested bacteria.

4. Effects of pH on the antibacterial activity of *Clitocybe nuda* extract

The original pH of the extract was 4.2. To study the effect of pH on the inhibitory activity of mushroom extract, the extract was mixed with two-fold strength culture media at 1:1 ratio (50% of original mushroom extract concentration). The pH of samples were adjusted of 4, 5, 6, 7, 8, 9, and 10 with 10 M HCl and 10 M NaOH. Bacteria were added to each sample to make 100 CFU/mL population. Cultural medium at the same pH without mushroom extract were used as

paired negative control at each pH. Triplicates were made for each treatment. Then, all samples were incubated at 37 ℃ with 200 rpm for 48 h. And the values of $O.D._{650nm}$ were measured using the same methods mentioned in the previous section for the analysis of antibacterial activity. The experiment was repeated twice for all tested bacteria.

5. Thermal stability of antibacterial activity of *Clitocybe nuda* extract

Mushroom extract was treated at 4, 72, 100, and 121 ℃ for 30 min. The sample stored at 4 ℃ was considered as the positive control. The treated samples were mixed with two-fold strength culture media at 1:1 ratio. The pH of all samples was adjusted to 7. Bacteria were added to each sample to make 100 CFU/mL population. Cultural media without bacteria were used as negative controls. Triplicates were made for each temperature treatment. The treated samples were incubated at 37 ℃ with 200 rpm shaking for 48 h. The antibacterial activities were analyzed from the $O.D._{650nm}$ values of all different temperature treated samples. The O.D. values at 650 nm were measured using the same protocol described in the previous section. The experiment was repeated twice for all four bacteria.

6. Molecular weight estimation of active compounds

Freeze dried samples were dissolved in deionized water to make 400 mg/mL concentration. The sample was dialyzed in 100-500, 1000, and 6000-8000 Dalton molecular weight cut-off (MWCO) dialysis tube (Spectrum, Shiga, Japan) in 1 L deionized (DI) water at room temperature for 24 h. The dialyzed water for 100-500 Dalton MWCO dialysis tube was freeze dried and resuspended in DI water to the same volume of the original sample. The sample at 400 mg/mL of mushroom extract without dialysis was used as the positive control. All samples were mixed with two fold strength of culture media at 1:1 ratio. The pH of all samples

was adjusted to 7. Bacteria were added to each sample to make 100 CFU/mL population. Cultural media without bacteria were used as negative controls. Triplicates were made for each treatment. All samples were incubated at 37 ℃ with 200 shaking rpm for 48 h. The antibacterial activities were analyzed by measuring the O.D. at 650 nm using the same protocol described before. The experiment was repeated twice for each bacterium.

7. Statistical Analysis

One-way ANOVA (SAS Enterprise Guide version 4.2, SAS Institute Inc., Cary, NC) was applied to determine the stability and estimate the molecular size of the key ingredients.

CHAPTER 4: RESULT AND DISCUSSION

1. Minimum inhibitory concentration of *Clitocybe nuda* extract

According to the extraction protocol, the concentration of the extract was 154.3 mg/mL. The concentrations used for the minimum inhibitory concentration test were 0, 7.72, 15.43, 30.86, 46.29, 61.72, 77.15, and 92.58 mg/mL. Linear regressions of mushroom extract concentration (mg/mL) versus bacterial growth ($O.D._{650nm}$) were made for all four tested bacteria (Table 1). Lower O.D. values indicate less bacterial growth and greater antibacterial activity. All r-square of linear regressions equations were larger than 0.85, which indicates good correlation between the concentrations of mushroom extract and the bacterial growth. The $O.D._{650nm}$ for the growth medium at 0 mg/mL mushroom extract was considered as 100% bacterial growth. Therefore, the MIC_{50} was calculated based on the linear regression equation at 50% of the $O.D._{650nm}$ at the 100% bacterial growth $O.D._{650nm}$. The mushroom extract showed antibacterial activity against *Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7, and *Staphylococcus aureus*. The MIC_{50} for *Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7 and *Staphylococcus aureus* were 79.20, 84.51, 105.86, and 143.60 mg/mL, respectively. This mushroom extract has lower MIC_{50} against *Listeria monocytogenes* and *Salmonella typhimurium*, and showed higher MIC_{50} against *Staphylococcus aureus* and *E. coli* O157:H7.The results also show that the antibacterial activity of the mushroom extract is non-selective to Gram (-) or Gram (+) bacteria.

Table 1-Antibacterial activities of *Clitocybe nuda* extraction at various concentrations.

Concentration (mg/mL)	*Listeria monocytogenes* (G3990)[1]	*Salmonella typhimurium* (ATCC 13311)[2]	*E. coli* O157:H7 (204P)[3]	*Staphylococcus aureus* (ATCC 12600)[4]
0.00	1.592 ±0.006[5]	1.320 ±0.008	1.670 ±0.004	1.517 ±0.017
7.72	1.434 ±0.027	1.146 ±0.026	1.430 ±0.042	1.319 ±0.013
15.43	1.334 ±0.025	1.015 ±0.055	1.327 ±0.013	1.331 ±0.035
30.86	1.178 ±0.025	0.889 ±0.008	1.243 ±0.009	1.206 ±0.030
46.29	1.222 ±0.035	0.811 ±0.014	1.162 ±0.004	1.172 ±0.005
61.72	0.923 ±0.019	0.787 ±0.007	1.123 ±0.048	1.084 ±0.009
77.15	0.856 ±0.029	0.724 ±0.014	1.018 ±0.006	1.058 ±0.006
92.58	0.634 ±0.014	0.681 ±0.011	0.986 ±0.011	1.043 ±0.003

[1]Linear regression equation: y = -0.0093x + 1.5326, R^2= 0.9577; MIC_{50}=79.20 mg/mL; n=3

[2]Linear regression equation: y = -0.0061x + 1.1755, R^2= 0.8626; MIC_{50}=84.51 mg/mL; n=3

[3]Linear regression equation: y = -0.0063x + 1.5019, R^2= 0.8663; MIC_{50}=105.86 mg/mL; n=3

[4]Linear regression equation: y = -0.0045x + 1.4047, R^2= 0.8724; MIC_{50}=143.60 mg/mL; n=3

[5]Measured at $O.D._{650nm}$

2. Thermal stability of *Clitocybe nuda* extract

Since the mushroom extract was stored at 4 ℃, antibacterial activity of the sample treated at 4 ℃ was used as the positive control. After treatments at 4, 72, 100, and 121 ℃ for 30 min, the bacterial growth in all treated samples for the four bacteria were significantly lower than the negative controls which had no mushroom extract, and the antibacterial activities at all temperatures for the treated samples were similar to the positive control without significant differences (Figure 1-4). Therefore, the antibacterial activity of the mushroom extract is thermally stable and will not be affected by high temperature treatments. This result agreed with Chen and Huang's study (2009) which showed that the inhibition efficacy on zoospore germination and mycelia growth of *Phytophthora capsici* and *Xanthomonas axonopodis* pv. *vesicatoria* by heat treated secondary metabolite of *Clitocybe nuda* at 25, 60, 100, and 121 ℃ are similar to each other.

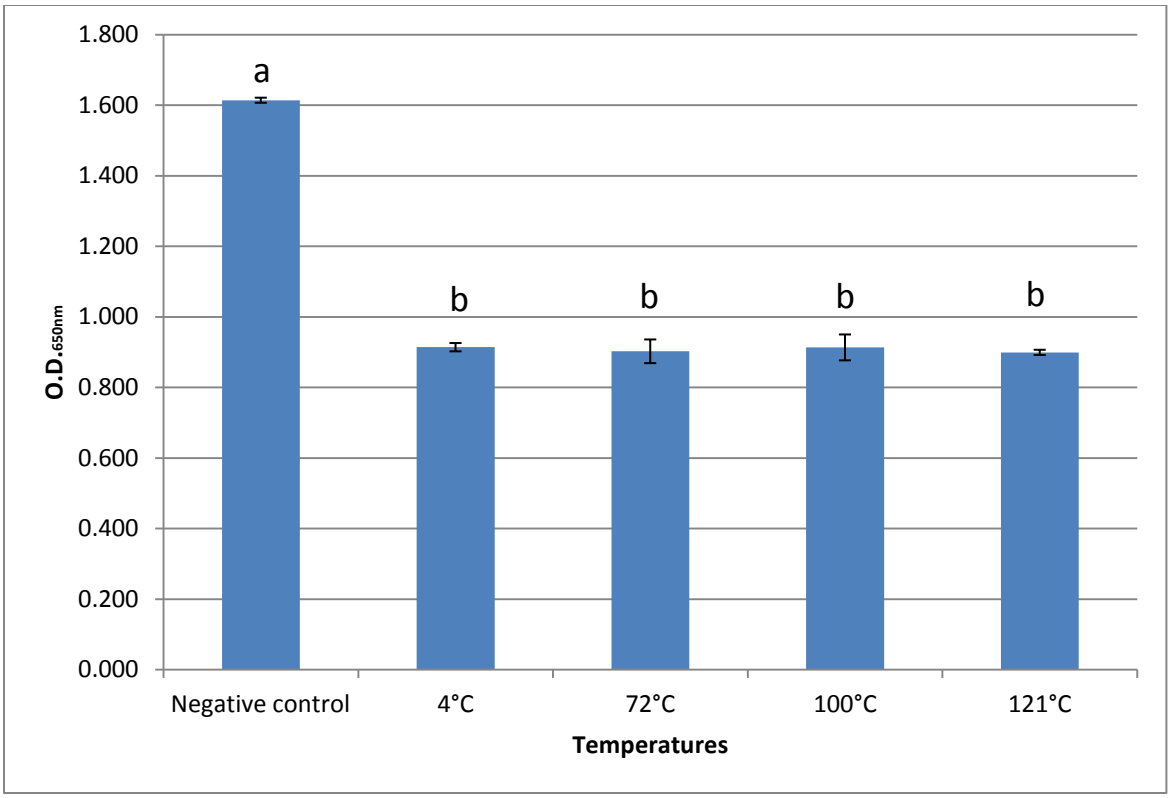

Figure 1-Thermal effect on antimicrobial activity of *Clitocybe nuda* extract against *Listeria monocytogenes* G3990. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

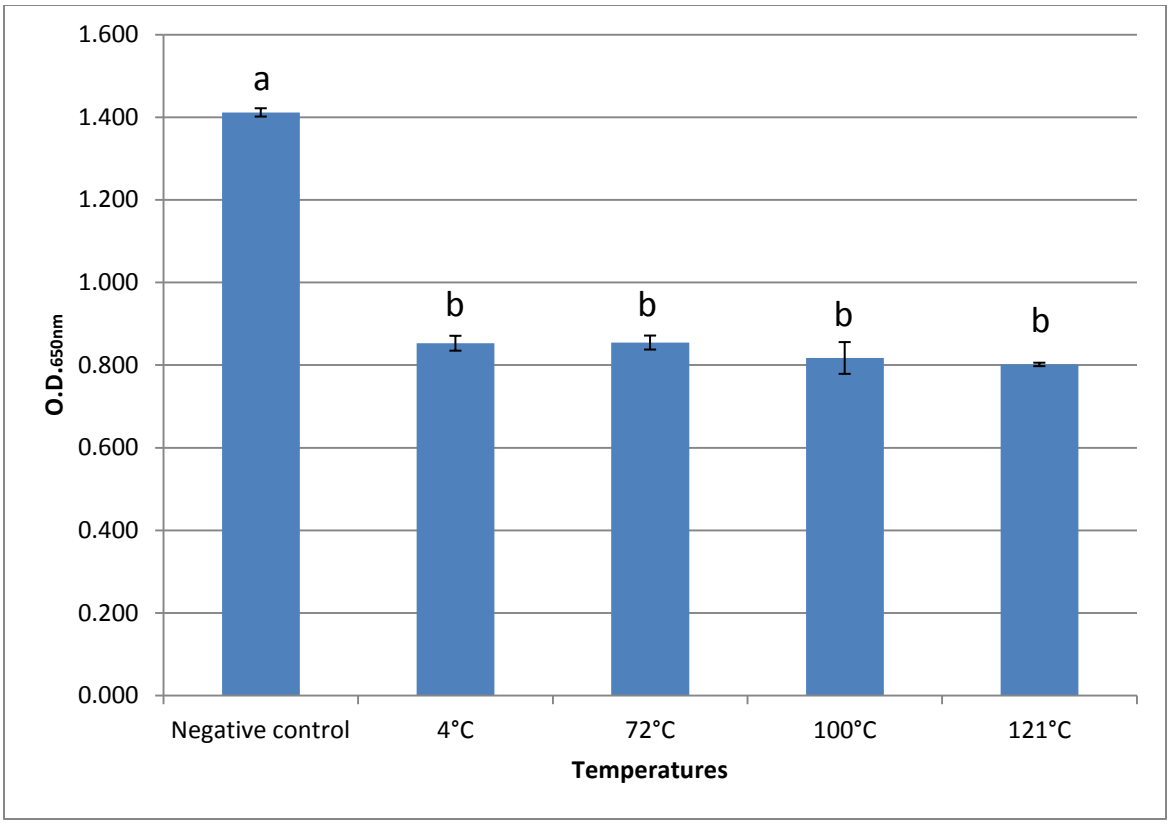

Figure 2-Thermal effect on antimicrobial activity of *Clitocybe nuda* extract against *Salmonella typhimurium* ATCC 13311. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

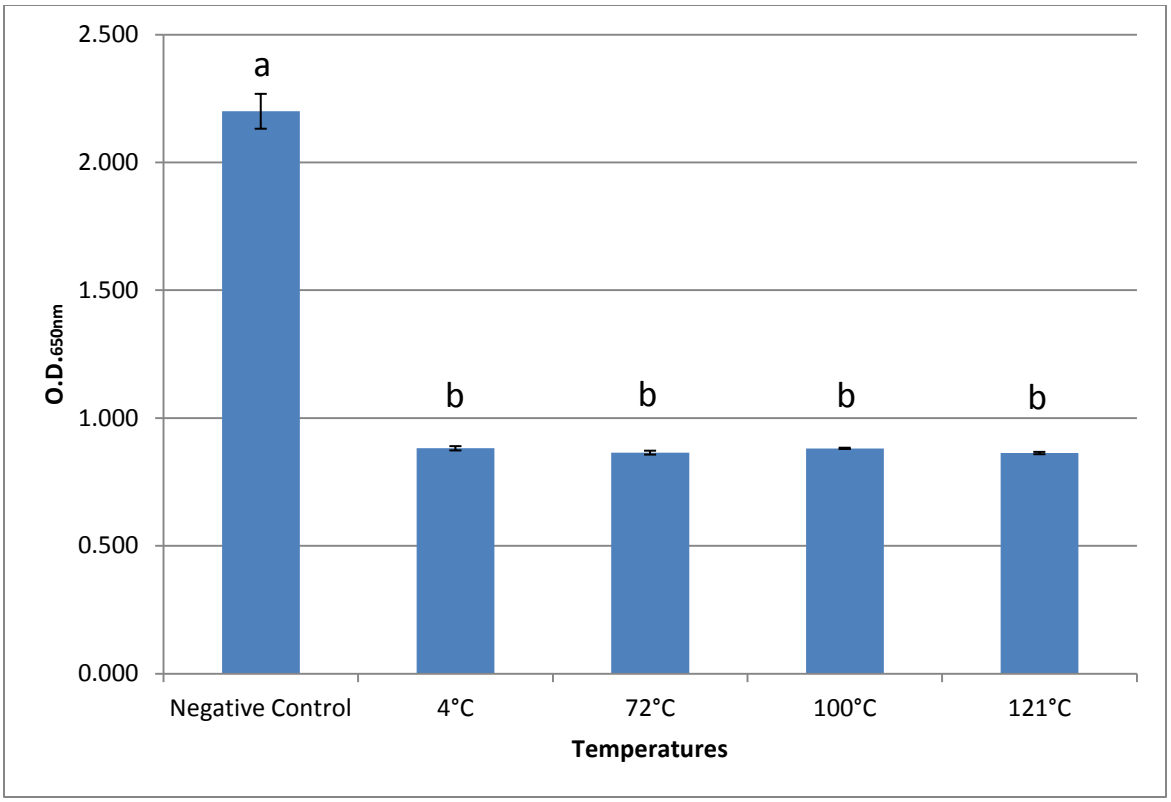

Figure 3-Thermal effect on antimicrobial activity of *Clitocybe nuda* extract against *E. coli* O157:H7 204P. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

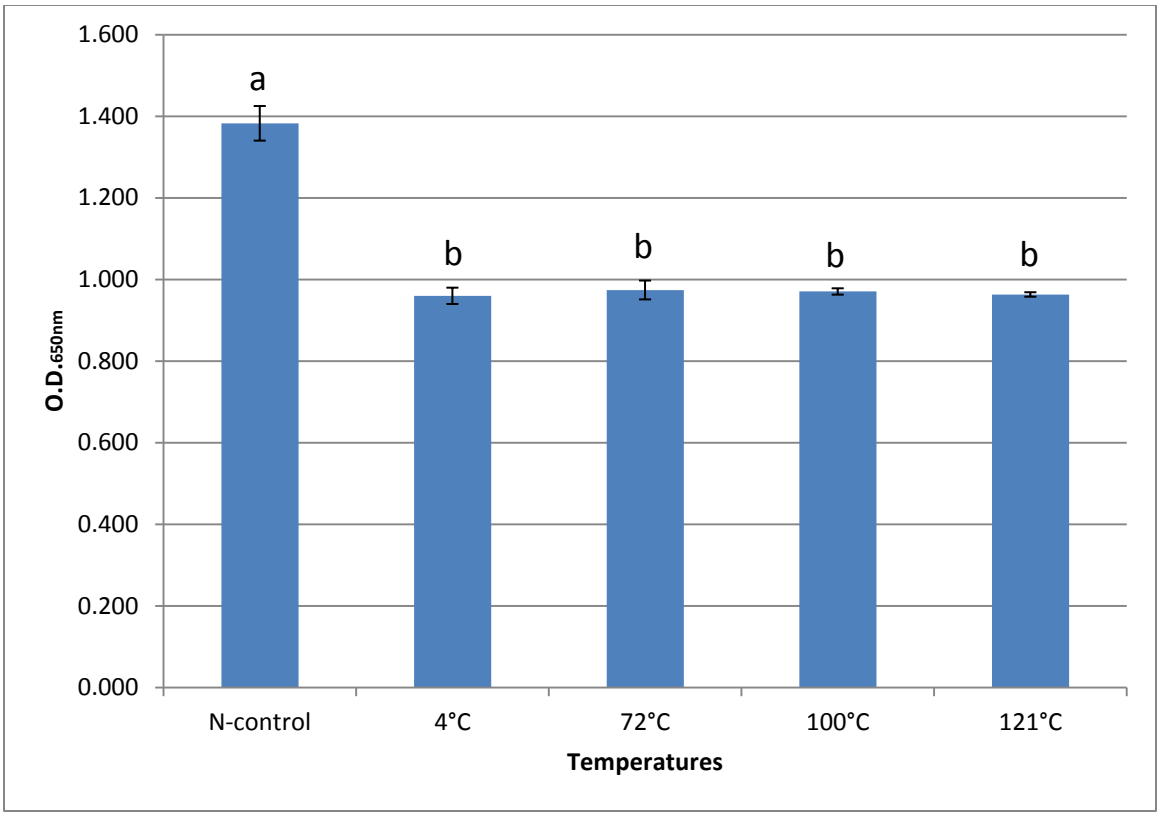

Figure 4-Thermal effect on antimicrobial activity of *Clitocybe nuda* extract against *Staphylococcus aureus* ATCC 12600. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

3. Effects of pH on the antimicrobial activity of *Clitocybe nuda* extract

3.1 To *Listeria monocytogenes* G3990

The anti-*Listeria monocytogenes* activity of the mushroom extract varied at different pHs (Figure 5). At pH 4, the bacteria didn't grow in the negative control and the mushroom extract sample which indicated that *Listeria monocytogenes* cannot grow at this pH. The antibacterial activity was higher when the pH was lower in the pH range of 5 to 8 and showed significant differences. On the other hand, growth on the controls at pH 5 and 6 were lower than those of the controls at pH 7 and 8. This result indicated that the antibacterial activity was caused by the mushroom extract and low pH. The $O.D._{650nm}$ in the samples at pH 9 and 10 were close to zero which meant that bacterial growth was almost completely inhibited. However, the bacterial growth in the control at pH 10 was lower than those of controls at pH 7-9, which indicated that the antibacterial activity at pH 10 was also caused by the mushroom extract and the extreme pH environment. The results agreed with Chen and Huang's (2009) study, which showed similar trend of pH effect of *Clitocybe nuda* culture filtrate on zoospore germination of *Phytophthora capsici.* Overall, the anti-*Listeria monocytogenes* activity is better at alkaline condition. Although there was a pH effect on antibacterial activity at extreme pH levels, the mushroom extract retained its antibacterial activity in a wide pH range of 5-10.

Table 2-Effects of pH on antimicrobial activity of *Clitocybe nuda* extract against *Listeria monocytogenes* G3990.

Treatments	O.D.$_{650nm}$[1]
pH 4 Control[3]	0.002 ±0.002[h2]
pH 4 Sample[4]	0.002 ±0.001[h]
pH 5 Control	1.413 ±0.033[c]
pH 5 Sample	0.612 ±0.030[g]
pH 6 Control	1.510 ±0.058[b]
pH 6 Sample	0.824 ±0.014[f]
pH 7 Control	1.735 ±0.032[a]
pH 7 Sample	0.925 ±0.023[e]
pH 8 Control	1.725 ±0.021[a]
pH 8 Sample	1.068 ±0.009[d]
pH 9 Control	1.699 ±0.010[a]
pH 9 Sample	0.001 ±0.001[h]
pH 10 Control	1.542 ±0.036[b]
pH 10 Sample	0.001 ±0.001[h]

[1]n=3

[2]Different letters represent significant differences among samples at p>0.05.

[3]Control means bacterial culture without mushroom extract at the same pH.

[4]Sample means bacterial culture with 75 mg/mL mushroom extract at each pH.

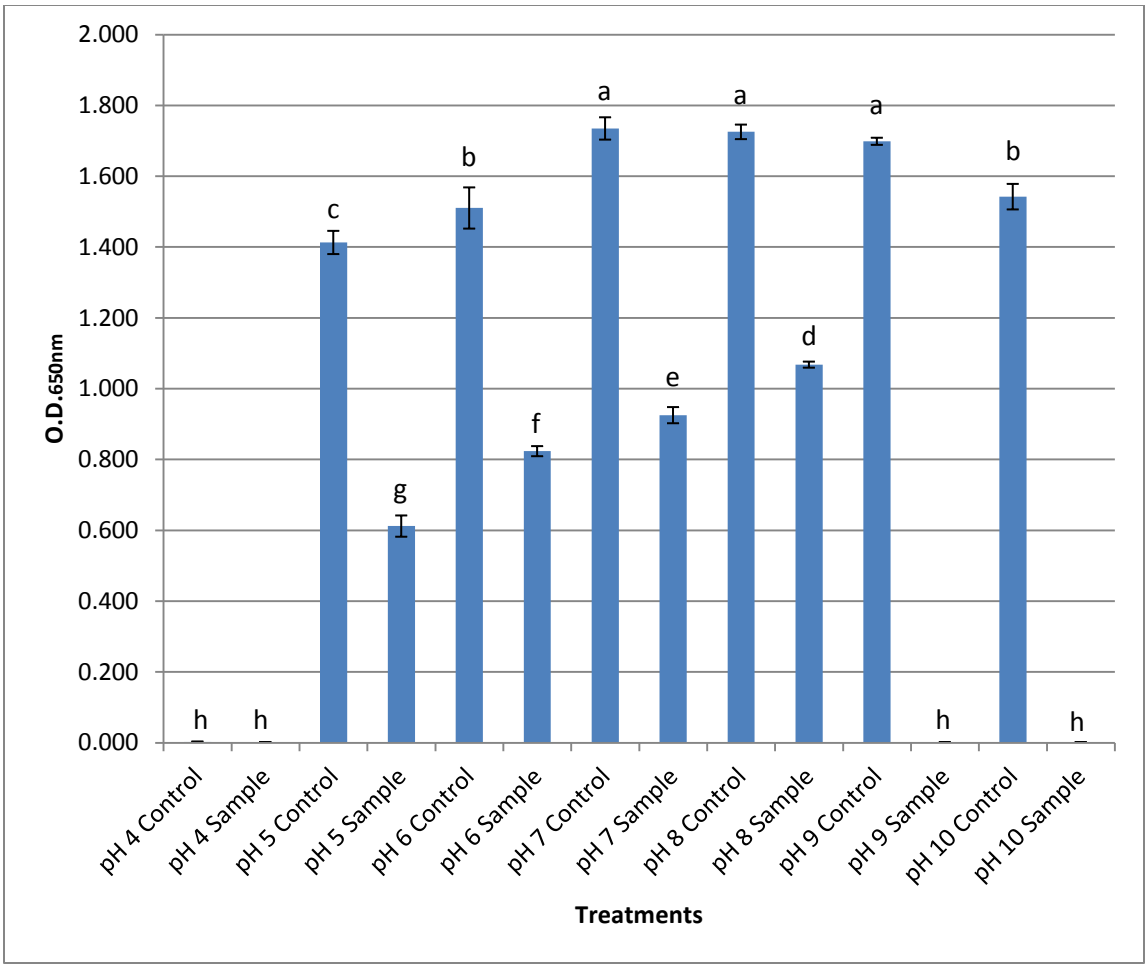

Figure 5-Effects of pH on antimicrobial activity of *Clitocybe nuda* extract against *Listeria monocytogenes* G3990. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

3.2 To *Salmonella typhimurium* ATCC 13311

The anti-*Salmonella typhimurium* activities of the mushroom extract also varied at different pHs (Figure 6). At pH 4 and 10, the bacterial growth was inhibited completely in both negative controls and mushroom extract samples. *Salmonella* did not grow in the samples at both pH 4 and 9, but grew in the controls. The bacterial growth in the control at pH 5 was lower than other controls at pH 6-9, which indicated that the anti-*Salmonella typhimurium* activity was caused by both the mushroom extract and pH. The anti-*Salmonella typhimurium* activities at pH 6 and 7 are similar to each other and higher than that at pH 8 significantly. The bacterial growth in the controls at pH 6-9 are close to each other without significant difference. However, the growth in the sample at pH 9 was completely inhibited which indicated that the anti-*Salmonella typhimurium* activity of mushroom extract was higher in alkaline condition. In general, the mushroom extract possesses good antibacterial activity against *Salmonella typhimurium* in the pH range of 5-8.

Table 3-Effects of pH on antimicrobial activity of *Clitocybe nuda* extract against *Salmonella typhimurium* ATCC 13311.

Treatments	O.D.$_{650nm}$[1]
pH 4 Control[3]	0.001 ± 0.001^{e2}
pH 4 Sample[4]	0.001 ± 0.001^{e}
pH 5 Control	1.369 ± 0.029^{b}
pH 5 Sample	0.000 ± 0.000^{e}
pH 6 Control	1.719 ± 0.024^{a}
pH 6 Sample	0.686 ± 0.006^{d}
pH 7 Control	1.729 ± 0.003^{a}
pH 7 Sample	0.716 ± 0.047^{d}
pH 8 Control	1.725 ± 0.050^{a}
pH 8 Sample	0.835 ± 0.008^{c}
pH 9 Control	1.718 ± 0.068^{a}
pH 9 Sample	0.000 ± 0.001^{e}
pH 10 Control	0.000 ± 0.001^{e}
pH 10 Sample	0.002 ± 0.002^{e}

[1]n=3

[2]Different letters represent significant differences among samples at p>0.05.

[3]Control means bacterial culture without mushroom extract at the same pH.

[4]Sample means bacterial culture with 75 mg/mL mushroom extract at each pH.

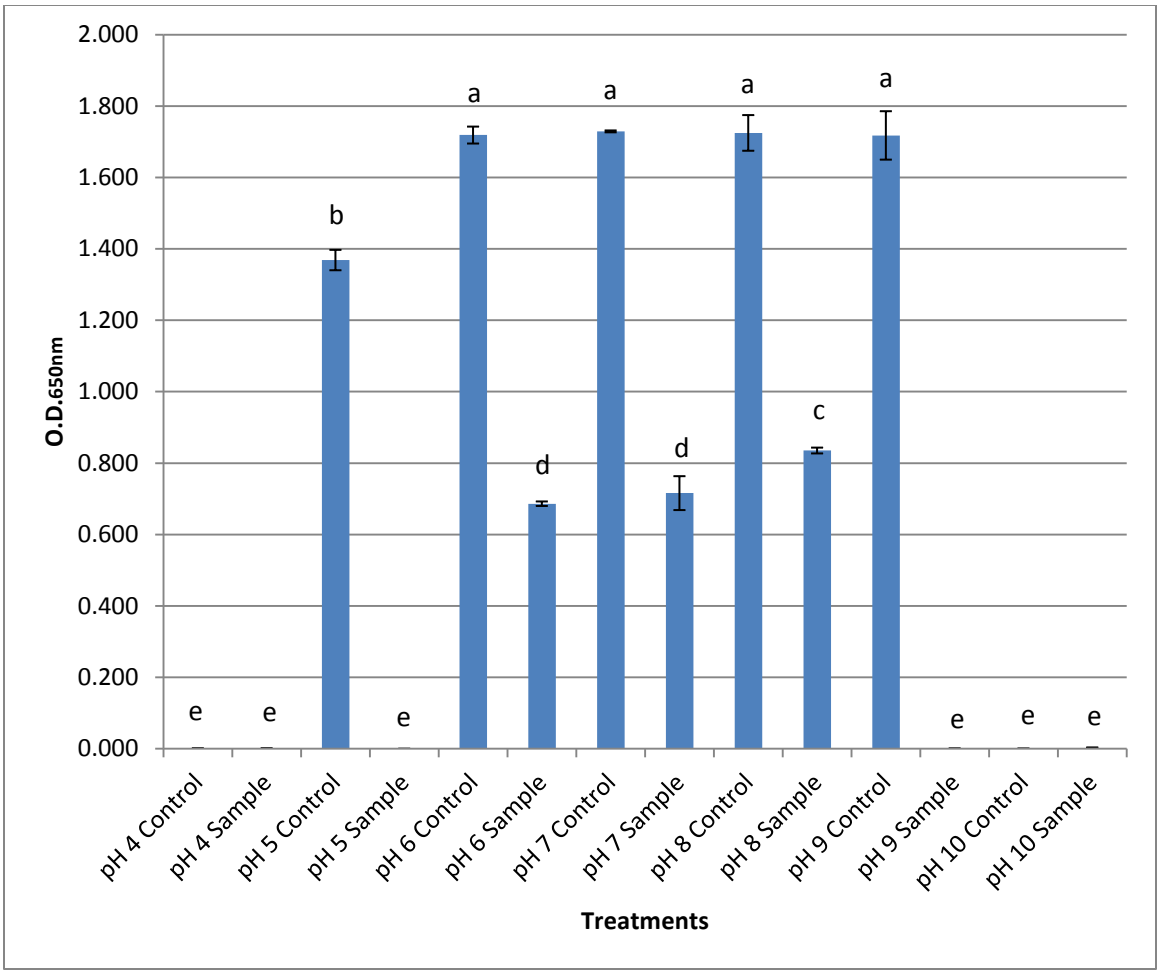

Figure 6-Effects of pH on antimicrobial activity of *Clitocybe nuda* extract against *Salmonella typhimurium* ATCC 13311. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05.

4. Molecular weights estimation of active compounds

After the mushroom extract was dialyzed in 1,000 and 6,000-8,000 Dalton MWCO dialysis tubes, the samples showed no antibacterial activity (data not shown). This indicated that the molecular weights of active compounds in the mushroom extract are smaller than 1,000 Dalton. After the 400 mg/mL sample was dialyzed in the 100-500 Dalton MWCO tube, both samples of inside the dialysis tube and the buffer outside the dialysis tube were used to test for antibacterial activity. The O.D. values of the buffer outside the dialysis tube to all tested bacteria were close to zero (Table 4). The result indicated that the outside dialyzed buffer performed excellent antibacterial activity, and the molecular weight of active compounds are smaller than 500 Dalton. The O.D. values of the sample inside the dialysis tube to *Listeria monocytogenes*, *E. coli* O157:H7, and *Staphylococcus aureus* were lower than negative controls which meant that after dialysis, some small active compounds (<100-500 Dalton) still stayed inside the tube and showed antibacterial activity.

Table 4 Molecular weights estimation of active compounds in *Clitocybe nuda* extract.

Treatments	*Listeria monocytogenes* (G3990)[1]	*Salmonella typhimurium* (ATCC 13311)[1]	*E. coli* O157:H7 (204P)[1]	*Staphylococcus aureus* (ATCC 12600)[1]
Negative control	1.493 ±0.022[a3]	2.196 ±0.092[a]	2.341 ±0.023[a]	1.416 ±0.032[a]
Positive control	0.003 ±0.002[c]	0.004 ±0.003[b]	0.001 ±0.001[c]	0.002 ±0.002[c]
MW>100-500 Dalton[2]	1.427 ±0.018[b]	2.131 ±0.017[a]	2.227 ±0.029[b]	1.220 ±0.025[b]
MW<100-500 Dalton[2]	0.004 ±0.002[c]	0.000 ±0.001[b]	0.002 ±0.001[c]	0.004 ±0.003[c]

[1]n=3

[2]MW>100-500 Dalton represents molecular weights (MW) of compounds are larger than 100-500 Dalton; MW<100-500 Dalton represents molecular weights are smaller than 100-500.

[3]Different letters represent significant differences among samples at p>0.05 within the column.

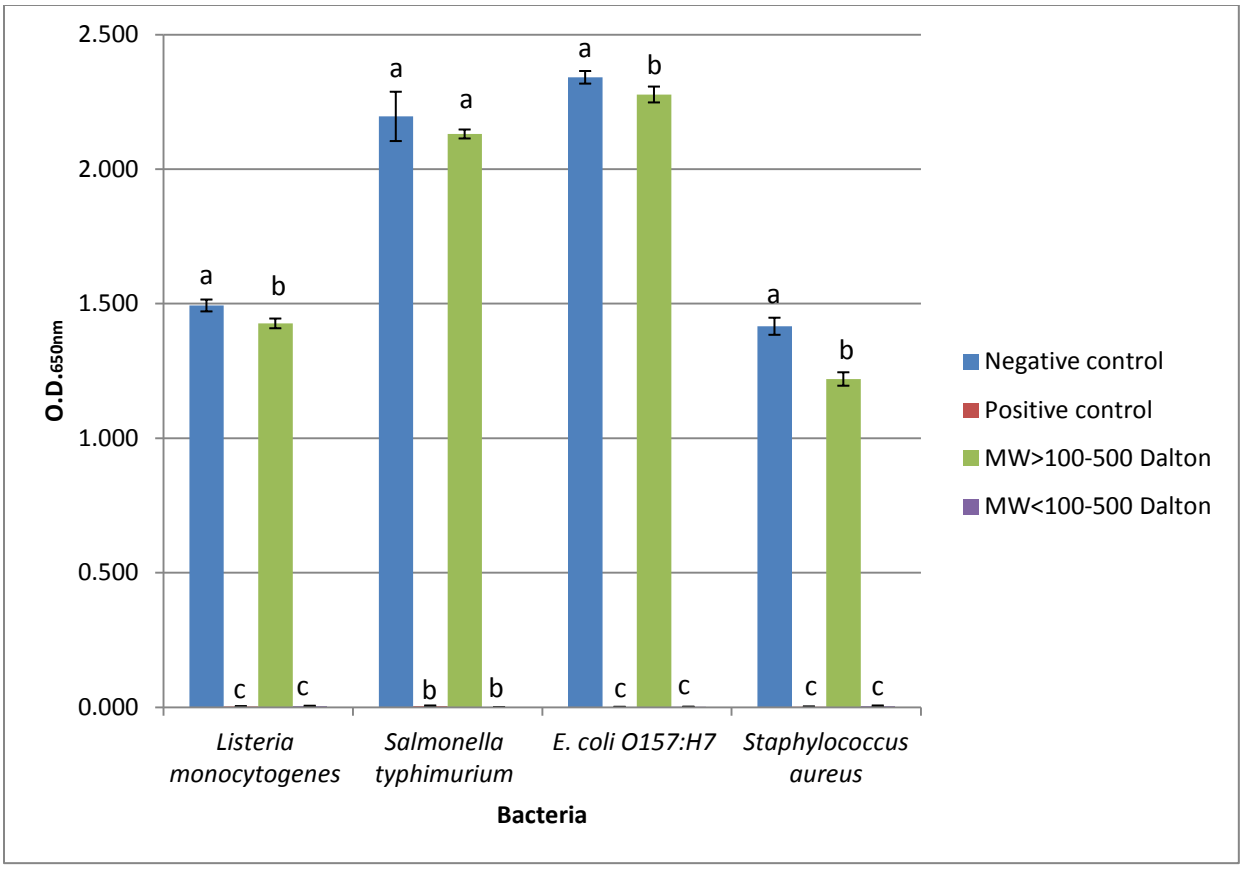

Figure 7-Molecular weights estimation of active compounds in *Clitocybe nuda* extract. Error bars represent standard deviations (n=3). Different letters represent significant differences among samples at p>0.05 within the same bacteria.

CHAPTER 5: CONCLUSION

Clitocybe nuda was extracted with 95% ethanol and the extract showed good antibacterial activity against four pathogenic foodborne bacteria: *Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7, and *Staphylococcus aureus*.

The extract had better antibacterial activity against *Listeria monocytogene* and *Salmonella typhimurium* than against *E. coli* O157:H7 and *Staphylococcus aureus*. The MIC_{50} for these four bacteria were 79.20, 84.51, 105.86, and 143.60 mg/mL, respectively. The mushroom extract has antibacterial activity to a broad range of bacteria including both Gram-positive and Gram-negative bacteria. These results should increase the potential application of this mushroom extract in foods.

The thermal stability was determined at four different temperatures: 4, 72, 100, and 121 ℃. It is important to study the stability of the extract at these four temperatures. The first temperature, 4 ℃, is a common temperature used for food refrigeration; 72 ℃ is the commonly used pasteurization temperature to process dairy products and fruit juices; 100 ℃ is the boiling point of water; and 121 ℃ is the elevating temperature, which are the common temperatures used to process foods for preservation. The results showed that the antibacterial activity of the mushroom extract was very stable at these four temperatures against the four bacterial pathogens. Therefore, there is a great potential for applying the mushroom extract in a broad range of foods processed under different conditions to control microorganisms.

Foods have a wide range of pHs. The food industry often produces food products at various pHs to control microbial growth and the quality or safety of foods. The effectiveness of some food preservatives is highly dependent on the pH of the food. This study showed that the antibacterial activity of the mushroom extract against *Listeria monocytogenes* and *Salmonella typhimurium* is very stable at a wide pH range of 5-10. Therefore, the mushroom extract can be applied in foods with different pH levels to control microbial growth improving the safety.

The molecular weights of the antibacterial compounds against all four pathogens were determined to be smaller than 500 Dalton. This information will be beneficial to scientists to further elucidate the antibacterial compounds in the *Clitocybe nuda* extract.

Overall, *Clitocybe nuda* extract can effectively inhibit the growth of four foodborne pathogens. Its antibacterial activity is very stable at high temperatures and over a wide range of pHs. These properties of the extract make the extract suitable for use in a wide range of foods and food ingredients.

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