

Antibacterial activities of secondary metabolites from *Clitocybe nuda*

by

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Abstract

The purpose of this study was to obtain a better understanding of the antibacterial activities of the secondary metabolites from *Clitocybe nuda*, one kind of edible mushroom. The antibacterial activity was determined by testing the Minimum Inhibitory Concentrations (MIC) of 50% log reduction of the secondary metabolites from *C. nuda* against four prevalent foodborne pathogens---*Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Escherichia coli* O157:H7, which were 27, 25, 23 and 40 mg/mL respectively. The thermal and pH-stability of the antibacterial activity of the secondary metabolites from *C. nuda* were also evaluated. They were stable at 121 °C after 30 min treatment and had good antibacterial activity under a broad pH range of 5-9. The antibacterial efficacies in liquid eggs and ground beef were also investigated. The secondary metabolites of *C. nuda* were able to reduce about 0.2 and 0.5 log CFU/g *L. monocytogenes* and *E. coli* O157:H7 in ground beef, respectively. A 1 log reduction of *Salmonella* Typhimurium in liquid eggs at 50 mg/mL treatment was found.

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List of Abbreviations or Symbols

ANOVA Analysis of Variance

BPB Butterfield's Phosphate Buffer

BSA Bismuth Sulfite Agar

CDC US Centers for Disease Control and Prevention

CFU Colony Forming Unit

FSIS USDA Food Safety and Inspection Service

HC Hemorrhagic Colitis

HEA Hektoen Enteric Agar

HUS Hemolytic-uremic Syndrome

MOA Modified Oxford Agar

NM Nonmotile

OD Optical Density

PDA Potato Dextrose Agar

PDB Potato Dextrose Broth

RTE Ready to Eat

SD Standard Deviation

SEA Staphylococcal Enterotoxin A

SED Staphylococcal Enterotoxin D

SMAC Sorbital MacConkey

STEC Shiga Toxin-producing *E. coli*

TC-SMAC Tellurite Cefixime-Sorbital MacConkey Agar

TSA Trypticase® Soy Agar

TSB Tryptic® Soy Broth

Chapter 1 Introduction

This study focuses on food safety concerns in the food processing industry and evaluates the potential use of the antibacterial activity of the secondary metabolites from *Clitocybe nuda* against *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella* Typhimurium.

1.1 Background

With the globalization of the food industry, food safety becomes a more important issue in the human health in the US. The Centers for Disease Control and Prevention (CDC) estimated that in the United States, 31 known pathogens and more unspecified pathogens together caused about 47.8 million illnesses in 2011 (CDC 2011). This data may be underestimated because some mild symptoms of foodborne illnesses were not reported.

Among those foodborne pathogens, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* are the major bacteria which cause a great deal of food safety problems. A *Listeria* outbreak linked to Crave Brothers Farmstead Cheeses caused 1

death and 6 hospitalizations in July, 2013 (CDC 2013). Another example is the multistate outbreak of *E. coli* O157:H7 in November, 2012 which involved in 33 people with 5 hospitalizations due to the consumption of contaminated organic spinach and spring mix blends (CDC 2012). Many outbreaks of *Salmonella* infections are linked to live poultry. From March to July, 2013, more than 300 persons were ill due to infections of *Salmonella* Typhimurium linked to live poultry in backyard flocks from 37 states all around the US (CDC 2013).

To provide consumers with safe foods, the addition of food antimicrobials is one of the strategies to protect foods from foodborne pathogen contamination. Due to the awareness of health issues, lately, consumers have concerns about the use of artificial food antimicrobials. In addition, the prevalence of antibiotics has led to the occurrence of drug-resistant foodborne pathogens. Thus, food scientists are looking for safe and effective naturally occurring food antimicrobials to replace artificial compounds.

1.2 Purpose of study

The use of food antimicrobials is an effective way to ensure the wholesomeness and safety of food products. In general, antimicrobials are divided into artificial and naturally occurring compounds (Davidson and others 2010). Since some consumers have concerns about the toxicity of synthetic chemicals added to their foods, naturally occurring antimicrobials

are becoming more attractive to the food industry. Naturally occurring antimicrobials can be isolated from animals, plants and of microbial origins (Ana and others 2010).

Certain mushrooms have been found to show considerable antimicrobial activities. *Clitocybe nuda*, formerly called *Lepista nuda*, is one of them (Kiran and others 2013). This is an edible mushroom mostly found in Europe and North America. In previous research, the methanolic extract of *C. nuda* was found to inhibit the growth of *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Alves and others 2012). Chen and others (2012) have isolated the components from the secondary metabolites of *C. nuda* which have been known to inhibit plant pathogens, such as *Phytophthora capsici* and *Xanthomonas axonopodis*.

The objectives of this study were (1) to evaluate the antibacterial activities of the secondary metabolites from *C. nuda* against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*, (2) to test the effects of pH and temperature on the antimicrobial activities of the secondary metabolites from *C. nuda*, and (3) to investigate the antibacterial effects of secondary metabolites from *C. nuda* on *Salmonella* in liquid eggs and *E. coli* O157:H7 and *L. monocytogenes* in ground beef.

1.3 Significance of study

Artificial food preservatives are raising issues related to the health concern. Some consumers expect less artificial chemicals to be added into their foods. It is also notable that the use of antibiotics has resulted in the increase of drug-resistant foodborne microorganisms (Threlfall and others 2000). Therefore, it becomes important for the food industry to find safe and effective antimicrobials from natural sources to replace artificial chemicals in foods.

The antimicrobial activities of mushrooms have been investigated for a long time. Both mushroom extract and the secondary metabolites show good inhibitory effects against bacteria and fungi. However, very few studies of the secondary metabolites from *C. nuda* on their antibacterial activities against foodborne pathogens have been done. This study will obtain a better understanding of the potential use of the secondary metabolites from *C. nuda* as food antimicrobials.

Chapter 2 Literature Review

2.1 Concern of food safety

With the great expansion and globalization of the food industry, consumers are more concerned with food safety issues. Food safety describes the proper handling, preparation and storage of food products to prevent them from becoming hazardous to consumers. Food products can get contaminated physically, chemically and biologically. The presence of pathogenic microorganisms in foods has long been a potential threat to public health, especially to the immune system compromised people, infants, the elderly and people with chronic diseases such as diabetes, AIDS and others. Good hygienic practices can reduce the hazards to some extent, but are never enough to ensure the safety of the final products. Although much has been done to prevent the broadcast of most well-recognized pathogens, trends are changing rapidly, such as the ever-fast growing population calls for larger and more industrialized production of food and the changing of eating habits. These changes result in more dining out. However, health conscious individuals are consuming more fresh produce. With the food industry trying to match the growing demand of food production, the use of antibiotics is increasing which may lead to

the emergence of gene-mutated pathogens. Yet, the globalized food market asks for better surveillance over imported food products (Newell and others 2010; Nyachuba 2010). These trends potentially increase the possibility of consumers coming in contact with contaminated food products and require stricter methods to improve food safety.

In the US, according to the data from the Center for Disease Control and Prevention (CDC) records, 31 major foodborne pathogens and other unspecified agents are responsible for about 47.8 million of illnesses, 128,000 cases of hospitalization and 3,000 deaths each year (Scallan and others 2011; Scallan and others 2011). However, this data can be largely underestimated by a variety of reasons; e. g. patients with minor symptoms may not be aware of the infections, outbreaks without serious illnesses are ignored, doctors fail to link the disease with foodborne pathogens.

Foodborne illnesses also cause great economic loss to the whole society. Robert L. Scharff (Scharff 2012) estimated that an annual cost of \$77.7 billion was spent on the health-related losses. Therefore, it is beneficial to produce safe food not only for the consumers, but also for the food industry and the government. These combined reasons help to explain why the concern of food safety has always been an important issue to the public.

Foodborne illness is classified into foodborne infection and intoxication. A foodborne agent which can invade or establish itself in the

host's intestinal tract after being ingested is called the infectious pathogen. Examples for such pathogens are *Salmonella*, *Campylobacter*, *Listeria monocytogenes* and *E. coli*. The incubation period for a foodborne infection could range from hours to several days before the first symptom occurs.

Foodborne intoxications (also known as food poisoning) occur when people consume food which contains toxins that have been formed before ingestion. *S. aureus* and *C. botulinum* are the most common toxin producers. A typical symptom for food poisonings is vomiting and the effects are generally acute.

The growth of bacteria in food can be influenced by many factors, such as pH, temperature, time, water activity, oxygen, preservatives and microbial interactions with the food. It is essential to control foodborne pathogens by changing the properties of the food products. However, the change of the properties could affect the sensory characteristics of certain products. The addition of antimicrobials in food production is commonly used.

2.2 *Listeria monocytogenes*

Listeria monocytogenes is a group of gram-positive foodborne bacteria which can cause listeriosis to both humans and animals. The genus *Listeria* consists of seven species (*monocytogenes*, *ivanovii*,

innocuar, *seeligeri*, *martii*, *welshimeri*, and *grayi*). Among all these species, only *L. monocytogenes* and *L. ivanovii* are pathogenic.

L. monocytogenes is pathogenic to both humans and animals while *L. ivanovii* cause diseases mostly to animals (Vázquez-Boland and others 2001). In the United States, *L. monocytogenes* alone causes about 1,600 cases of listeriosis infection each year, including 1,400 hospitalizations and 250 deaths. Listeriosis is more threatening to immune-compromized people, pregnant women, elder citizens, and newborns. Listeriosis infection can be extremely serious, especially to the populations who are at risk. In 2011, 72 people were infected with listeriosis after eating the cantaloupe grown at Jensen Farm in Colorado. In that outbreak, most of the sick people were older than 60, and 13 deaths were reported (CDC 2011). Although the infectivity of *L. monocytogenes* is low, the fatality rate of listeriosis (20-30%) can be a lot higher than vibriosis and salmonellosis.

L. monocytogenes is commonly found in the environment like soil, surface water, waste and food products including raw milk, dairy products and vegetables (Liu 2006). Plus, it has also been isolated from food-processing plants (Autio and others 1999; Destro and others 1996; Miettinen and others 1999). Once *L. monocytogenes* presents at a plant, eradication planning of the bacteria must be made immediately. *L. monocytogenes* can survive in extreme environmental conditions, like acid, base, low temperature and salt treatments (Liu and others 2005). Therefore, it is difficult to be eliminated from the environment. Hot water

and hot steam are usually involved in the eradication plan of *L. monocytogenes*.

L. monocytogenes can grow at refrigeration temperature which makes many ready-to-eat (RTE) foods vulnerable to the contamination of the bacteria. *L. monocytogenes* can re-contaminate the RTE foods by handling after the lethality treatment. A “zero tolerance” policy of *L. monocytogenes* in RTE foods was established in the US to address the importance of removing this fatal microorganism from RTE foods such as deli meat and hot dogs. The Food Safety and Inspection Service (FSIS) provides three alternatives used to control or eliminate *L. monocytogenes* contamination in RTE food products, including post-lethality treatments and/or the addition of antimicrobial agents.

2.3 *Escherichia coli* O157:H7

Escherichia coli is a group of bacteria which are mostly nonpathogenic. However, some of the serotypes have acquired genes which can cause severe diseases in humans. The Shiga toxin-producing *E. coli* (STEC) have emerged as a major type of food-borne pathogens which can cause symptoms ranging from uncomplicated diarrhea to hemorrhagic colitis (HC) and the life-threatening hemolytic-uremic syndrome (HUS) (Hughes and others 2006). *E. coli* O157:H7 is the most predominant serotype that has been well described over the years.

The STEC is categorized by serotyping of O & H antigens. With 174 O antigens and 56 H antigens in *E. coli*, there are many possible combinations of serotypes (Ørskov and others 1992). The specificity of the O antigen is based on the polysaccharide portion of cell wall lipopolysaccharide and H antigen is determined by the flagella protein. Many of the STEC serotypes are nonmotile (NM) mutants of strains with H antigens (Gyles 2007). The serotyping is the basis of differentiating the STEC and analyzing their properties. Since *E. coli* O157:H7 plays the predominant role in all the serotypes of STEC, the STEC serotypes are commonly divided into O157 and non-O157.

E. coli O157:H7 are gram-negative, rod-shaped bacterium and can be detected using the sorbitol MacConkey (SMAC) agar plate, because they have a unique phenotypic feature which is the failure of fermenting sorbitol within 24 h incubation.

E. coli O157:H7 is normally transmitted through fecal-oral route. Humans are most likely to get infected with STEC through the consumption of contaminated water or food. It usually starts with the colonization in the intestinal tract before resulting in diseases like watery/bloody diarrhea, hemorrhagic colitis or even hemolytic-uremic syndrome.

E. coli O157:H7 has been linked to many severe diseases and identified as a food borne pathogen carried frequently by cattle, as well as

other animals. Beef, especially ground beef, is the most frequently associated with *E. coli* O157:H7 outbreaks. Between 1982 and 2002, about 8,598 cases from 350 outbreaks were identified in 49 states (Rangel and others 2005). Among all the outbreaks, ground beef was recognized as the most common food vehicle which accounts for up to 41% of them. Other foods such as fresh produce, dairy products and drinking water have also been implicated in some of the outbreaks.

2.4 *Salmonella*

Salmonella is non-spore forming, gram-negative, rod-shaped bacterium which was discovered since the 19th century by a veterinary scientist named Salmon. Normally, *Salmonella* grow rapidly at temperatures between 25 and 43 °C. However, *Salmonella* can still grow at temperatures as low as 5.3 °C (Matches and others 1968). Therefore, it is important to avoid contact between raw and cooked foods even at refrigerated temperatures. The pH range of *Salmonella* is about 4.0-9.0, with an optimal growth pH at around 7.0 (Foster 1995, Chung 1970).

Salmonella are the cause of salmonellosis, one kind of foodborne disease, which causes approximately 40,000 cases of infection annually in the United States. Moreover, the number of the cases could be greatly underestimated because of the unreported minor symptoms. There are more than 2,400 serotypes of *Salmonella* (Brenner and others 2000). Among them, *Salmonella* Typhimurium is one of the most common

serotypes to cause infections in the United States. *Salmonella* is found in the intestinal tracts from humans, reptiles and birds. People can get infected after the ingestion of contaminated food products. The common food vehicles are eggs, poultry products, fresh produce, milk and beef. In 2009, a large multistate outbreak of *Salmonella* Typhimurium sickened more than 700 individuals (CDC 2009). Peanut butter was identified as the source of the outbreak. Salmonellosis generally has three distinct syndromes: typhoid fever, enteric fever and gastroenteritis. However, gastroenteritis is the most common one.

2.5 *Staphylococcus aureus*

S. aureus is a gram-positive cocci belonging to the Micrococcaceae family. The most obvious characteristic of the species is its capability in producing golden colonies. *S. aureus* is ubiquitous. It is present at skin, respiratory tract of humans. About 30% of healthy people are the carriers of *S. aureus* and the bacteria can survive for weeks or months if they are colonized on mucous membranes (Archer 1998).

Staphylococcal food poisoning occurs when individuals consume food products which have been contaminated by pre-formed toxins. Common symptoms are nausea, vomiting, abdominal cramps and diarrhea. The symptoms usually are rapid, even acute. It depends on the amount of toxins consumed and the health status of the person. Most patients can recover after 2-3 days. Severe cases usually occur among

the infants or elder people. With a toxin dose of 1.0 µg, it is enough to induce intoxication. In an outbreak of staphylococcal food poisoning related to chocolate milk, only a total of 200 ng of staphylococcal enterotoxin A (SEA) was found to produce illness (Evenson and others 1988).

Staphylococcal enterotoxins are a group of heat stable enterotoxins that can even withstand the processes of canned products (Balaban and others 2000). SEA and SED are two of the most common toxins related to staphylococcal food poisoning.

Most processed food products get contaminated through improper handling of the food. Since many healthy people could be the carriers of *S. aureus*, their contact with the food can be a great potential threat to the food safety. The proliferation of *S. aureus* in the food will lead to the accumulation of the enterotoxins which could cause disease after being consumed.

2.6 Mushrooms---Antimicrobial sources

Fungi are a group of organisms which are different from bacteria, plants and animals but widely distributed around the world also. The fungi kingdom includes yeasts, molds and mushrooms. The medicinal use of mushrooms has a long history in traditional Chinese medicine. To survive in the wild environment, mushrooms need to have the ability of producing bioactive compounds protecting themselves. Thus, it is possible for

humans to isolate those bioactive compounds for different uses. In early studies, mushrooms are found to have different functions, such as antibacterial (Hatvani 2001; Rosa and others 2003), antifungal (Ngai and others 2005; Wang and others 2004), antiviral (Piraino and others 1999), antitumor (Wang and others 1996; Wang and others 1996), antiallergic (Ellertsen and others 2009; Yoshino and others 2008), anti-inflammatory (Hirota and others 2002; Kim and others 2004), antioxidant (Cheung and others 2003; Song and others 2003), and hypolipidemic activities (Bobek and others 1991; Yang and others 2002).

Clitocybe nuda (wood blewit) is an edible mushroom mostly found in Europe and North America. The color of the mushroom can range from lilac to purple. The percentage of the chemical components based on the dry matter of *C. nuda* has been reported to be: 59.4% crude protein, 1.8% lipids, 18.5% ash and 20.3% carbohydrates (Barros and others 2008). The mushroom is high in protein content on dry weight basis with very little lipid. Therefore, it can be a good dietary food source. However, there is a potential to cause allergies to sensitive people after eating the mushroom, especially when consuming the mushroom raw.

Chen and others recently isolated three bioactive compounds from the 95% ethanol extract of the secondary metabolites of *Clitocybe nuda*: 2-methoxy-5-methyl-6-methoxymethyl-p-benzoquinone, 6-hydroxy-2H-pyran-3-carbaldehyde, and indole-3-carbaldehyde (Chen and others 2012). They also reported the antifungal ability of *C. nuda* in preventing the

Phytophthora capsici, a kind of plant pathogen which can cause severe disease in the earlier stage, such as peppers (Chen and others 2009).

Chapter 3 Materials and methods

3.1 Preparation of the secondary metabolites from *Clitocybe nuda*

The secondary metabolites from *C. nuda* were prepared by following Chen's (Chen and others 2009) methods with modification. The *C. nuda* was grown on potato dextrose agar (PDA) (NEOGEN, Lansing, MI) at 25 °C. Round *C. nuda* culture blocks (d=5 mm) were prepared by cutting the mycelium grown PDA plates with a sterilized cork-stopper borer. Each culture block was transferred into a 500-mL flask with 250 mL potato dextrose broth (PDB) (HIMEDIA, India). The inoculated PDB was incubated at 25 °C for 21 days with shaking at 120 rpm. The cultured PDB was collected through filtration with 25 µm filter paper (VWR filter paper 415, VWR, Radnor, PA). The volume of the filtrate was reduced from 600 mL to 100 mL by heating on a hot plate (CORNING, PC-420) at about 82 °C, and then continued to dry completely in an oven at 95 °C (PRECISION, Model 16, Thermo Fisher Scientific Inc., Walham, MA).

One liter of 95% ethanol was added to the dried sample for further extraction. The mixture was kept at room temperature with constantly stirring for 24 h and filtered through a 25 µm filter paper followed by

filtering using a 0.45 µm filter paper. The filtrate was dried at 40 °C to remove ethanol. The dried extract was then re-suspended in 5 mL sterile water and the water solution was dried at 42 °C in a vacuum oven (FISHER SCIENTIFIC, Model 282A). The dried extract was dissolved in sterile water to make a stock solution at 200 mg/mL. The pH of the stock solution was adjusted to 7 with 10 M HCl/NaOH and stored at 4 °C.

3.2 Bacterial preparation

In this study, *Listeria monocytogenes* G3990, *Escherichia coli* O157:H7 ATCC43894, *Salmonella* Typhimurium ATCC13311 and *Staphylococcus aureus* ATCC12600 were used. *E. coli* O157:H7, *Salmonella* Typhimurium and *S. aureus* were cultured in Tryptic[®] soy broth (TSB) (BD, Sparks, MD) and incubated at 37 °C with shaking at 150 rpm for 20 h. *L. monocytogenes* were cultured in TSB with 0.5% yeast extract (AMRESCO, Solon, OH) and incubated at the same condition with other bacteria. The cultures were centrifuged at 4,500 rpm for 3 min and washed twice with Butterfield's phosphate buffer (BPB). Then, the bacterial solution was diluted to an absorbance of 0.4-0.8 at O.D.640nm. The bacterial population was estimated based on preconstructed equations. The populations were adjusted to the levels for experimental use.

3.3 Minimum inhibitory concentration (MIC) tests for the secondary metabolites of *C. nuda*

The dried extract was diluted to various concentrations and mixed with double strength culture medium at 1:1 ratio for the minimum inhibitory concentration tests against the four bacteria. The negative control was culture medium only. The pH of all samples was adjusted to 7.0 ± 0.2 . Treatments were triplicated. The inoculum of the bacteria was designated to 25 CFU/mL sample solution. All tested samples were incubated at 37 °C with shaking at 150 rpm for 24 h. After 24 h incubation, 1 mL culture was taken from the sample and washed twice with BPB after centrifugation at 4,500 rpm for 3 min. The washed cultures were properly made using a 10-fold serial dilution to plate out on Trypticase[®] soy agar (TSA) (BD, Sparks, MD) plates for *E. coli* O157:H7, *Salmonella* Typhimurium and *Staphylococcus aureus* and Trypticase[®] soy agar with yeast extract (TSA+YE) plates for *L. monocytogenes* and all plates were incubated at 37 °C for 24 h. The colonies were enumerated and the bacterial population was calculated. The minimum inhibitory concentration of 50% log reduction (MIC₅₀) was calculated by the equation from the linear regression of log bacterial populations vs. concentrations (mg/mL). All experiments were repeated.

3.4 The pH effects on the antibacterial activity of the secondary metabolites of *C. nuda*

The dried extract of secondary metabolites from *C. nuda* was dissolved in sterile water and concentrations were adjusted to the levels of 2X MIC₅₀ for each tested bacteria. The prepared extracts were mixed with

double strength culture media at 1:1 ratio to have growth media contained the extract at MIC₅₀ levels. The negative control groups were culture media only without extract. The pH of samples and controls were adjusted to 4, 5, 6, 7, 8 and 9 with 10 M HCl/NaOH. After samples were inoculated with bacteria at 25 CFU/mL, they were incubated at 37 °C with shaking at 150 rpm. The bacterial populations of tested samples were enumerated using spread-plating method at 24 h incubation for further analysis. All experiments were repeated.

3.5 Thermal stability of the antibacterial activity of the secondary metabolites of *C. nuda*

The extract solution of *C. nuda* was placed at 4, 25, 72, 100 and 121 °C for 30 min. Sterile water was used as a negative control. After heat treatment, the secondary metabolites samples were mixed with same volume of double strength culture media. The pH of the samples was adjusted to 7 and the samples were inoculated with the test bacteria at the concentration of 25 CFU/mL. All inoculated samples were incubated at 37 °C with shaking at 150 rpm. After 24 h incubation, the bacterial populations were enumerated. All experiments were repeated.

3.6 The antibacterial efficacy of the secondary metabolites of *C. nuda* on ground beef and liquid egg samples

The antibacterial efficacies of the secondary metabolites of *C. nuda* to *L. monocytogenes* and *E. coli* O157:H7 on ground beef and to

Salmonella Typhimurium in liquid eggs were investigated. In this study, the ground beef and liquid egg were purchased from a local grocery store. The background of *L. monocytogenes* and *E. coli* O157:H7 on ground beef and *Salmonella* Typhimurium in liquid egg were checked.

3.6.1 Ground beef

One hundred and fifty grams of ground beef were weighed into a sterile plastic bag, and *L. monocytogenes* or *E. coli* O157:H7 were added at 10^4 CFU/ g ground beef level. Inoculated bacteria and the ground beef were mixed thoroughly and then the sample was put into 6 sterile bags of 20 g each. The 6 samples were divided into two groups, 3 in each group. One group was used as a negative control. In the treated group, 1.5 g of the dried secondary metabolites of *C. nuda* were added into a bag. All samples were stored at 4 °C. The bacterial populations of the samples were analyzed by using the spread-plating method on modified Oxford agar (MOA) plates and tellurite cefixime-sorbitol MacConkey (TC-SMAC) agar plates on day 1, 3 and 5. All experiments were repeated.

3.6.2 Liquid egg

Aliquots of 6 mL liquid egg were added to 50 mL sterile centrifuge tubes. Nalidixic acid resistant *Salmonella* Typhimurium was inoculated into the tube at 10^4 CFU/ mL. The bacteria and liquid egg were mixed thoroughly. In the treated group, 300 mg dried secondary metabolites of *C. nuda* were added in a tube and mixed well. The control group consisted of

the inoculum and liquid egg only, and all samples were stored at 4 °C for 1, 3 and 5 days. The bacterial populations of the samples were determined by spread-plating on TSA+100 ppm nalidixic acid. All experiments were repeated.

3.7 Statistical analysis

All the assays were carried out in triplicate and the results were expressed as mean values and standard deviations (SD). The pH effects of the *C. nuda* were analyzed by using analysis of variance (ANOVA) of the Statistical Analysis System (Version 9.2 for Windows, SAS Institute, Inc., 2012) with a significance level of $P = 0.05$.

Chapter 4 Result and Discussion

4. 1 Minimum inhibitory concentration (MIC) of secondary metabolites from *Clitocybe nuda*

The secondary metabolites from *C. nuda* showed different antibacterial activities against *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Escherichia coli* O157:H7. Various concentrations of the secondary metabolites from 0-50 mg/mL were tested for MIC test against *L. monocytogenes* and *E. coli* O157:H7. The MIC 50% log reduction was calculated using the linear regression of log bacterial number versus the concentration of the secondary metabolites after 24 h incubation. The MIC 50% log reduction for *L. monocytogenes* was 27 mg/mL (Table 1). For *E. coli* O157:H7, the inhibitory effects from 0-30 mg/mL were very low, but bacterial growth was completely inhibited at 50 mg/mL. The MIC 50% log reduction of *E. coli* O157:H7 was calculated from the inhibitory effects of 30 to 50 mg/mL, and the MIC₅₀ was 40 mg/mL (Table 2). The concentrations of the mushroom secondary metabolites from 0-30 mg/mL were used for MIC₅₀ test against *Salmonella* Typhimurium. Since the inhibitory effects from 0-15 mg/mL

were similar and minimal, however, and the bacterial growth was completely inhibited at 30 mg/mL, the MIC 50% was calculated from the inhibitory effects of 15-30 mg/mL. The MIC 50% log reduction of *Salmonella* Typhimurium was 25 mg/mL (Table 3). The inhibitory effects of the secondary metabolites against *S. aureus* were similar to those of *Salmonella* Typhimurium. The MIC 50% log reduction of *S. aureus* was 23 mg/mL (Table 4).

The results indicate that the secondary metabolites from *C. nuda* has better inhibitory effects against *Salmonella* Typhimurium and *S. aureus* than it does for *L. monocytogenes* and *E. coli* O157:H7. The secondary metabolites from *C. nuda* have been shown to have non-selective inhibitory effects against both Gram-positive and Gram-negative bacteria.

Table 1-Antibacterial activity on *L. monocytogenes* of the secondary metabolites of *C. nuda* at various concentrations.

Concentration (mg/mL)	Bacterial population (log CFU/mL)
0	9.711±0.037
5	9.313±0.071
10	9.135±0.088
15	8.250±0.167
20	7.178±0.242
25	5.475±0.111
30	3.579±0.274
40	1.709±1.480
50	0.000±0.000

*Linear regression was calculated from 0 to 50 mg/mL: $y = -0.214x + 10.670$, $R^2 = 0.968$; $MIC_{50} = 27.19$ mg/mL, $n=3$, $x =$ concentration, $y =$ log CFU/mL

Table 2-Antibacterial activity on *E. coli* O157:H7 of the secondary metabolites of *C. nuda* at various concentrations.

Concentration (mg/mL)	Bacterial population (log CFU/mL)
0	9.445±0.040
10	9.296±0.060
20	9.249±0.011
30	9.191±0.027
40	4.483±0.877
50	0.000±0.000

*Linear regression was calculated from 30 to 50 mg/mL: $y = -0.460x + 22.940$, $R^2 = 0.9998$; MIC₅₀=39.88 mg/mL, n=3, x= concentration, y= log CFU/mL

Table 3-Antibacterial activity on *Salmonella* Typhimurium of the secondary metabolites of *C. nuda* at various concentrations.

Concentration (mg/mL)	Bacterial population (log CFU/mL)
0	9.831±0.113
10	9.205±0.058
15	9.048±0.032
20	8.831±0.073
25	5.423±0.025
30	0.000±0.000

*Linear regression was calculated from 15 to 30 mg/mL: $y = -0.611x + 19.574$, $R^2 = 0.872$; MIC₅₀=24.63 mg/mL, n=3, x= concentration, y= log CFU/mL

Table 4-Antibacterial activity on *S. aureus* of the secondary metabolites of *C. nuda* at various concentrations.

Concentration (mg/mL)	Bacterial population (log CFU/mL)
0	8.887±0.041
5	8.880±0.106
10	8.758±0.027
15	8.708±0.100
20	5.841±0.758
30	0.000±0.000

*Linear regression was calculated from 15 to 30 mg/mL: $y = -0.581x + 17.439$, $R^2 = 1$; $MIC_{50} = 22.52$ mg/mL, $n=3$, $x =$ concentration, $y =$ log CFU/mL

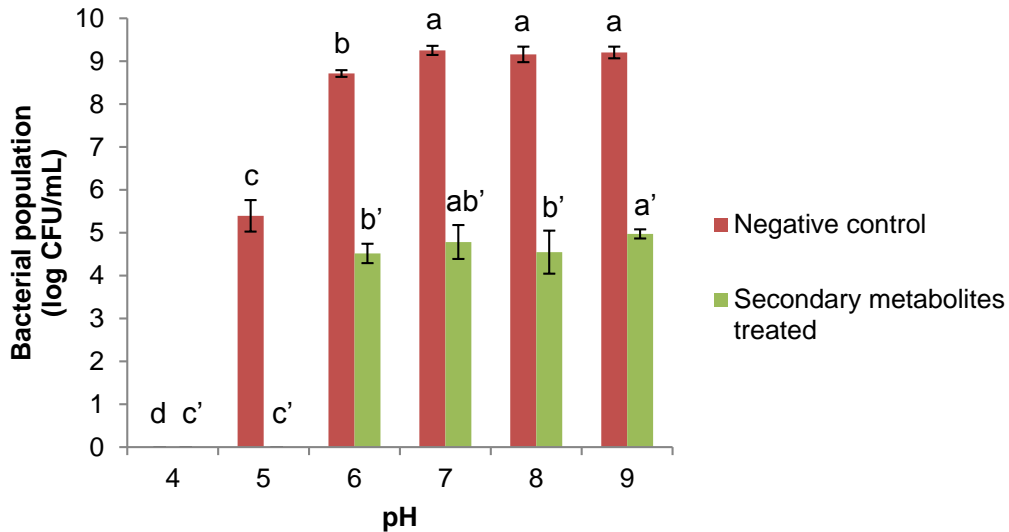
4.2 Effects of pH on the antibacterial activities of the secondary metabolites from *C. nuda*

The secondary metabolites from *C. nuda* showed different antibacterial activities at different pH levels. At all pH values evaluated, the concentrations of the secondary metabolites of *C. nuda* were determined at MIC as previously described. The secondary metabolites were replaced by sterile water in the negative control groups. In general, the optimal pH for the growth of the four bacterial pathogens was around 7. At pH 4, all four pathogens could not grow in either the negative controls or in sample solutions. At other pH levels, the bacterial populations in all sample solutions were lower than those in negative controls. The results indicate that the secondary metabolites from *C. nuda* exhibit antibacterial activity under a wide range of pH levels.

4.2.1 *Listeria monocytogenes*

L. monocytogenes grew similarly in control samples at pH 7-9. Bacterial growth was inhibited and the populations were significantly lower at pH 5-6 than at pH 7-9. The growth of *L. monocytogenes* was completely inhibited at pH 4 and 5 with the presence of the secondary metabolites of *C. nuda*. From pH 6 to 9, the bacterial populations with the secondary metabolite samples were similar to each other and they were all

significantly lower than those in the negative control groups (Figure 1). The results also indicate that the anti-*L. monocytogenes* activity of the secondary metabolites from *C. nuda* could be enhanced in acidic environments.



*Inoculum: 23 CFU/ mL sample

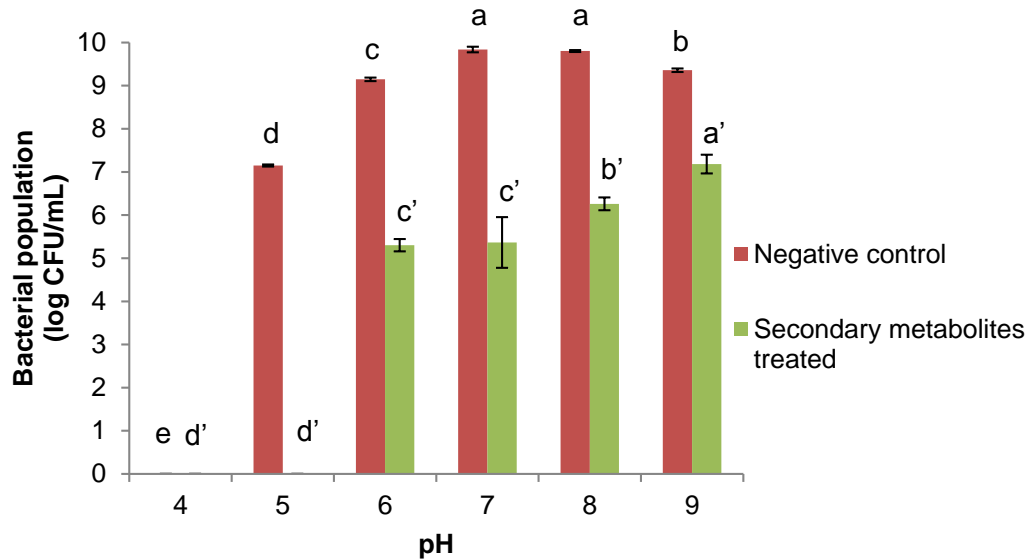
*Concentration of treated sample: 26 mg/mL

Figure 1-pH effect on antibacterial activity of secondary metabolites from *C. nuda* against *L. monocytogenes*.

4.2.2 *Salmonella* Typhimurium

In the controls, *Salmonella* Typhimurium populations were the highest in pH levels of 7 and 8, and then at pH 9, 6, and 5 in that order. In the samples with the secondary metabolites from *C. nuda*, the growth of *Salmonella* Typhimurium was inhibited and their bacterial populations were lower than those of the controls at various tested pH levels (Figure 2). Bacteria growth was completely inhibited in the samples at pH 5. In

treated samples bacteria grew significantly better in alkaline conditions than those in neutral and acidic environments. Thus, the anti-*Salmonella* Typhimurium activity of the secondary metabolites from *C. nuda* was enhanced by acid and weakened by alkaline.



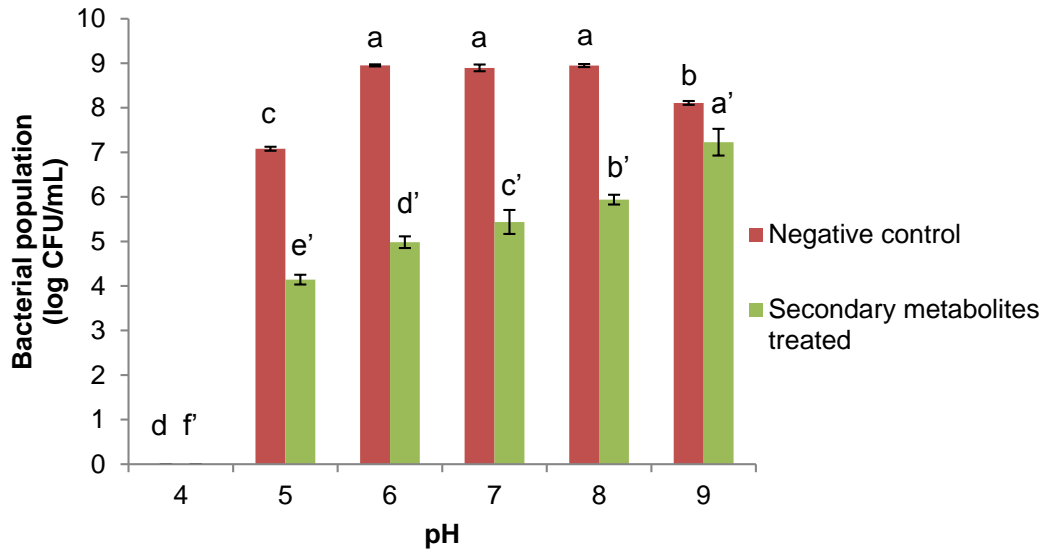
*Inoculum: 26 CFU/mL sample
 *Concentration of treated samples: 25 mg/mL

Figure 2-pH effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *Salmonella* Typhimurium.

4.2.3 *Staphylococcus aureus*

For *S. aureus*, the peak bacterial growth in the controls was at pH 6-8. *S. aureus* was able to grow in the secondary metabolites of *C. nuda* over the pH range of 5-9, and the growth was completely inhibited at pH 4. However, bacterial growth was significantly lower compared to the negative controls (Figure 3). Unlike the other three bacteria, the growth of *S. aureus* was not completely inhibited by the secondary metabolites of *C.*

nuda at pH 5. In the treated sample, the bacterial population increased significantly as the pH went up from 5 to 9. This indicated that the anti-*S. aureus* activity of the secondary metabolites from *C. nuda* was better at lower pH conditions.



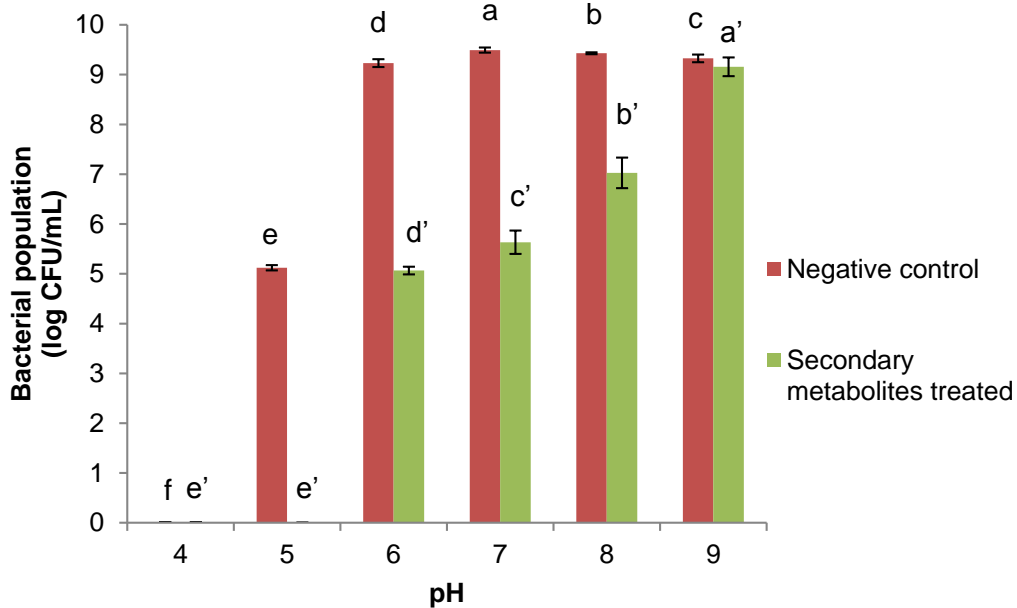
*Inoculum: 15 CFU/mL sample
 *Concentration of tested sample: 25 mg/mL

Figure 3-pH effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *S. aureus*.

4.2.4 *Escherichia coli* O157:H7

In the control groups, the growth of *E. coli* O157:H7 was the best at pH 7 and was completely inhibited at pH 4. For the secondary metabolite treated samples, the bacterial growth was completely inhibited at pH 5. The bacterial populations increased dramatically from pH 6 to 9, but were all lower than those in the negative controls at corresponding pH values (Figure 4). The trends of anti-*E. coli* O157:H7 activity at various pH levels

were similar to that for anti-*S. aureus* which were as the pH levels decreased so did the reduction of the bacteria.



*Inoculum: 24 CFU/mL sample

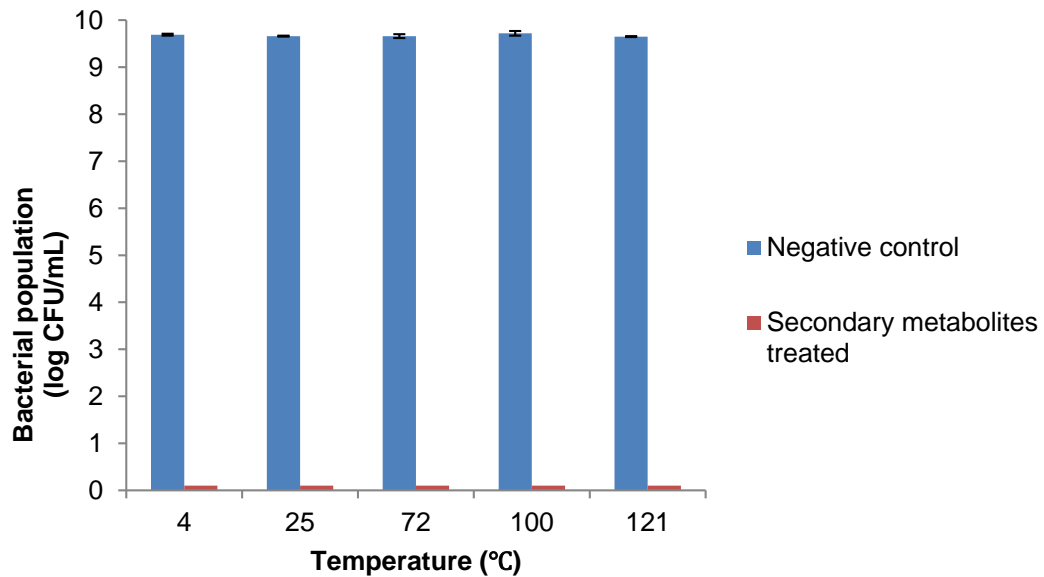
*Concentration of tested sample: 40 mg/mL

Figure 4-pH effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *E. coli* O157:H7.

4.3 Thermal stability of the secondary metabolites from *C. nuda*

The secondary metabolites from *C. nuda* were treated at 4, 25, 72, 100 and 121 °C for 30 min before treatment with the various bacteria. The concentrations of the secondary metabolites from *C. nuda* were 60, 40, 50 and 60 mg/mL for *L. monocytogenes*, *Salmonella* Typhimurium, *S. aureus* and *E. coli* respectively. These temperatures chosen were commonly used in the food industry for processing food and food products, such as refrigeration, pasteurization, cooking and retort heat treatment. Sterile

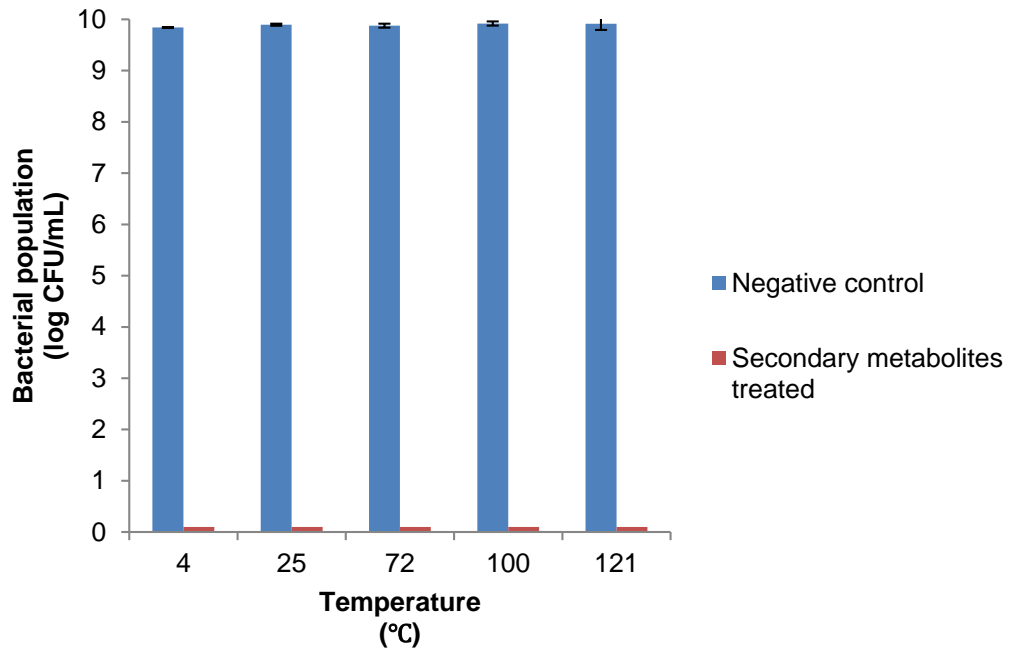
water was treated at the same conditions and used in negative controls. The results showed that all four bacteria grew well in the control samples and the populations reached to around 9.0-9.5 log CFU/mL. The growth of the four bacteria was completely inhibited in all treated samples with the secondary metabolites from *C. nuda* at various temperatures (Figure 5, 6, 7, 8). This indicates that the secondary metabolites from *C. nuda* are thermally stable.



*Inoculum: 23 CFU/mL sample

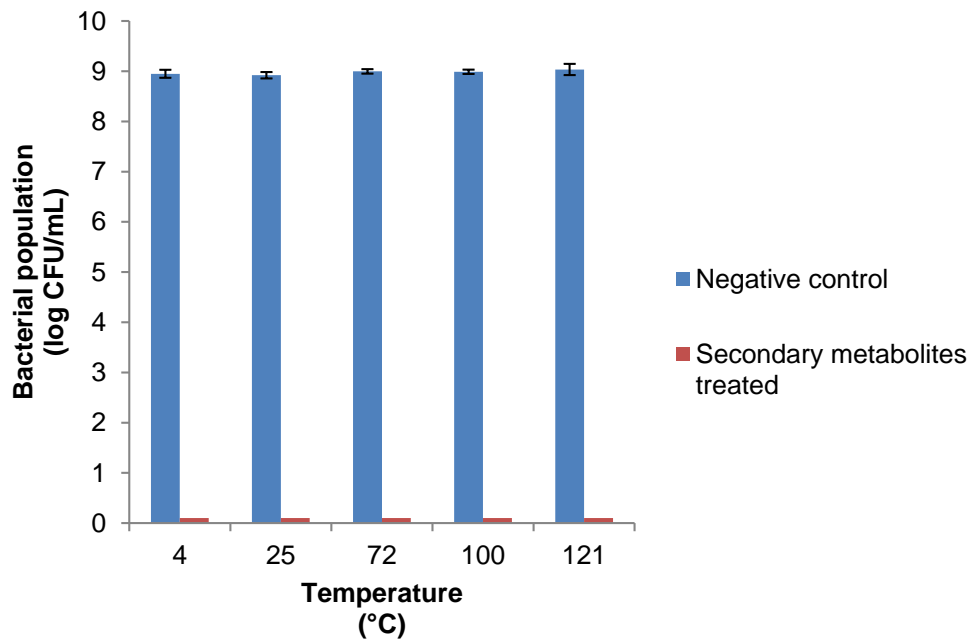
*Concentration of treated sample: 60 mg/mL

Figure 5 –Temperature effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *L. monocytogenes*.



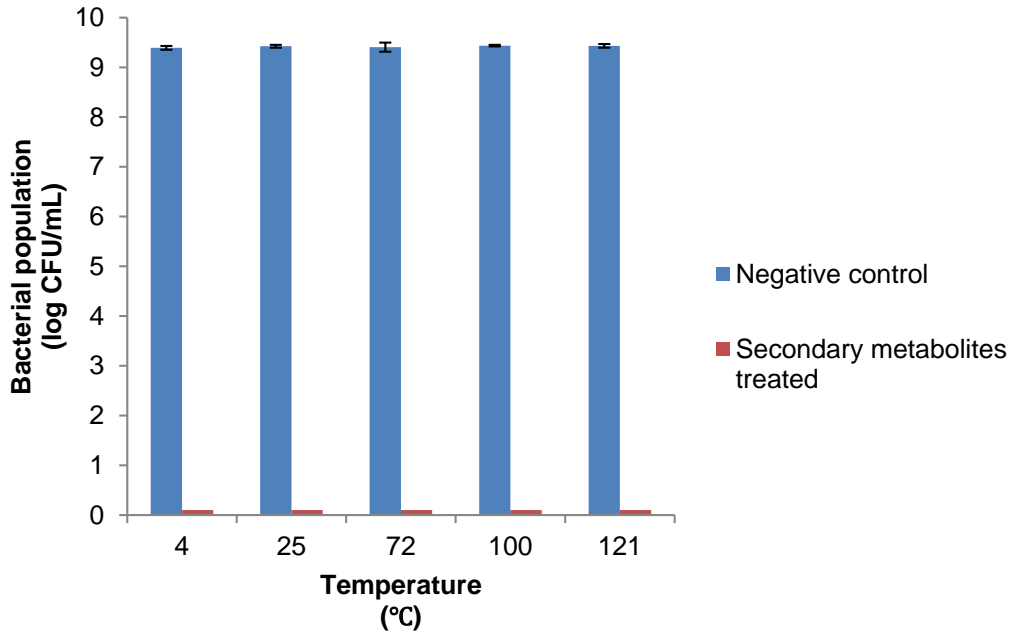
*Inoculum: 24 CFU/mL sample
 *Concentration of treated sample: 40 mg/mL

Figure 6-Temperature effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *Salmonella* Typhimurium.



*Inoculum: 12 CFU/mL sample
 *Concentration of tested sample: 50 mg/mL

Figure 7-Temperature effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *S. aureus*.



*Inoculum: 30 CFU/mL sample

*Concentration of tested sample: 60 mg/mL

Figure 8-Temperature effect on the antibacterial activity of the secondary metabolites of *C. nuda* on *E. coli* O157:H7.

4.4 Antibacterial effects of secondary metabolites from *C. nuda* on food samples

Approximately 10^4 CFU/g *L. monocytogenes* and *E. coli* O157:H7 were inoculated into the ground beef, respectively. Liquid egg was inoculated with *Salmonella* Typhimurium at the same level. Antimicrobial efficiencies of the secondary metabolites from *C. nuda* were analyzed on Day 0, 1, 3 and 5.

In the negative control group, the population of *L. monocytogenes* increased slightly during the test period, which was from 4.292 to 4.537 log CFU/mL. The bacterial populations in the secondary metabolite treated samples were kept at about the same level and were lower than those in the control groups (Figure 9). The secondary metabolites from *C. nuda* inhibited the growth of *L. monocytogenes* in ground beef and resulted in about a 0.2 log CFU/mL reduction. However, the inhibitory efficiency of the secondary metabolites from *C. nuda* against *L. monocytogenes* in ground beef was low.

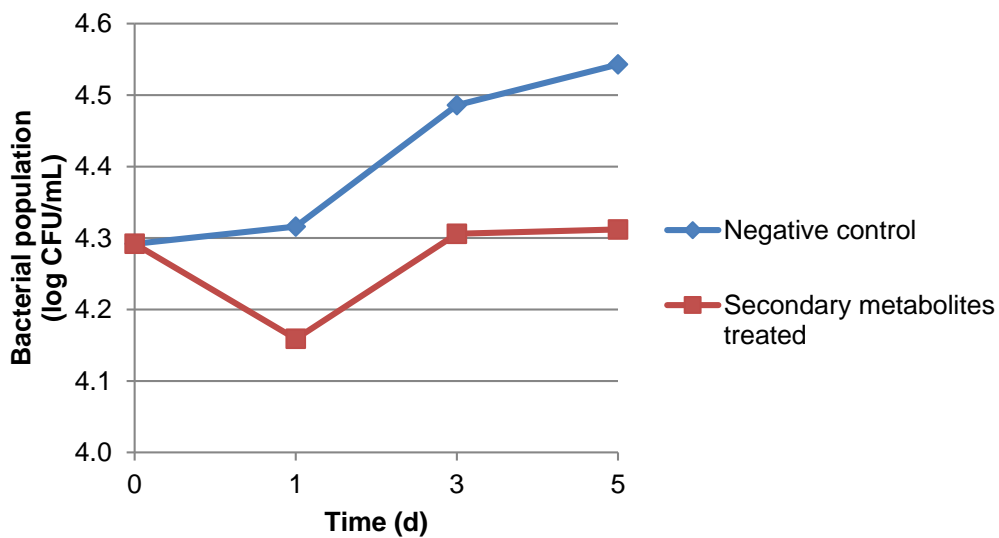


Figure 9- Anti-*L. monocytogenes* activity of the secondary metabolites from *C. nuda* in ground beef.

The bacterial populations of *E. coli* O157:H7 increased from 4.004 to 4.434 log CFU/mL from day 0 to day 5 in the negative control group. During the first day, the bacteria grew slightly lower than from day 1 to day 5. The bacterial population of the treated sample was reduced from 4.004

to 3.619 log CFU/mL after one day treatment. Then the bacterial population increased steadily from day 1 to day 5, reaching 4.075 log CFU/mL (Figure 10). The inhibitory efficiency of the secondary metabolites against *E. coli* O157:H7 in ground beef was about 0.4 log CFU/mL reduction, but was not as effective as in the culture medium.

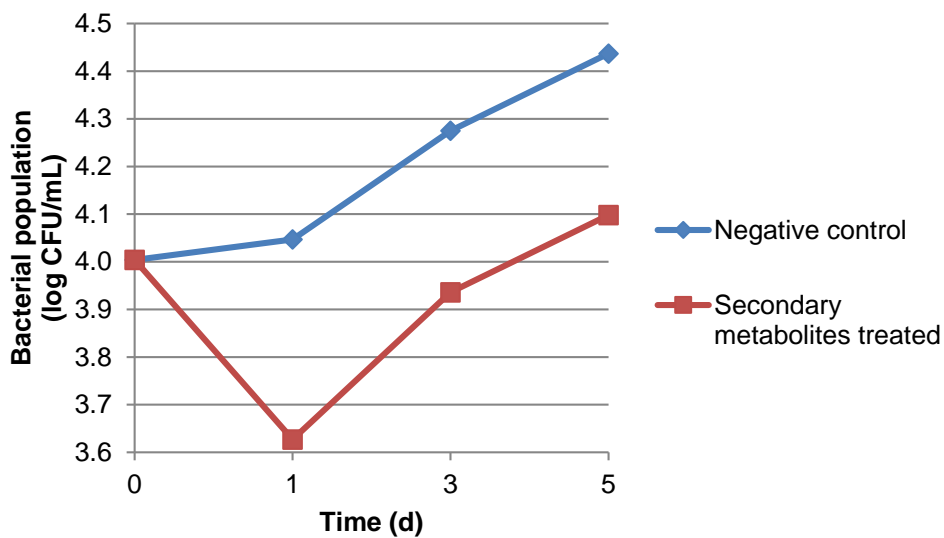


Figure 10-Anti-*E.coli* O157:H7 activity of the secondary metabolites from *C. nuda* in ground beef.

In liquid egg, the *Salmonella* Typhimurium population dropped in both the treated samples and the negative control groups over the 5-day test period. The drop in the negative control samples from 3.9 to 3.7 log CFU/mL might be caused by the presence of the lysozyme in eggs. The *Salmonella* Typhimurium count decreased in the treated samples from 3.9 to 3.0 log CFU/mL which might be caused by both the secondary metabolites and lysozyme (Figure 11). The secondary metabolites of *C.*

nuda against *Salmonella* Typhimurium can cause 0.7 log CFU/mL reduction.

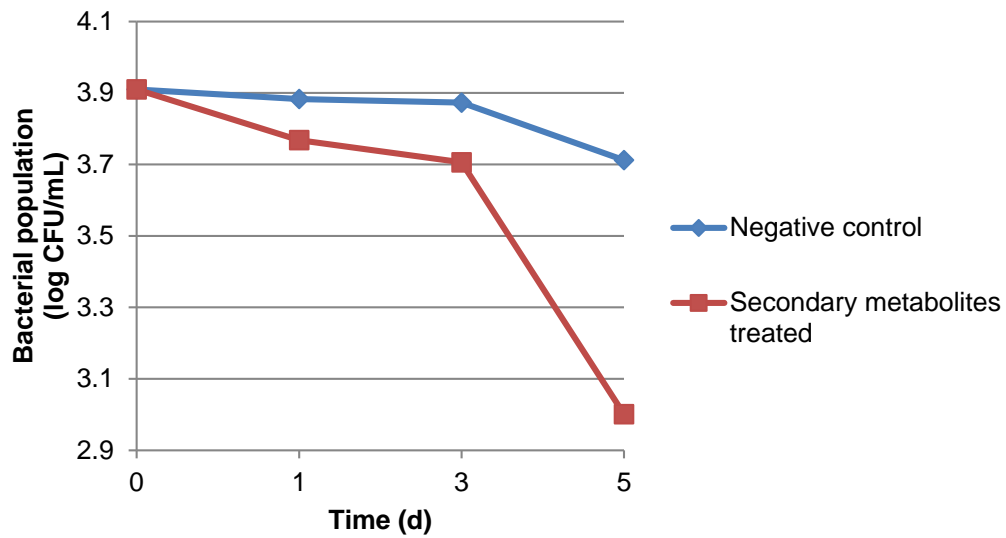


Figure 11-Anti-*Salmonella* Typhimurium activity of the secondary metabolites from *C. nuda* in liquid eggs.

Chapter 5 Conclusion

In general, the secondary metabolites from *Clitocybe nuda* showed inhibitory effects against the four tested foodborne pathogens---*Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Escherichia coli* O157:H7.

The secondary metabolites from *C. nuda* showed better inhibitory effects against *Salmonella* Typhimurium and *S. aureus* than *L. monocytogenes* and *E. coli* O157:H7. The MIC 50% log reductions were 27, 40, 25, 23 mg/mL for *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* Typhimurium and *S. aureus*, respectively.

The pH effect tests, results showed that the secondary metabolites of *C. nuda* had better antibacterial activity in acidic environments. At pH 4-5, the growth of *L. monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157:H7 were completely inhibited. However, when pH increased up to 8 and 9, the antibacterial activities decreased, except to *L. monocytogenes*. The results indicated that acid and the secondary metabolites of *C. nuda* were able to synergistically inhibit the bacterial growth.

The secondary metabolites of *C. nuda* were stable at all tested temperatures of 4, 25, 72, 100 and 121 °C at pH 7. These results indicated that there is the potential for applying the secondary metabolites of *C. nuda* in thermal and non-thermal food processing without losing their antibacterial activities.

In ground beef samples, the addition of the secondary metabolites of *C. nuda* at 75 mg/mL showed limited inhibitory efficiencies against *L. monocytogenes* and *E. coli* O157:H7 after 5 days of treatment. The complexity of ground beef matrix may provide bacteria a better environment from contacting with the secondary metabolites. The further investigation of applying the secondary metabolites to ground beef for controlling foodborne pathogens is needed.

The secondary metabolites of *C. nuda* can inhibit the growth of *Salmonella* Typhimurium in liquid eggs and resulted in a 1 log CFU/mL reduction at 50 mg/mL concentration. The lysozyme in eggs appears to have a synergistic antibacterial activity with the metabolites. However, the secondary metabolites of *C. nuda* may cause the changes in the appearance and flavor of the liquid eggs. Therefore, the sensory evaluations of adding the secondary metabolites of *C. nuda* to liquid eggs will be necessary.

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