



**UNIVERSITI PUTRA MALAYSIA**

**ANTIBIOTIC SENSITIVITY OF SELECTED BACTERIA AND  
MICROBIOLOGICAL QUALITY OF *LACTOBACILLUS*-FED BROILER  
MEAT IN COMPARISON WITH COMMERCIAL BROILER MEAT**

**STEPHANIE TAN YIN YI**

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FPV 2016 26**

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**UNIVERSITI PUTRA MALAYSIA**

**SERDANG SELANGOR**

**2016**

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BROILER MEAT**

**BY**

**STEPHANIE TAN YIN YI**

**A project paper submitted to the  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
In partial fulfilment of the requirement for  
THE COURSE VPD 4999 – FINAL YEAR PROJECT  
Universiti Putra Malaysia  
Serdang, Selangor Darul Ehsan.**

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## CERTIFICATION

It is hereby certified that we have read the project paper entitled “Antibiotic Sensitivity of Selected Bacteria and Microbiological Quality of *Lactobacillus*-Fed Broiler Meat In Comparison With Commercial Broiler Meat” by Stephanie Tan Yin Yi and in our opinion, it is satisfactory in terms of scope, quality and presentation as a partial fulfillment of the requirements for the course VPD 4999 – Final Year Project.

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## **ABSTRAK**

Abstrak dari kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek Ilmiah Tahun Akhir.

### **KEPEKAAN BAKTERIA TERTENTU TERHADAP ANTIBIOTIK DAN KUALITI MIKROBIOLOGI DAGING AYAM PEDAGING *LACTOBACILLUS* BERBANDING DENGAN DAGING AYAM PEDAGING KOMERSIAL**

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Penggunaan probiotik sebagai pengganti antibiotik dalam makanan ternakan ayam telah diperkenalkan sejak beberapa tahun kebelakangan ini. Penggunaan probiotik dipercayai memberikan kesan peningkatan kesihatan yang sama tanpa menyebabkan perkembangan kerintangan antibiotik, terutamanya antibiotik penting. Pembekalan probiotik juga dipercayai menghasilkan daging yang lebih baik dari segi kualiti mikrobiologi. Projek ini adalah untuk mengkaji Standard Plate Count (SPC), Coliform Plate Count (CPC) dan kepekaan antibiotik bakteria tertentu dalam ayam

pedaging *Lactobacillus* untuk dibandingkan dengan ayam pedaging komersial. Dua puluh ayam pedaging *Lactobacillus* dan dua puluh lima ayam pedaging komersial telah diperoleh daripada tujuh pasar raya yang terletak di Selangor dan Kuala Lumpur. Pemencilan bakteria *Salmonella sp.*, *Escherichia coli* dan *Staphylococcus aureus* telah dilakukan pada sampel daging ayam. Empat (20%) sampel daging ayam pedaging *Lactobacillus* didapati positif mengandungi *Salmonella sp.*; 11 (55%) sampel didapati positif mengandungi *E. coli*, dan hanya satu (5%) sampel didapati positif mengandungi *Staphylococcus aureus*. Untuk daging ayam pedaging komersial, satu (4%) sampel didapati mengandungi *Salmonella sp.*, 10 (40%) sampel mengandungi *E. coli*, dan satu (4%) sampel mengandungi *Staphylococcus aureus*. Perbezaan dalam prevalen ketiga-tiga bakteria tersebut antara dua jenis daging adalah tidak ketara. Ujian kepekaan antibiotik telah dijalankan terhadap semua isolat bakteria. Untuk *Salmonella sp.*, semua isolat daripada kedua-dua jenis daging menunjuk kerintangan terhadap ceftriaxone dan oxytetracycline. *E. coli* daripada kedua-dua jenis daging menunjuk kerintangan terhadap ceftriaxone, ampicillin, streptomycin dan oxytetracycline manakala kerintangan terhadap ciprofloxacin hanya terdapat dalam satu isolat daripada daging ayam pedaging komersial. Isolat *Staphylococcus aureus* daripada daging ayam pedaging komersial menunjuk kerintangan terhadap oxytetracycline manakala isolat daripada daging ayam pedaging *Lactobacillus* adalah sensitif kepada semua antibiotik. Perbezaan dalam kepekaan antibiotik ketiga-tiga bakteria antara dua jenis daging ayam pedaging adalah tidak ketara, kecuali kepekaan ampicillin dalam *E. coli* yang didapati lebih rendah dalam daging ayam pedaging komersial, berbanding dengan isolat daripada daging ayam pedaging *Lactobacillus*.

Min SPC adalah  $17 \times 10^4$  dan  $44 \times 10^4$  cfu per gram dalam daging ayam pedaging *Lactobacillus* dan daging ayam pedaging komersial. Min CPC adalah  $23 \times 10^3$  dan  $30 \times 10^3$  cfu per gram dalam daging ayam pedaging *Lactobacillus* dan daging ayam pedaging komersial. Berbanding dengan daging ayam pedaging komersial, SPC daging ayam pedaging *Lactobacillus* adalah lebih rendah secara signifikan ( $P < 0.05$ ). Walau bagaimanapun, perbezaan dalam CPC antara dua jenis daging adalah tidak berbeza secara signifikan ( $P > 0.05$ ). Projek ini mencadangkan bahawa kualiti mikrobiologi daging ayam pedaging *Lactobacillus* dan daging ayam pedaging komersial adalah setanding.

Kata kunci: *Lactobacillus*, ayam pedaging, *Salmonella sp.*, *Escherichia coli*, *Staphylococcus aureus*, kepekaan antibiotik, kualiti mikrobiologi

## ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

### ANTIBIOTIC SENSITIVITY OF SELECTED BACTERIA AND MICROBIOLOGICAL QUALITY OF *LACTOBACILLUS*-FED BROILER MEAT IN COMPARISON WITH COMMERCIAL BROILER MEAT

By

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2016

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The use of probiotics to replace antibiotics in poultry feed had been introduced in recent years. It is believed that probiotics may give the same overall health effect to livestock, without the undesirable development of antibiotic resistance to vital antibiotics. It is also believed that chickens fed with probiotics may have better overall microbiological quality. This study was performed to evaluate the Standard Plate Count (SPC), Coliform Plate Count (CPC) and antibiotic sensitivity of selected bacteria of *Lactobacillus*-fed broiler meat, and to compare them with those of commercial broiler meat. Twenty *Lactobacillus*-fed broiler meat and twenty-five commercial broiler meat were purchased from seven retail outlets within Selangor and

Kuala Lumpur. Bacterial isolation of *Salmonella sp.*, *Escherichia coli* and *Staphylococcus aureus* were performed on the meat samples. Four (20%) of the *Lactobacillus*-fed broiler meat samples were found to be positive for *Salmonella sp.*; 11 (55%) samples were positive for *E. coli*, and only one sample (5%) was positive for *Staphylococcus aureus*. For commercial broiler meat, one sample (4%) was positive for *Salmonella sp.*; 10 samples (40%) were positive for *E. coli*, and one sample (4%) was positive for *Staphylococcus aureus*. The difference in the prevalence of all three bacteria between the commercial and *Lactobacillus*-fed chickens was not significantly different. All isolates were subjected to antibiotic sensitivity test. For *Salmonella sp.*, all isolates from both types of meat were found to be resistant to ceftriaxone and oxytetracycline. *E. coli* from both types of chicken were resistant to ceftriaxone, ampicillin, streptomycin and oxytetracycline, while resistance towards ciprofloxacin was observed in one isolate from commercial broiler meat. *Staphylococcus aureus* isolate from commercial broiler meat was resistant to oxytetracycline, while the isolate from *Lactobacillus*-fed broiler meat was susceptible to all antibiotics. The antibiotic sensitivity of *Salmonella sp.*, *E. coli* and *Staphylococcus aureus* isolates between the two types of broiler meat were similar, with the exception to ampicillin sensitivity of *E. coli* that was significantly lower from commercial broiler meat, as compared to isolates from *Lactobacillus*-fed broiler meat. The mean SPC in *Lactobacillus*-fed broiler meat and commercial broiler meat were  $17 \times 10^4$  and  $44 \times 10^4$  cfu per gram of chicken meat respectively. The mean CPC were  $23 \times 10^3$  and  $30 \times 10^3$  cfu per gram of meat in *Lactobacillus*-fed broiler meat and commercial broiler meat respectively. SPC of *Lactobacillus*-fed broiler meat was

significantly lower than that of commercial broiler meat ( $P < 0.05$ ). However, the difference in CPC between the two types of broiler meat was not significant ( $P > 0.05$ ). This study suggests little difference in the microbiological quality of commercial and more expensive *Lactobacillus*-fed broiler meat.

Keywords: *Lactobacillus*, broiler meat, *Salmonella sp.*, *Escherichia coli*, *Staphylococcus aureus*, antibiotic sensitivity, microbiological quality

## CHAPTER 1

### INTRODUCTION

#### 1.1 Introduction

Broiler meat is a popular source of protein for all ethnic groups in Malaysia. According to USDA GAIN: Malaysia Broiler Meat Sector 2014 report, per capita consumption for broiler meat in Malaysia would reach over 40 kilograms per year and is among the highest in the world. Therefore, poultry production is considered a vital part of Malaysia's livestock production industry.

In poultry production, bacterial diseases can significantly affect its productivity. Therefore, large amounts of antimicrobial agents are being used in poultry production for therapeutic and prophylaxis purposes (Akbar *et al.*, 2014). This practice had led to the emergence and development of antibiotic-resistant bacteria and presence of antibiotic residues in meat. This issue should not be taken lightly, as resistant bacteria can be carried by food animal and transmitted to humans through food consumption, direct contact, and environmental spread (Aidara-Kane, 2012).

In recent years, public awareness of antibiotic resistance had increased. Therefore, a trend to reduce the use of antibiotics in the livestock industry is now seen worldwide. It has also led to the development of interest towards the use of probiotics to substitute antibiotics in feed. In recent studies, it had been reported that

chickens fed with probiotic produced better carcass microbiological quality (Fritts *et al.*, 2000; Khaksefidi & Rahimi, 2005).

Being a product recently introduced commercially in Malaysia, there is a lack of study to ascertain the microbiological quality of *Lactobacillus*-fed broiler meat. Differences in microbiological quality between *Lactobacillus*-fed broiler meat to the commercial broiler meat should be investigated, especially when the market price of such broiler meat is significantly more expensive. Since *Salmonella sp.*, *Escherichia coli* and *Staphylococcus aureus* are among pathogenic bacteria that can cause food poisoning in human, high prevalence of such bacteria, especially antibiotic resistant strains in poultry meat is of public health concern. Therefore, in this study, the microbiological quality of *Lactobacillus*-fed broiler meat, in terms of the Standard Plate Count (SPC), Coliform Plate Count (CPC), prevalence of selected bacteria and their antibiotic sensitivity, was assessed in comparison with those of commercial broiler meat.

## 1.2 Objectives

The objectives of this project are:

- i) To determine the SPC and CPC per gram of meat from *Lactobacillus*-fed poultry meat and to compare them with commercial broiler meat.
- ii) To evaluate the antibiotic sensitivity of selected bacteria isolated from *Lactobacillus*-fed poultry meat and to compare them with those isolated from commercial broiler meat.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Probiotics

Probiotics is defined by Food and Agricultural Organization of the United Nations as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO & WHO, 2001). Probiotics can be grouped into three main categories of organisms, which are lactic acid bacteria, spore-forming *Bacillus spp.*, and yeast (Ahmed *et al.*, 2013). Examples of lactic acid bacteria are *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, with *Lactobacillus* and *Streptococcus* being the most common organisms used for the production of probiotics (Jadhav *et al.*, 2015). Members of genus *Lactobacillus* are non-pathogenic, Gram-positive bacteria that possess the ability to colonize the gastrointestinal tracts of human and animals. They are suitable for the development of probiotics as they benefit the host by improving properties of the normal flora of the gut (Mahroop Raja *et al.*, 2009).

The supplementation of probiotics is believed to result in prevention of colonization of intestinal pathogens, such as *Escherichia coli* and *Salmonella sp.*. Inhibition of gut colonization by probiotics occurs through several mechanisms, which include production of inhibitory substances, blocking of adhesion sites, competition for nutrients, degradation of toxin receptors and stimulation of host immunity (Rolfe, 2000).

The use of probiotics in poultry production had been increasing in recent years. For instance, top producer, Tyson Food Inc, which supplies to well-known fast-food chains in America has initiated the incorporation of probiotics into poultry feed. Plus, there had been recent studies which reported the benefits of probiotics-fed poultry meat. A study by Fritts *et al.*, 2000, which involved the evaluation of effects of *Bacillus subtilis* on live performance and microbiological status of broiler chickens, reported that it resulted in an increase in 42-day body weight, improvement in feed conversion, as well as reductions in SPC, number of coliforms and prevalence of *Campylobacter* in processed carcasses.

## 2.2 Antibiotics

Antibiotics was first defined by Nobel Prize Winner, a microbiologist named S.A. Waksman, as “chemical substances that are produced by microorganisms and that have the capacity, in dilute solution, to selectively inhibit the growth of and even to destroy other microorganisms”. Antibiotics are classified into groups based on several criteria. For instance, they can be grouped according to their range of susceptible bacterial groups, where they can have a broad, intermediate or a narrow spectrum of activity. They can also be classified to bactericidal agents, which are capable of killing the target bacteria, or bacteriostatic agents, which can only inhibit bacterial growth. Besides that, antibiotics can be classified into various classes based on their chemical structure. Classification of the antibiotics used in this project based on their chemical classes and type of activity is expressed in Table 1.

**Table 1: Classification of Antibiotics Used in this Project**

<b>Class</b>	<b>Active ingredient</b>	<b>Type of Activity</b>
<b>Aminoglycosides</b>	Streptomycin	Bactericidal
	Gentamicin	
<b>Cephalosporin</b>	3 <sup>rd</sup> Generation	Bactericidal
	Cephalosporin – Ceftriaxone	
<b>Glycopeptide</b>	Vancomycin	Bactericidal
<b>Lincosamide</b>	Clindamycin	Bactericidal
<b>Macrolides</b>	Erythromycin	Bacteriostatic
<b>Penicillin</b>	Ampicillin	Bactericidal
<b>Quinolones</b>	Ciprofloxacin	Bactericidal
<b>Sulfonamides + diaminopyrimidines</b>	Trimethoprim- sulfamethaxazole	Bactericidal
<b>Tetracycline</b>	Oxytetracycline	Bacteriostatic

### 2.3 Antibiotic Resistance

According to Guardabassi & Courvalin, 2006, the definition of resistance is when a strain under in vitro conditions is able to grow in the presence of higher concentration of drug, while under in vivo or clinical conditions, a resistant strain can resist and survive through antimicrobial therapy (Guardabassi & Courvalin, 2006). The development of antibiotic resistance is a bacteria's evolutionary adaptation to antibiotic selective pressure (Acar & Moulin, 2012). The development of antibiotic

resistance involves various biochemical mechanisms, which can be classified to six categories: active drug efflux, which pumps the drug out of the cell; reduction of cell permeability, where antibiotic is prevented from entering the cell; enzymatic inactivation of antibiotics; alteration of target of antibiotics; duplication of drug target with a resistant form; and target amplification due to regulatory mutations (Rowe-Magnus & Mazel, 2006).

The most common mechanism of acquired resistance is through horizontal gene transfer, which can occur when resistant bacteria is in contact with a susceptible one (Acar & Moulin, 2012). There are three principle modes for horizontal gene transfer: transformation, where free DNA molecule is picked up from the environment; transduction, where a transfer is initiated by phages, and finally, conjugation, where DNA is transferred between cells in the form of a plasmid or a sex pillus (Madden, 2009). Conjugation is the most important mechanism for the spread of antibiotic resistance between bacteria, as it allows transferred genes to be incorporated into the chromosome (Madden, 2009).

The frequent use of antibiotics for therapeutic purposes in livestock production and human medicine are contributing to the development of resistant strains of bacteria (Akbar *et al.*, 2014). Neela *et al.*, 2013 stated that feed in Malaysia poultry farms tend to contain antibiotics, like chloramphenicol, erythromycin, sulfonamide, penicillin, lincomycin, spectinomycin, oxytetracycline, endofloxacin and tylosin. Recent studies have also proved the existence of environmental reservoir of resistance genes (Acar & Moulin, 2012).

## 2.4 Meat Microbiological Quality

Broiler meat is highly susceptible to microbial contamination. Complete absence of contamination is impossible but the number of pathogenic microorganism needs to be minimized. The microbiological quality of meat depends on various factors, which include slaughter process, hygiene during processing and packaging process, adequate refrigeration from processing line to retail level, handling and finally, the consumer (Selvan *et al.*, 2007). Rania & Qasem, 2014 also referred to microbiological quality of broiler meat as an indication to the overall hygiene condition from slaughterhouse, packaging, to storage and distribution of meat product (Rania & Qasem, 2014). Effects of any form of compromise in the hygiene standard during production line would be manifested in the meat microbiological quality. Microbiological quality of meat can be evaluated through total count of aerobic mesophilic bacteria and isolation of certain indicator organisms. For instance, Enterobacteria, such as *Salmonella sp.* and *Escherichia coli*, as well as *Staphylococcus aureus* are considered as good microbiological indicators (Capita *et al.*, 2002; Odwar *et al.*, 2014).

## 2.5 *Salmonella sp.*

*Salmonella sp.* is a Gram-negative, non-spore forming, motile bacillus that are facultative anaerobes. According to the Centers for Disease Control (CDC) Food Net Surveillance Network, 2006, the most important source of *Salmonella sp.* infection in human is food animals (CDC, 2006). Tham, 2012 also reported that in recent years, there has been an increase in incidence of non-typhoidal salmonellae, where pathogens

were found in multiple animal reservoirs (Tham, 2012). It has also been reported that chicken products are reservoirs for *Salmonella sp.* (Jorgensen & Willis, 2014). In a study involving identification of *Salmonella* serotypes in meat samples submitted to Veterinary Research Institute (VRI), Malaysia (Roseliza *et al.*, 2011), most common serotypes isolated from poultry meat included *S. Enteritidis* (23.3%), *S. Corvallis* (21.8%), *S. Indiana* (15.9%) and *S. Typhimurium* (13.4%). It has been suggested that the antibiotic use in food animal production contributed to the occurrence of antibiotic resistant *Salmonella sp.* in human infections (Soomro *et al.*, 2010). Resistance towards fluoroquinolones and cephalosporins, such as ceftiofur and ceftriaxone is especially alarming (Hur *et al.*, 2012). In recent years, multi-drug resistant strains had been prevalent among food sources in South East Asia (Akbar & Anal, 2013).

## **2.6 *Escherichia coli***

*Escherichia coli* is a Gram-negative, non-spore forming, motile bacillus that are facultative anaerobes. Among the food-borne pathogens, *E. coli* together with *Salmonella* are commonly linked to human food-borne infections (Akbar *et al.*, 2014). In poultry production, colibacillosis is a disease of significance, where it affects the poultry industry greatly by causing major losses through mortality and decreased productivity. It is also one of the most commonly communicable diseases to humans (Khoo *et al.*, 2010). Being a member of the normal intestinal flora, meat contamination with *E. coli* is normally associated with fecal contamination, which frequently occurs at slaughterhouse (Odwar *et al.*, 2014; Sáenz *et al.*, 2001). Antimicrobial resistance of *E. coli* is not a new phenomenon and had been reported worldwide. A study by

Österblad *et al.*, 2000 indicated the *E. coli* is the main carrier of resistance and has the capability to transfer multi-drug resistance to other bacteria in the human gastrointestinal tract (Österblad *et al.*, 2000).

### **2.7 *Staphylococcus aureus***

*Staphylococcus aureus* is a Gram-positive, non-spore forming, non-motile cocci that are facultative anaerobes and appear in grape-like clusters under microscopic examination (Harris, 2002). Being ubiquitous bacteria, *Staphylococcus aureus* is impossible to be eradicated from the environment. *Staphylococcus aureus* is a pathogenic bacteria that causes Staphylococcal food-poisonings in human through the production of heat-stable enterotoxins (Schelin *et al.*, 2011). In recent years, the interest in *Staphylococcus aureus* escalated due to reports on increased frequency of isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from animals (Kwon, 2005; Kwon *et al.*, 2006). Datta *et al.*, 2012 also reported that the development of resistant or multi-resistant strains of *Staphylococcus sp.* was linked to therapeutic failures in human medicine (Datta *et al.*, 2012).

## **CHAPTER 3**

### **MATERIALS AND METHODS**

#### **3.1 Sample Collection**

Twenty *Lactobacillus*-fed broiler meat and twenty-five commercial broiler meat were sampled in this study. Samples of raw whole chicken were obtained at random from seven different retail outlets, which were located in Selangor and Kuala Lumpur. All samples were kept in separate plastic bags and stored in an ice box while being transported to Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia on the same day. Bacterial analysis was initiated as soon as the samples arrived at the laboratory.

#### **3.2 *Salmonella* sp.**

##### **3.2.1 Pre-enrichment and Selective enrichment**

Two grams of meat were taken from five parts of the whole chicken each, namely wings, both thighs, and both breasts to get a total of 10 grams of chicken meat. The knife and cutting board were disinfected with 70% alcohol after each use. The sample was then placed into a bag containing 90 ml of Buffered Peptone Water (Oxoid CM 0509), a non-selective, pre-enrichment medium, which facilitated the recovery and growth of *Salmonella* sp.. It was then incubated at 37°C for 24 hours. 1 ml of aliquots from the pre-enrichment culture was pipetted into 9 ml of Rappaport-Vassiliadis Soya Peptone Broth (Oxoid CM 0866) for enrichment and was incubated at 42°C for 24 hours.

### 3.2.2 Selective Plating

A loopful of the culture from Rappaport-Vassiliadis Soya Peptone Broth was streaked onto Xylose-lysine-deoxycholate (XLD) Agar (Oxoid CM0469) and Brilliant Green Agar (BG) (Oxoid CM 0263), which were then incubated at 37°C for 24 hours. Colonies resembling *Salmonella sp.* appeared as black or black-centered colonies on XLD agar and red-centered pink colonies on BG agar. Two to three colonies were sub-cultured onto Nutrient Agar (Oxoid CM0003).

### 3.2.3 Gram Staining

A presumptive *Salmonella sp.* colony was mixed with a drop of saline on a slide to produce a dense suspension. The slide was heat fixed and stained according to standard procedure.

### 3.2.4 Biochemical Tests

#### Urease Test

A colony from the pure culture was streaked onto the surface of the agar and incubated at 37°C for 48 hours. Positive result was seen as no changes in the medium's yellow colour.

#### Triple-Sugar Iron (TSI) Test

A colony from the pure culture was stabbed into the agar, streaked onto the surface of slant and incubated at 37°C for 24 hours. The expected result was acid butt (yellow),

alkaline slant (red), with hydrogen sulfide production, and with or without gas production.

#### Lysine Iron Agar (LIA) Test

The same procedure was repeated as in Triple-Sugar Iron Test. The expected result was alkaline butt (violet), alkaline slant (violet), with the presence of hydrogen sulfide gas.

#### Sulphide Indole Motility (SIM) Test

The colony was stabbed into the agar and incubated at 37°C for 24 hours. The expected result was positive for motility, where there was diffuse turbidity of the culture medium surrounding the stab line, as well as the presence of hydrogen sulfide gas.

#### 3.2.5 Slide Agglutination Test

Presumptive *Salmonella sp.* isolates were subjected to slide agglutination tests with polyvalent O *Salmonella* antisera (Remel R30858201). One drop of normal saline was dropped onto a clean glass slide. A single presumptive *Salmonella sp.* colony was selected from the pure culture with a wire loop and was mixed with the normal saline to produce a dense suspension. A drop of polyvalent O *Salmonella* antisera was placed onto the bacterial suspension. The glass slide was held and rocked gently for at least 1 minute. The expected positive result was formation of agglutination.

#### 3.2.6 Serotyping Test

Suspected *Salmonella sp.* isolates were inoculated onto Nutrient Agar Slants (Oxoid CM0003) and sent to Veterinary Research Institute (VRI) for serotyping test. At the time this thesis is written, the results are still pending.

### **3.3 *Escherichia coli***

#### **3.3.1 Selective Plating**

By using different cotton swabs, the meat was swabbed at five parts, namely the wings, both thighs and both breasts. The swabs were placed into 10 ml of sterile Peptone Water (Oxoid CM0009). Streaking was performed on Eosin Methylene Blue Agar (EMBA) (Merck 1.01347.0500). Plates were incubated at 37°C for 24 hours. Presumptive *E. coli* colonies appeared as dark blue-black colonies with metallic green sheen. Subculture on another EMBA was performed in order to obtain more isolated colonies. Two to three isolated colonies were selected and streaked onto Nutrient Agar (Oxoid CM0003) to obtain pure cultures.

#### **3.3.2 Gram Staining**

The procedure was performed as described in 3.2.3. Gram-negative bacillus was characteristic of *E. coli*.

#### **3.3.3 Biochemical Test**

##### **Sulphide Indole Motility (SIM) Test**

Presumptive *E. coli* colony was stabbed into the SIM agar. The agar was incubated at 37°C for 24 hours. The expected result was positive for motility and positive for

Indole, where there was a red colour change when Kovac's Indole Reagent (Thermo 1657028) was added, with absence of hydrogen sulfide gas production.

### **3.4 *Staphylococcus aureus***

#### **3.4.1 Selective Plating**

The procedure was performed as described in 3.3.1. Two to three loopfuls of Peptone Water were streaked onto Mannitol Salt Agar (Oxoid CM0085), a selective agar for members of the genus *Staphylococcus sp.* Plates were incubated at 37°C for 24 hours. Presumptive *Staphylococcus aureus* colonies will appear as yellow colonies, surrounded by a bright yellow zone. Two to three isolated colonies were selected and subcultured onto Blood Agar (Oxoid CM027). Blood Agar plates were incubated at 37°C for 24 hours. Presumptive *Staphylococcus aureus* colonies appeared as creamy white colonies, surrounded by zones of clear beta-hemolysis.

#### **3.4.2 Gram Staining**

The procedure was performed as described in 3.2.3. Gram-positive cocci in clusters was characteristic of *Staphylococcus sp.*

#### **3.4.3 Biochemical Test**

##### **Catalase Test**

A colony from the pure culture was picked with a wire loop and placed onto a drop of hydrogen peroxide on a slide. Positive result showed presence of bubbling, which indicated the production of oxygen during degradation of hydrogen peroxide.

### Coagulase Test

A colony from the pure culture was picked with a wire loop and mixed with normal saline on a slide. A drop of rabbit plasma (Remel R21050) was dropped onto the bacterial suspension. The slide was gently rocked and formation of clumps were observed for after 30 seconds. Tube coagulase test was also performed to confirm the results of slide test, if they were negative. A loopful of culture was inoculated into 0.5cc of plasma, and incubated at 37°C. Clotting was observed for after 4 hours of incubation. When there was no clotting observed, incubation period was extended to another 20 hours. Further biochemical testing was performed onto isolates that were positive for catalase and coagulase test.

### Hemolysin Test

A colony from the pure culture was inoculated into the blood broth and incubated at 37°C for 24 hours. Expected positive result was seen as the appearance of three layers, which included a plasma layer, a buffy coat layer, and erythrocyte layer.

### Acetoin Production (Voges-Proskauer)

A colony from the pure culture was inoculated into the Voges-Proskauer broth and incubated at 37°C for 24 hours. After incubation, 6 drops of alpha-naphthol followed by 2 drops of 40% potassium hydroxide were added, and the mixture was shaken. After half an hour, the broth was examined. A definite reddening of the culture supernatant indicated production of acetoin, which was a positive result.

### Maltose and Mannitol

A colony from the pure culture was inoculated into the Maltose broth and Mannitol broth. The broths were incubated at 37°C for 24 hours. Development of a yellow colour in the medium indicated acid production by maltose and mannitol fermentation.

### Arginine Dihydrolase (ADH)

A colony from the pure culture was inoculated into the Arginine Dihydrolase broth. 6 drops of mineral oil were added into the broth after bacterial inoculation. The broth was incubated at 37°C for 24 hours. Development of a purple colour in the medium indicated a positive result.

## 3.5 Antibiotic Sensitivity Test

Colonies from pure cultures of all three bacterial isolates were inoculated into Tryptic Soy Broth (Oxoid CM0129) and incubated at 37°C for 4 hours. After incubation, it was adjusted to McFarland Standard 0.5. The standardized culture was plated onto Mueller-Hinton agar (Oxoid CM0337) using a sterile cotton swab, streaking in 3 different directions by rotating the plate at 60° after each streaking. Commercial available antibiotic discs (Oxoid) were dispensed with the use of an antibiotic disc dispenser (Oxoid). The antibiotics used for each type of bacteria were selected by including three antibiotics of clinical significance and three antibiotics of veterinary significance (Table 2).

**Table 2: Antibiotics used for Antibiotic Sensitivity Test**

<b>Bacteria</b>	<b>Human Medicine</b>	<b>Veterinary Medicine</b>
<i>Salmonella</i>	Trimethoprim- Sulfamethaxazole 25µg	Gentamicin 10µg
	Ceftriaxone 30µg	Streptomycin 10µg
	Ciprofloxacin 5µg	Oxytetracycline 30µg
<i>Escherichia coli</i>	Trimethoprim- Sulfamethaxazole 25µg	Ampicillin 10µg
	Ceftriaxone 30µg	Streptomycin 10µg
	Ciprofloxacin 5µg	Oxytetracycline 30µg
<i>Staphylococcus aureus</i>	Trimethoprim- Sulfamethaxazole 25µg	Erythromycin 15µg
	Vancomycin 30µg	Streptomycin 10µg
	Clindamycin 2µg	Oxytetracycline 30µg

The plates with antibiotic sensitivity discs were incubated at 37°C for 19 hours. After incubation, the zone of inhibition diameter was measured with the aid of a caliper. Zone of inhibition were interpreted as resistant, intermediate or sensitive, as recommended by Clinical and Laboratory Standards Institute (CLSI, 2014). In this study, intermediate resistance was considered as resistant.

### 3.6 Microbiological quality

#### 3.6.1 Standard Plate Count

Five grams of meat were taken from five parts of the whole chicken each, namely wings, both thighs, and both breasts to get a total of 25 grams of chicken meat. The knife and cutting board were disinfected with 70% alcohol after each use. The sample was placed into a stomacher bag containing 225 ml of sterile Peptone Water (Oxoid CM0009) and was homogenized by a stomacher. 1 ml from the  $10^{-1}$  dilution was pipetted into 9 ml sterile Peptone Water to make a  $10^{-2}$  dilution and so on until  $10^{-6}$  dilution. Dilutions  $10^{-3}$  to  $10^{-6}$  were used for both Standard Plate Count and Coliform Plate Count. 0.1 ml of each dilution were pipetted into 4 different Standard Plate Count agars (Oxoid CM0452). L spreader was used to spread the aliquot evenly over the agar. The plates were incubated at  $37^{\circ}\text{C}$  for 48 hours. Enumeration was performed, according to standard methods from U.S. Food and Drug Administration Bacteriological Analytical Manual.

### **3.6.2 Coliform Plate Count**

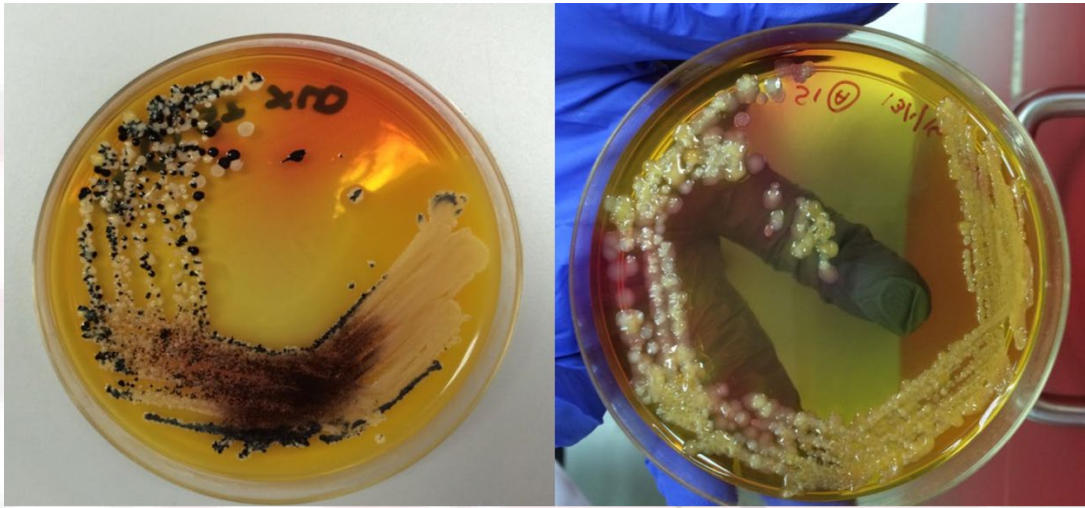
One ml of each dilution was pipetted onto Coliform Count Plate (3M™ Petrifilm™) and was incubated at  $37^{\circ}\text{C}$  for 24 hours, followed by enumeration of the bacteria.

### **3.7 Statistical analysis**

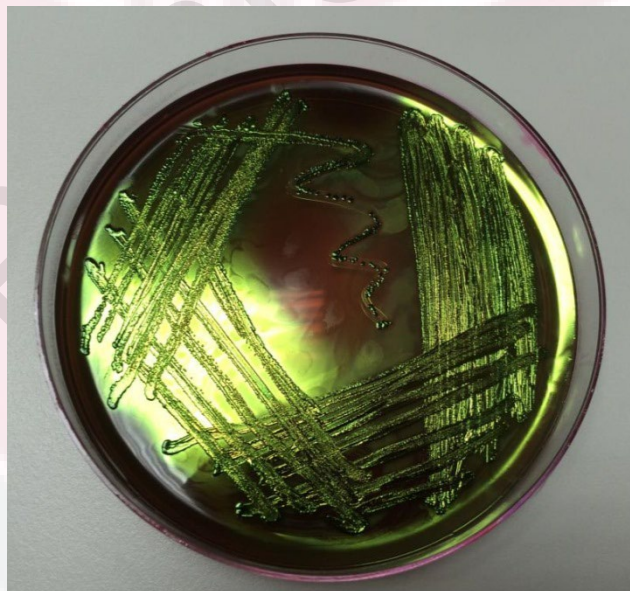
IBM SPSS Statistics Version 22 was used to perform statistical analysis. Statistical analysis with Chi-Square was performed to compare the prevalence of bacteria between the two groups of meat. For comparison of antibiotic sensitivity of isolated bacteria between both groups of chicken, Chi-square and Fischer's Exact Test were

performed, depending on suitability of data. To compare the results of SPC and CPC, Mann-Whitney test was used. P-value of less than 0.05 was considered statistically significant.

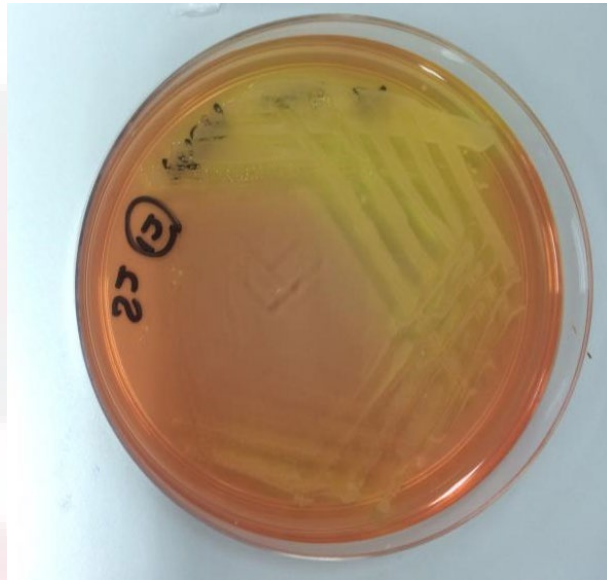
**Plate 1: Typical presumptive *Salmonella sp.* colonies on Xylose-lysine-oxycholate (XLD) agar and Brilliant Green (BG) agar**



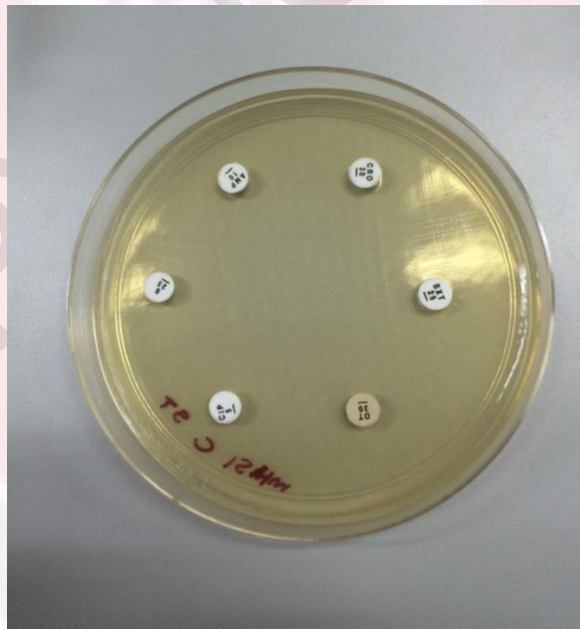
**Plate 2: Typical presumptive *Escherichia coli* colonies on Eosin Methylene Blue agar**



**Plate 3: Typical presumptive *Staphylococcus aureus* colonies Mannitol Salt Agar**



**Plate 4: Mueller-Hinton Agar**

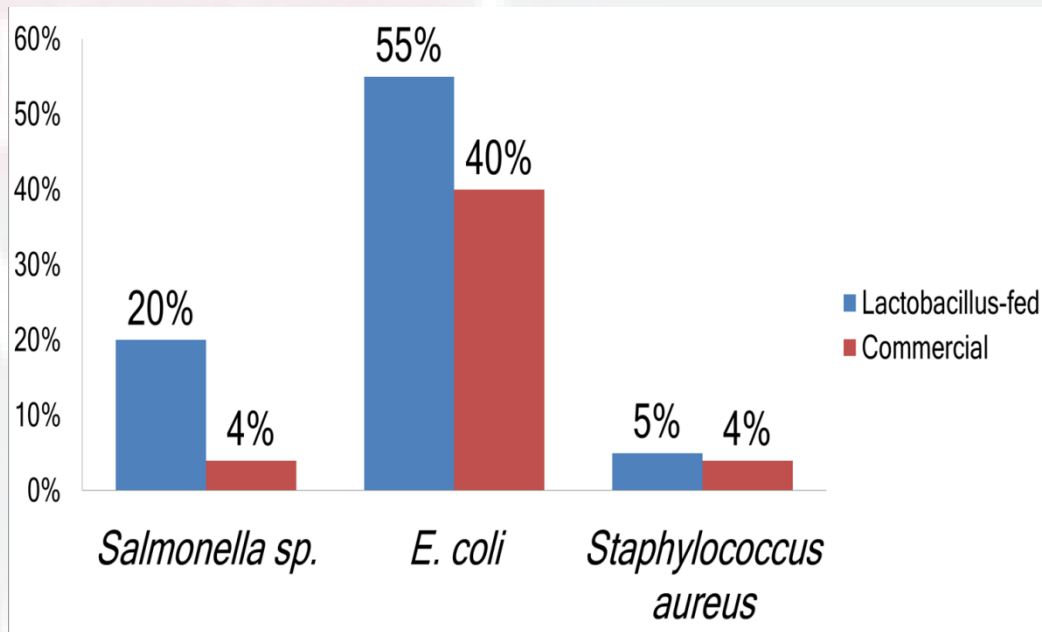


## CHAPTER 4

### RESULTS

A total of 20 *Lactobacillus*-fed broiler meats and 25 commercial broiler meats were examined for the presence of *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus*. In this study, the overall occurrence rate of *Salmonella sp.*, *Escherichia coli* and *Staphylococcus aureus* in all meat samples was 11.1%, 46.7% and 4.4%, respectively. Four (20%) of the *Lactobacillus*-fed broiler meat samples were found to be positive for *Salmonella sp.*; 11 (55%) samples were positive for *E. coli*, and only one sample (5%) was positive for *Staphylococcus aureus*. For commercial broiler meat, one sample (4%) was positive for *Salmonella sp.*; 10 samples (40%) were positive for *E. coli*, and one sample (4%) was positive for *Staphylococcus aureus* (Figure 1). Chi-square analyses and Fischer Exact test (*Salmonella sp.* -  $\chi^2$  (1,  $N = 45$ ) = 2.880,  $p = 0.090$ ; *E. coli* -  $\chi^2$  (1,  $N = 45$ ) = 1.004,  $p = 0.316$ ; *S. aureus* -  $\chi^2$  (1,  $N = 45$ ) = 0.026,  $p = 0.872$ ) revealed that the prevalence of all three bacteria between the two broiler meat types did not differ significantly.

In terms of antibiotic sensitivity, all *Salmonella sp.* isolates from both types of meat had multiple-drug resistance, where they were resistant to both ceftriaxone and oxytetracycline. (Table 3). The difference in ceftriaxone and oxytetracycline sensitivity of isolates between both type of meat was not statistically significant ( $p = 0.375$ ).

**Figure 1: Prevalence of selected bacteria in two types of chicken meat**

Three *E. coli* isolates from *Lactobacillus*-fed broiler meat were susceptible to all antibiotics, while all isolates from commercial broiler meat showed resistance towards one or more antibiotics. Among the resistant isolates of *Lactobacillus*-fed broiler meat, 50% were multi-drug resistant, while for commercial broiler meat, 60% were multi-drug resistant. For isolates of both types of meat, resistance towards ceftriaxone, ampicillin, streptomycin and oxytetracycline was observed. There was high prevalence of ampicillin and oxytetracycline resistance in isolates of both types of meat. Resistance against ciprofloxacin was only observed in one isolate of commercial broiler meat (Table 4). The difference in ceftriaxone, ciprofloxacin, streptomycin, and oxytetracycline sensitivity of isolates between both types of meat did not differ significantly (ceftriaxone -  $\chi^2(1, N=21) = 0.403$ ,  $p = 0.525$ ; ciprofloxacin

-  $\chi^2$  (1, N=21) = 1.155,  $p$  = 0.283; streptomycin -  $\chi^2$  (1, N=21) = 0.403,  $p$  = 0.525; oxytetracycline -  $\chi^2$  (1, N=21) = 2.376,  $p$  = 0.123). However, ampicillin sensitivity of isolates in commercial broiler meat was found to be significantly lower than those of *Lactobacillus*-fed broiler meat ( $\chi^2$  (1, N=21) = 4.677,  $p$  = 0.031).

*Staphylococcus aureus* isolate from *Lactobacillus*-fed broiler meat was susceptible to all antibiotics while the isolate from commercial broiler meat was resistant to oxytetracycline (Table 5). The difference in oxytetracycline sensitivity between both types of meat was not statistically significant ( $\chi^2$  (1, N=2) = 2.000,  $p$  = 0.157).

The SPC of *Lactobacillus*-fed broiler meat and commercial broiler meat ranged from 3 – 100 x 10<sup>4</sup> and 5 - 250 x 10<sup>4</sup> cfu per gram of chicken meat respectively. The mean SPC of *Lactobacillus*-fed broiler meat and commercial broiler meat were 17 x 10<sup>4</sup> and 44 x 10<sup>4</sup> cfu per gram of chicken meat respectively. Based on the results of Mann Whitney U Test,  $Z$  = 2.694,  $p$  = 0.007, there was sufficient evidence to reject the null hypothesis, and it can be concluded that the SPC in *Lactobacillus*-fed broiler meat was significantly lower than commercial broiler meat.

The CPC of *Lactobacillus*-fed broiler meat and commercial broiler meat ranged from 1 – 31 x 10<sup>3</sup> and 25 – 63 x 10<sup>3</sup> cfu per gram of meat respectively. The mean CPC in *Lactobacillus*-fed broiler meat and commercial broiler meat that was 23 x 10<sup>3</sup> and 30 x 10<sup>3</sup> cfu per gram of meat respectively was not statistically different (Mann Whitney U Test,  $Z$  = 1.201,  $p$  = 0.422).

**Table 3: Antibiotic sensitivity of *Salmonella* sp. in two types of chicken meat**

Drugs	<i>Lactobacillus</i> -fed broiler meat		Commercial broiler meat	
	No. of resistant	%	No. of resistant	%
	isolates	resistance	isolates	resistance
<b>Trimethoprim-sulfamethaxazole</b>	0/4	0	0/1	0
<b>Ceftriaxone</b>	4/4	100	1/1	100
<b>Ciprofloxacin</b>	0/4	0	0/1	0
<b>Gentamicin</b>	0/4	0	0/1	0
<b>Streptomycin</b>	0/4	0	0/1	0
<b>Oxytetracycline</b>	4/4	100	1/1	100

**Table 4: Antibiotic sensitivity of *Escherichia coli* in two types of chicken meat**

Drugs	<i>Lactobacillus</i> -fed broiler meat		Commercial broiler meat	
	No. of resistant	%	No. of resistant	%
	isolates	resistance	isolates	resistance
<b>Trimethoprim-sulfamethaxazole</b>	0/11	0	0/10	0
<b>Ceftriaxone</b>	2/11	18.2	3/10	30
<b>Ciprofloxacin</b>	0/11	0	1/10	10
<b>Ampicillin</b>	5/11	45.5	9/10	90

<b>Streptomycin</b>	2/11	18.2	3/10	30
<b>Oxytetracycline</b>	4/11	36.4	7/10	70

**Table 5: Antibiotic sensitivity of *Staphylococcus aureus* in two types of chicken meat**

<b>Drugs</b>	<b><i>Lactobacillus</i>-fed broiler meat</b>		<b>Commercial broiler meat</b>	
	No. of resistant isolates	% resistance	No. of resistant isolates	% resistance
	<b>Trimethoprim-sulfamethaxazole</b>	0/1	0	0/1
<b>Clindamycin</b>	0/1	0	0/1	0
<b>Vancomycin</b>	0/1	0	0/1	0
<b>Erythromycin</b>	0/1	0	0/1	0
<b>Streptomycin</b>	0/1	0	0/1	0
<b>Oxytetracycline</b>	0/1	0	1/1	100

## CHAPTER 5

### DISCUSSION

#### Prevalence of selected bacteria in broiler meat

The presence of *Escherichia coli* contamination from both types of broiler meat was not surprising as *E. coli* is a commensal bacteria in the gut of chicken and fecal contamination might have occurred during evisceration process. Presence of high percentage of *E. coli* could indicate either poor evisceration or improper washing of carcass after the process (Alonso *et al.*, 2011). In this study, the level of *E. coli* presence can be considered low, as compared to reported contamination rates of 98% observed in the poultry meat samples in India, 100% in Cameroon and 100% in Vietnam (Odwar *et al.*, 2014).

*Salmonella sp.* contamination was also lower than those observed in previous studies. A study on prevalence of *Salmonella* in broilers at retail outlets, processing plants and farms in Malaysia yielded a result of 36% (Rusul *et al.*, 1996). A study in Thailand reported prevalence of 25% and a study in Spain reported 22.7% (Minami *et al.*, 2010; Fernandez *et al.*, 2012). A study by Zhao *et al.*, 2001 also found that retail raw meats tend to be less contaminated with *Salmonella* (Zhao *et al.*, 2001).

Previous study on *S. aureus* in chicken meat in Turkey found 13.3% (Yurdakul *et al.*, 2013), and in Thailand found 18.2% (Akbar & Anal, 2013) prevalence as compared to 5% in *Lactobacillus*-fed meat and 4% in commercial broiler meat in this study. High amount of *S. aureus* contamination normally indicates improper food handling practices (Kadariya *et al.*, 2014).

The prevalence of *Salmonella sp.*, *E. coli* and *S. aureus* was not significantly different between *Lactobacillus*-fed broiler meat and commercial broiler meat. The result of a study by Jin *et al.*, 1996 had a similar result where there was no significant difference found between control group and treatment group, which was treated with probiotics (*Bacillus Subtilis* and *Lactobacillus*) in the occurrence of cecal *Salmonella* and *Campylobacter* (Jin *et al.*, 1996). A study done by Afshin *et al.*, 2012, produced a similar result to the present study, where there was no significant effect of probiotics on *S. aureus* properties in meat (Afshin *et al.*, 2012). However, studies by Fritts *et al.*, 2000 and Kaksefidi & Rahimi, 2005 reported lower incidence of *Salmonella* and coliforms (Fritts *et al.*, 2000; Kaksefidi & Rahimi, 2005). Otutumi *et al.*, 2012 also acknowledged that the effects from the use of probiotics in animal production have been inconsistent in the past, which became a constraint for the promotion of their use (Otutumi *et al.*, 2012).

There are several possible sources of contaminations that might have occurred during and after slaughter, which include equipment and environment during processing line, transport, as well as handlers (FAO, 2007). These sources would increase or introduce contaminations to the produce. The supplementation of probiotics in chicken feed will not prevent post-harvest meat contaminations.

The efficacy of probiotics given to chickens may be affected by several factors. According to Jin *et al.*, 1996, stressor factors, such as excessive heat or humidity can affect the equilibrium of the gut microflora and therefore reduce the efficacy of probiotics (Jin *et al.*, 1996). Afshin *et al.*, 2012 stated that potential difference of

probiotic bacterial strains, even within the same species, could result in difference in areas of adherence and immunological effects (Afshin *et al.*, 2012).

### **Antibiotic resistance of isolated bacteria**

In this present study, all *Salmonella sp.* isolates from both types of meat were resistant to ceftriaxone and oxytetracycline. Resistance to ceftriaxone, a third generation cephalosporin is a significant finding, as this is a drug-of choice in treatment of invasive *Salmonella sp.* infections (Bush, 2016). This result was in agreement with recent findings of sharp increases in resistance to ceftriaxone, together with ceftiofur, and amoxicillin/clavulanic acid in *Salmonella sp.* isolates (Foley & Lynne, 2008). Resistance to cephalosporins by *Salmonella* is due to acquired resistance, where production of  $\beta$ -lactamase enzymes allows degradation of antibiotics, while the transfer of resistance and multi-resistance gene among strains is most probably through plasmid (Foley & Lynne, 2008). In this present study, 100% resistance to oxytetracycline among *Salmonella sp.* isolates may indicate the uncontrolled use of tetracycline in poultry. Resistance of *Salmonella* isolated from poultry meat to tetracycline (94.73%; 72.72%) was also seen in previous studies in Pakistan and Thailand (Soomro *et al.*, 2010, Akbar & Anal, 2013). High resistance is probably due to possible diffusion of *tet* resistance gene, which leads to expression of resistance efflux pumps which was observed in an epidemiological study of *Salmonella* isolated from animals (Pezella *et al.*, 2004). Resistance towards other antibiotics were not observed, unlike a study in Thailand, where 45.45% isolates were

resistant to streptomycin, 36.36% resistant to gentamicin, and 27.27% were resistant to ciprofloxacin and trimethoprim-sulfamethoxazole (Akbar & Anal, 2013).

Prevalence of antibiotic resistance in *E. coli* isolates from both groups of broiler meat was highest in ampicillin, followed by oxytetracycline, streptomycin and ceftriaxone. Resistance towards ciprofloxacin was only observed in an isolate from commercial broiler meat. Low incidence of resistance towards ciprofloxacin is considered good, as it is one of the medications used in treatment of human *E. coli* infections. However, there was resistance towards ceftriaxone, especially among isolates from commercial broiler meat (30%). High resistance towards medications of human medicine importance is alarming. The prevalence of ampicillin, oxytetracycline and streptomycin resistance seen in this study was comparable with results of previous studies (Altahi *et al.*, 2010; Akbar *et al.*, 2014; Odwar *et al.*, 2014). High prevalence of ampicillin and tetracycline resistance among *E. coli* isolates (92%) from poultry meat was also observed in a study by Akbar *et al.*, 2014. The spread of antibiotic resistance among *E. coli* occurs through conjugation, a mechanism of horizontal gene transfer (Madden, 2009). Besides transferring antibiotic resistance to other *E. coli* strains, they possess the ability to spread resistance to other bacteria within the gastrointestinal tract of humans by acting as reservoirs of resistant genes (Österblad *et al.*, 2000). Therefore, the occurrence of individual and multiple antibiotic resistance in *E. coli* seen in both groups of meat was worrying, as it meant that such broiler meat would be sources of resistant bacteria to humans.

Resistance towards oxytetracycline was observed in *S. aureus* isolate in commercial broiler meat. Such resistance was also reported in previous studies (Akbar

& Anal, 2013; Otalú *et al.*, 2011). The occurrence of resistance towards oxytetracycline is most probably due to frequent use of tetracycline for staphylococcal and other infections in poultry. It is good to note that resistance towards other antibiotics, especially vancomycin, drug of choice for cases of Methicillin-resistant *S. aureus* infections, was not present in this study. Resistance to vancomycin has been emerging worldwide (Pesavento *et al.*, 2005). A study by Geidam *et al.*, 2012 in Malaysia, yielded 85% resistance towards erythromycin, while a study by Datta *et al.*, 2012 in Bangladesh yielded 100% resistance towards streptomycin (Geidam *et al.*, 2012; Datta *et al.*, 2012). In another study, resistance to ampicillin (58.33%), tetracycline (8.33%), trimethoprim-sulphamethoxazole (8.33%), erythromycin (8.33%) and clindamycin (8.33%) were observed in *S. aureus* isolates from poultry meat (Pesavento *et al.*, 2005).

### **Microbiological quality**

In this study, the SPC of *Lactobacillus*-fed broiler meat was significantly lower than commercial broiler meat. In a study by Afshin *et al.*, 2012, the effect of probiotics on meat microbial properties of quail produced the same results (Afshin *et al.*, 2012). In regards to coliform properties, however, the results of this study was not in agreement with previous studies (Fritts *et al.*, 2000; Khaksefidi & Rahimi, 2005) that have reported that chicken carcasses fed with probiotic had significantly lower numbers of coliforms. As mentioned before, the effects of probiotics in animal production in the past had been inconsistent, therefore necessitate further studies on probiotics use in animal production.

## CHAPTER 6

### CONCLUSION AND RECOMMENDATIONS

The presence of bacteria, *Salmonella*, *Escherichia coli* and *Staphylococcus aureus* in broiler meat samples from retail markets suggests the importance of these pathogens in chicken. Based on the results of this project, it can be concluded that the SPC of *Lactobacillus*-fed broiler meat is significantly better than commercial broiler meat. However, there is little difference in the microbiological qualities in terms of coliform properties, prevalence of selected bacteria and their level of antibiotic sensitivity between both types of broiler meat. Given the significantly higher price for probiotic-fed chickens, customers need to be educated about the potential value for money in consuming these chickens.

Multi-drug resistance was observed in *Salmonella* and *E. coli* isolates of both groups of broiler meat. This finding is of public health concern as *E. coli* is a reservoir of antibiotic resistance genes. Therefore, hygiene standards from farm to table, especially in abattoir need to be improved. There is also a need for rational use of antimicrobials as well as regular antibiotic sensitivity surveillance in the livestock industry.

For further continuation of this study, the author would like to recommend to perform evaluation of antibiotic residues in probiotic-fed broiler meat, and to make a comparison with commercial broiler meat.

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<http://dx.doi.org/10.1128/aem.67.12.5431-5436.2001>



APPENDIX

Plate 5: Enumeration of Standard Plate Count Agar

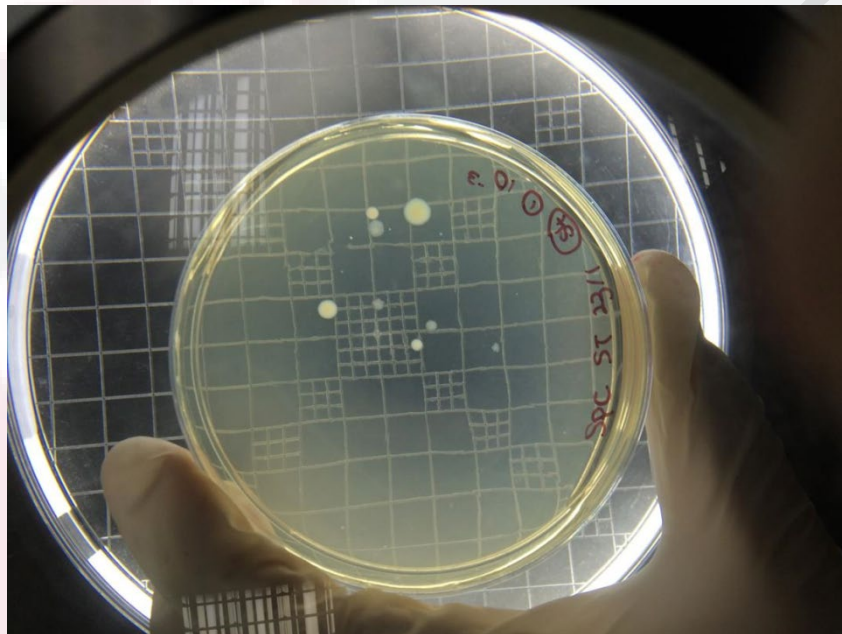
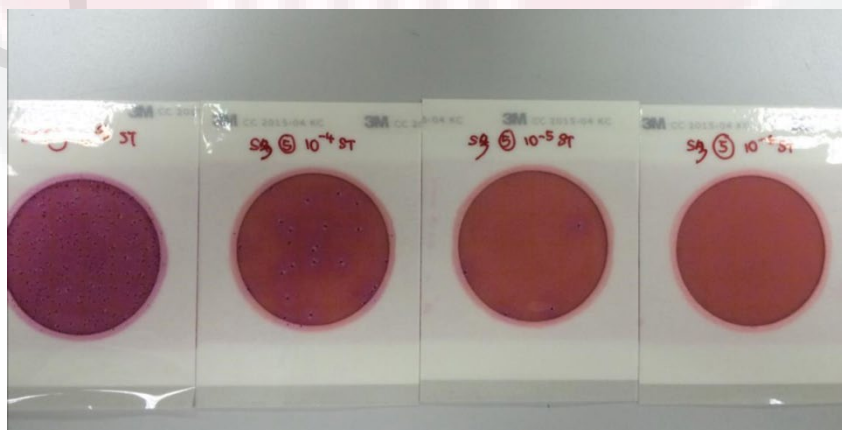
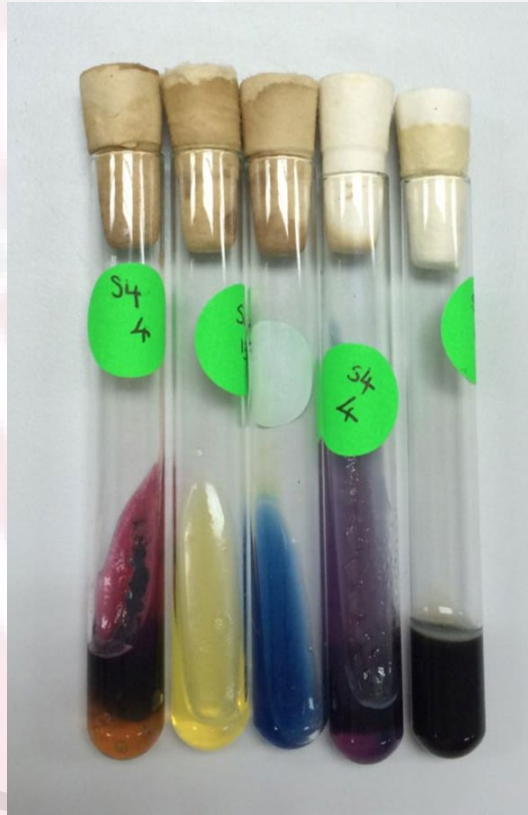


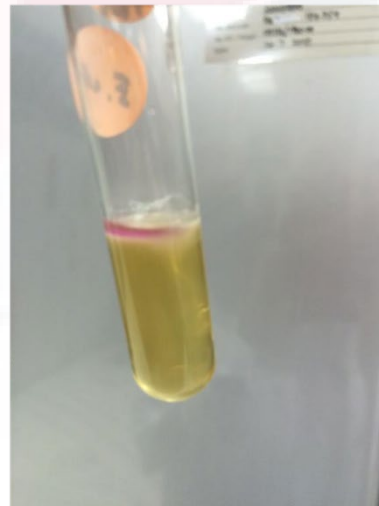
Plate 6: 3M™ Petrifilm™ of Coliform Plate Count



**Plate 7: Typical positive biochemical results of *Salmonella sp.***



**Plate 8: Typical positive biochemical results of *Escherichia coli***



**Plate 9: Typical positive biochemical results of *Staphylococcus aureus***

