ผลของกรดอินทรีย์รวมต่อการทำงานของลำไส้ และการต้านอนุมูลอิสระในไก่เนื้อที่ได้รับเชื้อซัลโมเนลล่า เอนเทอริกา ซีโรวาร์ เอนเทอริติดิส

นางสาว มัลลิกา ผ่องผิว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาสรีรวิทยาการสัตว์ ภาควิชาสรีรวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

EFFECTS OF MIXED ORGANIC ACIDS ON INTESTINAL FUNCTION AND ANTIOXIDANT ACTIVITY OF BROILER CHALLENGE WITH *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Animal Physiology
Department of Veterinary Physiology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2010

Thesis Title	Effects of mixed organic acids on intestinal function and antioxidant activity of broiler challenge with *Salmonella Enterica* serovar Enteritidis
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มัลลิกา ผ่องผิว : ผลของกรดอินทรีย์รวมต่อการทำงานของลำไส้ และการต้านอนุมูลอิสระในไก่เนื้อ ที่ได้รับเชื้อซัลโมเนลล่า เอนเทอริกา ซีโรวาร์ เอนเทอริติดิส (EFFECTS OF MIXED ORGANIC ACIDS ON INTESTINAL FUNCTION AND ANTIOXIDANT ACTIVITY OF BROILER CHALLENGE WITH *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. นสพ. ดร. กฤษ อังคนาพร: อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. สพญ. อินทิรา กระหม่อมทอง , ผศ. นสพ. ดร. นพดล พิฟารัตน์, 54 หน้า.

Salmonellosis เป็นโรคติดเชื้อแบคทีเรียในอาหารสำคัญที่ติดต่อจากสัตว์สู่คน ซึ่งเกี่ยวข้องกับอุตสาหกรรมการผลิตสัตว์ปีก การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาถึงผลของการใช้กรดอินทรีย์รวม (กรดฟอร์มิก, กรดโพรพิโอนิก และ HMTBa) ต่อการทำงานของลำไส้ และการต้านอนุมูลอิสระในไก่เนื้อ ที่ได้รับเชื้อซัลโมเนลลา เอนเทอริกา ซีโรวาร์ เอนเทอริติดิสที่ต้านต่อกรดนาลิไดลิก โดยทำการทดลองในไก่กระทงเพศผู้อายุ 1 วัน จำนวน 588 ตัว แบ่งออกเป็น 4 กลุ่มคือ กลุ่มควบคุม, กลุ่มที่ได้รับเชื้อซัลโมเนลล่า, กลุ่มที่ได้รับกรดอินทรีย์รวม (กรดฟอร์มิก และ กรดโพรพิโอนิก) และ กลุ่มที่ได้รับกรดอินทรีย์รวม และ HMTBa ตามลำดับโดยกลุ่มที่ 3 และ 4 ได้รับกรดอินทรีย์ในน้ำใน 2 สัปดาห์แรก และ สัปดาห์สุดท้ายของการทดลอง อีกทั้งได้รับเชื้อ ซัลโมเนลล่าเช่นเดียวกับกลุ่มที่ 2 ที่ความเข้มข้น 10^5 cfu/ml ในวันที่ 1 และ 7 โดยทำการป้อนเชื้อครึ่งหนึ่งของจำนวนไก่ในแต่ละกลุ่มการทดลอง มีการให้อาหารพื้นฐานที่มีองค์ประกอบของข้าวโพดและกากถั่วเหลือง และน้ำ อย่างเพียงพอตลอดการทดลอง 42 วัน ทำการวิเคราะห์เชื้อซัลโมเนลล่าเชิงคุณภาพและกึ่งปริมาณ การย่อยได้ของสารอาหารที่ลำไส้เล็กส่วนปลาย การศึกษาการเปลี่ยนแปลงทางจุลกายวิภาคของลำไส้ส่วนต่าง ๆ และ ปริมาณเอนไซม์ต้านออกซิเดชั่นในตับและซีรัม

จากการศึกษาพบการแพร่กระจายเชื้อซัลโมเนลล่าจากกลุ่มที่ได้รับเชื้อไปยังกลุ่มที่ไม่ได้รับเชื้อทั้งบริเวณตับ-ม้าม และลำไส้ส่วนปลาย ในวันที่ 14 ของการทดลอง นอกจากนี้พบเชื้อซัลโมเนลล่าสูงสุดที่ลำไส้ส่วนปลายในกลุ่ม SE group และไม่พบเชื้อซัลโมเนลล่าที่บริเวณตับ-ม้าม ในกลุ่ม SE+ (ORAs+HMTBa) การย่อยได้ของโปรตีนและ ไขมันในกลุ่มที่ได้รับกรดอินทรีย์ทั้งสองกลุ่มการทดลอง (กลุ่มที่ 3 และ กลุ่มที่ 4) มีอัตราการย่อยได้สูงกว่ากลุ่มที่ได้รับเชื้อซัลโมเนลล่าในวันที่ 21 ของการทดลอง ($P<0.05$) และในวันที่ 42 พบอัตราการย่อยได้ของโปรตีนและไขมันในแต่ละกลุ่มการทดลองให้ผลไม่แตกต่างกัน และพบว่าลักษณะของเยื่อบุลำไส้เล็กมีความสูงของวิลไลมากขึ้น และ ความลึกของคริปที่ลดลงในกลุ่ม SE + (ORAs+ HMTBa) ในวันที่ 14 และ 21 ของการทดลอง และในระยะ grower-finisher เอนไซม์ต้านอนุมูลอิสระ (GSH-px และ GSH) และ MDA ไม่แตกต่างกัน ($P>0.05$) ในวันที่ 14, 21 และ 42 ในแต่ละกลุ่มการทดลอง

จากการศึกษาสรุปได้ว่า การให้กรดอินทรีย์รวมที่มี 2-Hydroxy-4-Methylthio Butanoic acid (HMTBa) ร่วมกับกรดฟอร์มิก และ กรดโพรพิโอนิค สามารถป้องกันการแพร่กระจายของเชื้อซัลโมเนลล่าที่ตับ-ม้าม และ บริเวณลำไส้ส่วนปลายโดยให้ประสิทธิภาพในการลดจำนวนเชื้อได้ดีกว่าการใช้กรดฟอร์มิกร่วมกับกรดโพรพิโอนิค นอกจากนี้การให้กรดอินทรีย์รวม (กรดฟอร์มิก, กรดโพรพิโอนิค และ HMTBa) ใน 14 วันแรกช่วยให้การย่อยของโปรตีนและไขมันดีขึ้นในไก่อายุ 21 วัน และพบว่าการให้กรดอินทรีย์รวมไม่มีผลต่อเอนไซม์ต้านออกซิเดชั่น และการลดลงของ MDA บริเวณตับ และพบว่ามีผลไม่มากต่อการเปลี่ยนแปลงลักษณะของเยื่อบุลำไส้เล็กส่วนต่างๆ

ภาควิชา สรีรวิทยา
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ลายมือนิสิต.....มัลลิกา ผ่องผิว.....
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##5075571831: MAJOR ANIMAL PHYSIOLOGY

KEYWORDS : *SALMONELLA* / ORGANIC ACID/ HMTBa/ ANTIOXIDANT/ BROILER

MULLICA PONGPIEW: EFFECTS OF MIXED ORGANIC ACIDS ON INTESTINAL FUNCTION AND ANTIOXIDANT ACTIVITY OF BROILER CHALLENGE WITH *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS. ADVISOR: ASSOC. PROF. KRIS ANGKANAPORN, D.V.M., Ph.D.; CO-ADVISOR: ASSOC. PROF. INDHIRA KRAMOMTONG, D.V.M., M.S., ASST. PROF. NOPPADON PIRARAT, D.V.M., Ph.D., 54 pp.

Salmonellosis is widely known as major food-born pathogen involved with poultry production. The objectives of this study were to determine the effect of (ORAs+ HMTBa) (formic acid, propionic acid and 2-Hydroxy-4-Methylthio Butanoic acid) on intestinal function and antioxidant enzyme activity of broilers challenged with nalidixic resistant *Salmonella* Enteritidis (SE). Five hundred eighty-eight, one day old, male, Arbor acre broilers were divided into 4 treatment groups; CON group, SE group, SE+ ORAs group and SE+ (ORAs+ HMTBa) group, respectively. For SE+(ORAS) and SE+ (ORAs+ HMTBa) groups were received organic acid in water at the first two weeks and the last week of experiment and were challenged with SE (10^8 cfu/ml) at days 1 and 7 as similar to SE group. In each replicate, SE was inoculated to half of the chicks. Corn-soybean meal basal feed and water were given *ad libitum* for 42 days. Qualitative and semi-quantitative SE examination, ileal digestibility and intestinal morphological study including liver and serum antioxidant enzyme activities were examined.

The result demonstrated the horizontal transmission from inoculated chicks to non-inoculated chicks in liver-spleen pool and ileo-cecal content at day 14. Furthermore, the highest SE count was found in the ileo-cecal content of positive control group (SE) and no SE count was found in SE+ (ORAs+ HMTBa) group at day 21. Ileal digestibility of protein and fat in both ORA groups (groups 3 and 4) were higher than SE group at day 21 ($P<0.05$). At day 42, there was no significant change in digestibility among groups. It is discovered that villus height was higher and crypth depth was lower in SE+ (ORAs+ HMTBa) group at days 14 and 21. There were no significant changes in antioxidant enzyme activity (GSH-px and GSH) and MDA concentration in all experiment groups at day 14, 21 and 42.

In conclusion, the present study showed that combination of 2-Hydroxy-4-Methylthio Butanoic acid (HMTBa), formic acid and propionic acid were more efficient to prevent the horizontal transmission of SE in liver-spleen pool and ileo-cecal contents than conventional mixed organic acids. Furthermore, the ileal digestibility of protein and fat in chicks receiving ORAs+ HMTBa improved significant in the starter period of SE treatment. ORAs+ HMTBa neither elicited the antioxidant effect nor reduced the pro-oxidant MDA in liver. The gut morphology was less influenced by effects of enhanced mixed organic acids.

Department: Veterinary Physiology... Student's Signature....Mullica Pongpiew.....

Field of Study: Animal Physiology..... Advisor's Signature.....

Academic Year: 2010..... Co-advisor's Signature.....

Co-advisor's Signature.....

ACKNOWLEDGEMENTS

This thesis would not be success without the contribution and assistance from my advisor, co-advisors, my friend and my family.

To my family, I am most grateful for their patience, encourage, cheerful all times and everything to help of my study and special thanks to my friend for their help of my laboratory.

My grateful also go to my kind advisor, Associate Professor Dr. Kris Angkanaporn and my co-advisor, Associate Professor Indhira Kramomtong and Assistant Professor Noppadon Pirarat for their helpful suggestion and support during period of my study.

I wish to express my sincere thanks to all my teachers at the Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University for all their loving helps during the time I was studying.

My appreciations are also devoted to my dear to Miss Siripen Komonvanish, Miss Petcharat Nampimoon and Mr. Chutchai Donkotjan for their help and good suggestion on my experimental work and laboratory technique. Mrs. Waree Niyomtham, Miss Pensuda Hongpu and Mrs. Kanchana Chantornvivat are acknowledged for their kind help and care in microbiology and nutrition laboratory.

Finally, I would like to thank Novus International for their financial support on the study.

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LIST OF ABBREVIATIONS

ADG	average daily gain
AIA	acid insoluble ash
ANOVA	analysis of variance
BHT	butyrate hydroxyl
BPW	buffer peptone water
BW	body weight
CFU	colony forming unit
DFI	daily feed intake
FCR	feed conversion ratio
HMTBa	2-hydroxy-4-(methylthio)butanoic acid
ID	ileal digestibility
kcal	kilocalorie
kg	kilogram
MDA	malondialdehye
mg	milligram
mg%	milligram percent
MIL	motility indole lysine medium
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mol	mole
MSRV	modified semi-rappaport vassiliadis
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
nmol	nanomole
ORA	organic acid
rpm	round per minute
SCFA	short chain fatty acid

SE	*Salmonella* Enteritidis
TBA	thiobarbituric acid
TSA	tryptic soy agar
TSI	triple sugar iron
U	unit
U/mg protein	unit per milligram protein
XLT_4	xylose lysine tergitol 4
µg	microgram
µl	microliter
µmol	micromole

CHAPTER I

INTRODUCTION AND AIMS

In the past decade,*Salmonella enterica* serovar Enteritidis (SE) became one of the important cause of foodborne human illnesses worldwide (Guan et al., 2006). Water and feed contaminations are major causes of *Salmonella* spp. infection in broiler (van Immerseel et al., 2004). European Union (EU) legislation aimed to control human food safety hazards and required feed manufacturers to adopt Hazard Analysis and Critical Control Points (HACCP) to control of *Salmonella* and other pathogens in feed (Williams, 2002). In 2002, the Netherlands issued new Good Manufacturing Practice (GMP) regulations aiming to control *Salmonella* in poultry (Jones and Richardson, 2004). In Thailand, *S.*Enteritidis was the most serovar found (19.9%) among the14,599 *Salmonella* isolates from chickens (Bangtrakulnonth, 2004; Chotikatam, 2005). The ability of *Salmonella* to adapt and survive acid stress is fundamental to their pathogenesis (Jonathon et al., 2001). *Salmonella* infection is mostly confined to the gastrointestinal tract of chicks. After invasion, the bacteria can multiply in the liver and spleen and spread to other organs, producing a systemic infection. The cause of death has a combination of anorexia and dehydration (Poppe, 2000).

Antibiotics have been used in the food and drinking water to control bacterial infection for decades. In 2003, Parry founded that the antibiotics growth promoters lead to Salmonella-resistant strains. Mead (2000) showed that antibiotic used to control *Salmonella* infections may induce cross-resistance to other bacterial species and they do not eliminate infection in infected animals.

Organic acids were proposed as alternative supplements for decreasing pathogenic bacteria (Meimandipour et al., 2010). Mode of actions of organic acids includes pH reduction in digesta, increased pancreatic secretion, and trophic effects of the gastrointestinal mucosa (Dibner and Buttin, 2002). In 2005, Byrd et al. found that supplementing acetic, lactic and formic acid in the drinking water of broilers reduced crop pH and decreased the recovery of *Salmonella* from crop.

DL-2-hydroxy-4-(methylthio)-butanoic acid (HMTBa) is one of organic acid that can reduce *Salmonella* spp. (Schasteen et al., 2007). Furthermore, HMTBa is the precursor of amino acid L- Methionine (Yi et al., 2007). Methionine (Met) is the first limiting amino acid for poultry (Schuttle et al., 1997). In 2000, Garcia and Mack found that Met can improve growth performance and metabolism of tissue skin and feed conversion rate. In addition Met, as part of sulfur amino acid, can transform to L-Cys, which in turn, is a precursor of glutathione synthesis or catabolized to taurine (Venegas et al., 2006). As a glutathione precursor, L-Cys have a key role in intestinal epithelial antioxidant functions, and regulate epithelial cell proliferation via modulation of redox status (Shoveller et al., 2005).

It is interesting that HMTBa not only act as acidifier to control *Salmonella* infection but can elicit antioxidant effect via the sulfur amino acid metabolism also. Therefore, the objectives of this experiment were:

1). to examine the effect of HMTBa and mixed organic acids on growth performance of broiler chickens.

2). to investigate the effect of HMTBa and mixed organic acids on qualitative and quantitative examination *S.*Enteritidis colonization in broiler chickens.

3). to examine the effect of HMTBa and mixed organic acids on apparent ileal digestibility and gut integrity in broiler chickens.

4). to study the effect of HMTBa and mixed organic acids on antioxidant activity in broiler chickens.

CHAPTER II

LITERATURE REVIEW

2.1 *Salmonella* spp.

2.1.1 The Organism

Salmonella is able to exist in different habitats as it passes from the dry, external environment, through the acidity of the stomach, the lumen of the gut, the extracellular space of host tissues and the inside of the macrophage, the surface components provide a protective and yet porous shield against the outside world (Rycroft, 2000). The genus *Salmonella* is a member of the bacteria in the family Enterobacteriaceae, *Salmonella* spp. Are gram-negative, nonsporing (Dunkley et al., 2008). Gram-negative bacteria, there are three layers: the cytoplasmic membrane (inner membrane), the peptidoglycan (murein) and the outer membrane. The compartment between the two membranes is referred to as the periplasmic space.

The cytoplasmic membrane of *Salmonella* is composed of phospholipids and proteins. As in other Gram-negative bacteria, it transports nutrients and it is the site of oxidative phosphorylation and the synthesis of phospholipid, peptidoglycan units and lipopolysaccharide (LPS) (Rycroft, 2000).

The periplasm contains the peptidoglycan and also numerous soluble proteins, which usually have one of three functions. These are: (i) catabolic functions, such as alkaline phosphatase, where solutes for which no transport system exists are converted to a form that can be transported though the cytoplasmic membrane; (ii) binding proteins which fasten on to nutrients, such as amino acids, ions and sugars, and assist their transport; and (iii) proteins which degrade or modify harmful substances, such as antibiotics (Rycroft, 2000).

The outer membrane is a highly complex lipid-bilayer membrane structure, which surrounds the peptidoglycan layer and shields the periplasm from the external environment. It also prevents leakage of the periplasmic proteins away from the immediate environment of the cytoplasmic membrane (Rycroft, 2000).

2.1.2 *Salmonella* Infection

Avian systemic salmonellosis has three distinct phases during each of which there is significant interaction with the immune system. The first is invasion via the gastrointestinal tract. The second phase is the establishment of systemic infection mainly as an intracellular infection of macrophages. Finally, infection may be cleared by the immune response (Chappell et al., 2008).

Poultry are commonly infected with a wide variety of *Salmonella* serovars. The infection is mostly confined to the gastrointestinal tract. Poultry and many other animals are often unapparent carriers, latently infected or less frequently, clinically ill. They may excrete *Salmonella* in their faeces and form a large reservoir and source of contamination for other animals, humans and the environment. Poultry often become infected via horizontal transmission by litter, faeces, feed, water, fluff, dust, shavings, straw, insects, equipment etc contaminated with *Salmonella* and by contact with other chicks or poultry, rodents, pets, wild birds, other domestic and wild animals and personnel contaminated with *Salmonella*. Vertical transmission occurs when follicles in the ovary are infected or the developing eggs become infected in the oviduct (Bäumler et al., 1998).

The alimentary tract is a hostile environment, which imposes severs stress upon invading bacteria (Bäumler et al., 1998). Within minutes of injecting *Salmonella* into ligated ileal loops, *Salmonella* can be seen to invade both M cells and enterocytes that overlie domed villi associated with lymphoid follicles and absorptive villi respectively (Frost et al., 1997). The surviving bacteria then reach the small intestine, which contains

bactericidal compounds such as bile salts. *Salmonella* can adapt to cope with these stress conditions (Bäumler et al., 1998) (Figure 2.1).

Salmonella causes illness in humans by passing from the intestinal tract into the epithelium, where it causes inflammation and systemically releases an enterotoxin and a potent endotoxin. *Salmonella* exists in a typical fecal-oral life cycle, although it can be spread through the nasal cavity to the gut (Callaway et al., 2008). Adaptation allows *Salmonella* to exist as a pathogen in a suitable host environment, or as a transient member of the gastrointestinal population in a less than ideal host environment (Callaway et al, 2008).

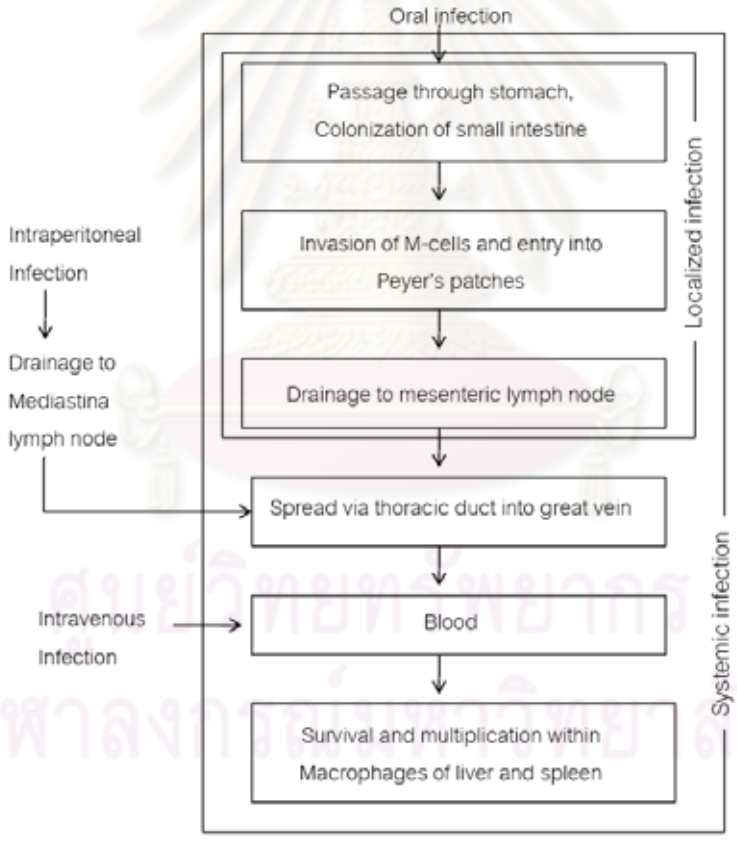


Figure 2.1 *Salmonella* infection (Bäumler et al., 1998).

2.1.3 Pathogenesis of *Salmonella* infections

Non-typhoidal *Salmonella* spp. such as *S.*Enteritidis most often causes gastroenteritis with watery diarrhea. There is gross structural damage to the mucosa, which causes generalized impairment of absorption. These factors include (1) epithelial invasion (2) synthesis of an enterotoxin (3) and cytotoxin (4) and induction of an inflammatory response (Mehta et al., 1998b). A prominent histological feature of *Salmonella* spp. infection is a dense infiltration of inflammatory cells in the intestinal epithelium and the underlying lamina propria. The dense accumulation of inflammatory leukocytes in the intestinal mucosa during the *Salmonella* spp. mediated intestinal infection suggests that these cells participate in the pathogenesis and resolution of tissue damage either by releasing enzymes or generating ROS. A range of ROS including superoxide radical, hydrogen peroxide, hypochlorus acid and monochloramines are released by the inflammatory leukocytes (Mehta et al., 1998a). The ROS are highly chemically reactive molecules and can damage all kinds of biochemical substances present in the cell. The most important cellular damage caused by ROS is lipid peroxidation, wherein these reactive species attack the lipids in the membrane and cause peroxidation, resulting in complete destruction of the membrane. In addition, reactive metabolites of oxygen are also believed to contribute to diarrhea by acting as secretagogues(Mehta et al.,1998a). A product of lipid peroxidation such as malondialdehyde (MDA) and conjugated diene is an indication of the extent of peroxidation (Mehta et al., 1998b). Rishi et al.(2008) have indicated that *Salmonella* cause liver damage through the involvement of tumor necrosis factor alpha (TNF-α) and lipid peroxidation in broiler.

Pathogenic microfloras in the small intestine are competitive with the host for nutrients and also reduce the digestion of fat and fat-soluble vitamins due to deconjugating effects of bile acids (Engberg et al., 2000). This depresses growth performance and increase incidence of desease (Hassan et al., 2010). Furthermore, *Salmonella* can pass from the intestinal tract into the epithelium, where it causes inflammation and systemically releases an enterotoxin and a potent endotoxin

(Callaway, et al. 2008). The mediated intestinal infection is accompanied by gross structural damage to the mucosa which causes generalized impairment of absorption (Mehta et al., 1998a). It is infection associated with gastrointestinal inflammation resulted in diarrhea (Cheminay and Hensel, 2008). *Salmonella* was shown to induce changes in the brush border membrane known as “membrane ruffles”. After lysis of the host cell, *Salmonella* bacteria are observed in macrophage-like cells and free in the lumen of lamina propria blood vessels to disseminate to other tissues (Desmidt et a., 1998)

Many methods have been used to reduce the level of *Salmonella* infection including the use of antimicrobial agents such as antibiotic (Dibner and Richards, 2005). The European Union (EU) banned the use of sub-therapeutic levels of antibiotics to prevent disease or promote growth, starting with a ban on avoparcin in 1997 and a ban on virginiamycin, bacitracin, spiromycin, and tylosin in 1999. Subsequently, antimicrobials banned by January 2006 included avilamycin, bambermycin, salinomycin and monensin (Hassan et al., 2010). Therefore, alternatives to antibiotics are of great interest to the poultry industry. These alternatives such as prebiotic (Cummings et al., 2002), probiotic (Jung et al., 2010), enzymes, herbal products and organic acid etc (Hassan et al., 2010) were studied extensively.

2.2 Organic Acids

As a group of chemicals, organic acids are considered to be any organic carboxylic acid, including fatty acids and amino acids, of the general structure R-COOH. The organic acids associated with specific antimicrobial activity eg. short-chain fatty acids (C1-C7) and medium-chain fatty acids (C6-C10) are either simple monocarboxylic acids such as formic, acetic, propionic and butyric acids, or carboxylic acids bearing an hydroxyl group (Dibner and Buttin, 2002).The use of short chain fatty acids (SCFA), medium chain fatty acids (MCFA) and other organic acids was largely based on their antimicrobial activity outside the intestinal tract (van Immerseel et al., 2006). SCFAs are important anions in the colonic lumen that influence gut morphology and function. In the Figure 2.2 show the impotant part in SCFA such as

colonic mucosal blood flow, ileal motility and mucosal cell proliferation (Scheppach, 1994). The effect of pH and acid concentration warrants particular attention. In the two-phase process, pH value is linked with acid concentration, which makes investigation of a separate effect of pH and acid concentration on pathogen survival difficult and found that to use high concentration of organic acid at pH 5.5-6.0 prevents a decrease of *Salmonella* spp. Concentration (Fukushi et al., 2003). Schineitz et al. (1998) demonstrated in rats that intraluminally infused VFA accelerated the crypt cell production rate and increased the gut-wall mass and the stimulation was most efficient with butyrate. The higher level of organic acids in combination with a low pH in crop and gizzard possibly form an improved bactericidal upper intestinal barrier (Heres et al., 2004).

Many researchers have been used to organic acids such as formic acid and propionic acid to reduce gut pH in broiler chickens (Iba and Berchieri, 1995; Alshawabkeh and Tabba, 2002). The antibacterial activity of these organic acids is achieved by influencing the cell structure and the cell metabolism as a result of reducing the pH in the alimentary tract below the growth range of the pathogenic

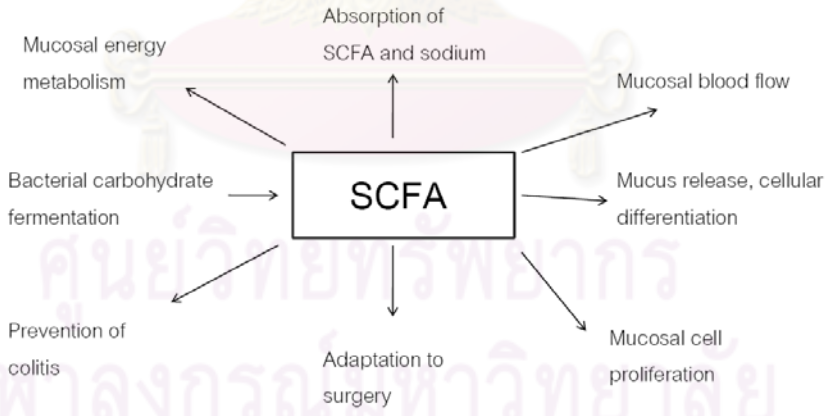


Figure 2.2 Effects of SCFA on colonic morphology and function (Scheppach, 1994)

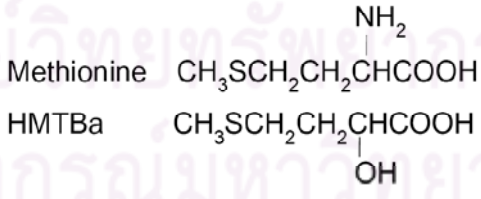
bacteria cells (Thompson and Hinton, 1997). Incorporation of formic acid and propionic acid had a disinfecting effect on contaminated feed and had sufficient antibacterial effect in the alimentary tract (Iba and Berchieri, 1995). In addition, it is effective in

preventing intestinal colonization of *Salmonella* from naturally or artificially contaminated feed (Tarazi and Alshawabkeh, 2003).

The antimicrobial mechanism of acids is related to the reduction in pH of the environment, which limits the growth of bacteria less tolerant to acidic pH. Moreover, undissociated organic acids can easily penetrate the lipid membrane of bacteria and moulds. In the cell, the organic acids release the protons in the alkaline cytoplasm, resulting in the decrease of intracellular pH. This alters enzymatic reactions and the nutrient transport system, forcing the bacterial cell to use energy to release protons and causes an intracellular acid anion accumulation (Rick, 2003; Hernández et al., 2006). Inside the cell, organic acids inhibit bacterial growth by breaking oxidative-phosphorylation and inhibiting the exchange of adenosine triphosphate (ATP). Export of excess protons requires consumption of cellular ATP and may result in depleting of cellular energy (Ricke, 2003).

2.3 Two-hydroxy-4-methylthio butanoic acid (HMTBa)

Two-hydroxy-4-methylthio butanoic acid or HMTB is a source of L-methionine in poultry diets (Dibner et al., 1990). HMTBa can be absorbed in the gastrointestinal (GI) tract of an animal, converted to L-methionine, and being used in protein synthesis (Richards et al., 2005). Knight and Dibner (1984) reported that the rate of HMTBa



absorption is equal to or greater than L-methionine uptake in vivo using intestinal uptake model. The liver is the major site of methionine metabolism, an amino acid vital for protein synthesis (Wang et al., 2001). Additionally, the liver is the major site for conversion of DL-HMTBa and D-methionine to the biochemically active form, L-methionine (Knight and Dibner, 1984).

HMTBa supplementation improved feed utilization and humoral and nonspecific immunocompetence of broiler chickens (Zhang and Guo, 2008). Furthermore, HMTBa is one of organic acid used to reduce *Salmonella* in broiler (Schasteen et al., 2007).

2.4 Methionine

Methionine (Met) is considered to be the most limiting amino acid in commercial corn-soy bean based broiler chicken diets (Zhang and Guo, 2008). Diet methionine deficiency led to impaired development of lymphoid organs and normal function (Carew et al., 2003). Furthermore Met is essential to proper growth performance in poultry diets (Richard et al., 2005). As study of Baker (2009) showed that small of cysteine are growth depressing in chicks fed methionine deficient diets.

The primary source of sulfur in broiler feces is dietary sulfur amino acids, with Met being the major dietary sulfur amino acid (Chavez et al., 2004). Venegas et al. (2006) showed that Cys and taurine synthesis after incubation with HMTBa is higher when compared with L-methionine incubation. Therefore, the data indicate that Cys and taurine formation by chicken enterocytes could be favored when HMTBa is used as a methionine source, thereby suggesting that the HMTBa might be preferentially diverted to the transsulfuration pathway.

2.5 Mechanism of sulfur amino acids on oxidative status

Moreover, studies conducted during the last 10 years indicate that amino acids act as regulators of metabolic pathways (i.e., the concept of “nutrient signal”), with, for instance, an effect targeted on mRNA translation (Métayer et al., 2008).

Methionine and cysteine hold very significant places among amino acids by playing numerous roles in protein metabolism. Like other amino acids, they are the components of tissue proteins and therefore serve as substrates for protein synthesis. They are also precursors of important molecules. For example, methionine participates

in methyl group metabolism and synthesis of other sulfur amino acids, notably cysteine (Figure 2.3). Cysteine is required for the synthesis of glutathione (GSH) and taurine, which are essential compounds for host defense against oxidative stress (Métayer et al., 2008).

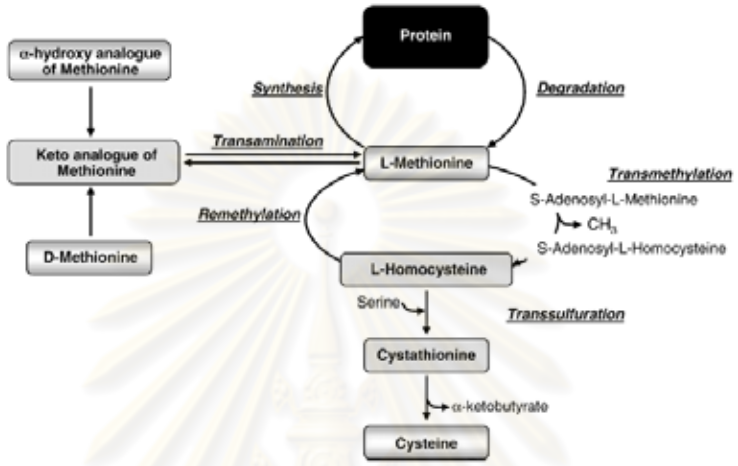


Figure 2.3 Methionine-cysteine metabolic pathways (Métayer et al., 2008).

Cysteine, taurine and GSH have a crucial role in oxidative stress conditions with their role mainly on cellular redox status (Shovller et al., 2005). GSH is an important intracellular antioxidant of animal body (Figure 2.4). GSH and cysteine had specific function as scavengers of reactive oxygen species (ROS) (Métayer et al., 2008).

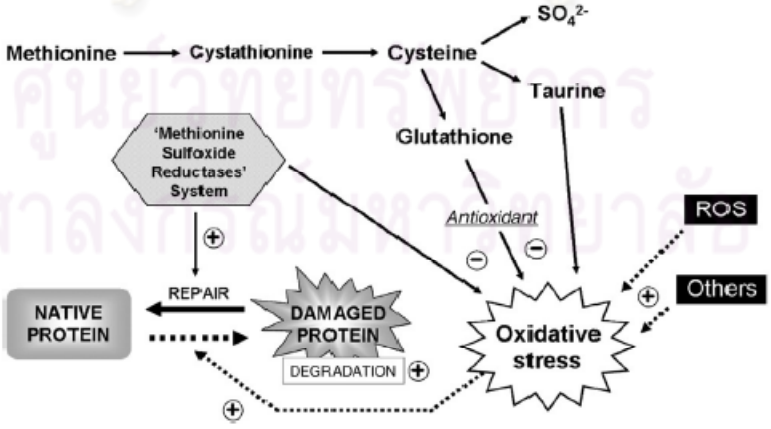


Figure 2.4 Sulfur amino acids: their role in the control of oxidative status (Métayer et al., 2008).

Almost all forms of reactive oxygen species (ROS) oxidize methionine residues of proteins to a mixture of the R- and S-isomers of methionine sulfoxide. Because organisms contain methionine sulfoxide reductases (Msr's) that can catalyze the thioredoxin dependent reduction of the sulfoxides back to methione. Surface-exposed methionine residues of proteins are readily oxidized to methionine sulfoxide [Met(O)] residues by many different forms of ROS. Reaction1where H_2O_2 is used as the ROS. However, unlike the oxidation of all other amino acid residues (except cysteine residues), the oxidation of Met residues is readily reversed by the action of an enzyme methionine sulfoxide reductase (Msr) that catalyzes the thioredoxin [$Th(SH)_2$] dependent reduction of Met (O) to form Met and oxidized thioredoxin [Th(S-S)] (Reaction 2).

$$\text{Met} + H_2O_2 \longrightarrow \text{Met (O)} + H_2O \quad (1)$$

$$\text{Met (O)} + \text{Th(SH)}_2 \xrightarrow{Msr} \text{Met} + \text{Th(S-S)} + H_2O \quad (2)$$

$$\text{Th(S-S)} + \text{NADPH} + H^+ \xrightarrow{ThR} \text{NADP}^+ + \text{Th(SH)}_2 \quad (3)$$

Sum 1.2.3:

$$H_2O_2 + \text{NADPH} + H^+ \longrightarrow \text{NADP}^+ + 2\,H_2O \quad (4)$$

It follows that when Reaction 1 and 2 are coupled with the NADPH dependent regeneration of $Th(SH)_2$ by the action of thioredoxin reductase (ThR) (Reaction 3), then the overall reaction is described by Reaction 4 (Stadtman et al., 2002).

In summary, cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism as followed (1) Methionine residues of proteins are readily oxidized to methionine sulfoxide [Met(O)] by any one of a number of different reactive oxygen species (ROS). (2) Organism contain methionine sulfoxide reductase (Msr's) that catalyze the thioredoxin [$Th(SH)_2$] dependent conversion of Met(O) to Met. (3) The cyclic interconversion of protein methionine residues between oxidized and reduced forms can lead to the scavenging of multiple forms of ROS. (4) Though not yet established, the cyclic interconversion of Met and Met (O) residues of some proteins may also have an important role in organisms either under normal or oxidative stress conditions (Stadtman et al., 2002)

CHAPTER III

MATERIALS AND METHODS

This study was approved by Animal Care and Use committee of the Faculty of Veterinary science, Chulalongkorn University.

3.1 Animals and Diets

The experiment was performed in a private broiler farm, Nakornpathom province. Total of 588 day-old, male, Arbor Acre broilers were obtained from an integrated company. Before the start of the experiment, cloacal swabs were randomly performed on sixty chicks (one sample/chick) and all samples were tested negative for all *Salmonella* spp. Total body weights of all chicks in each group were similar. Feed and water were given *ad libitum* for 42 days in all treatments. Eleven chicks in each replicate (groups 2, 3 and 4) were inoculated with 0.3 ml *S.*Enteritidis culture (10^8cfu/ml) at day 0 and 1 ml of 10^8 cfu/ml at day 7 post hatching, respectively. The chicks were fed on high-energy starter (day 1 to day 21), grower (day 22 to day 35), and finisher (day 36 to day 42) diets composed of corn and soybean meal as major ingredients. At days 18 to 21 and days 39 to 42, Celite, a source of acid-insoluble ash (AIA), was added to all diets (20 g/kg feed) as an indigestible marker. Drinking water was changed daily. Continuous fluorescence lighting was maintained throughout the experiment. The average max/min temperature and relative humidity in broilers house were 30/27 °C and 84.6/71.5%, respectively. The total experimental period was 42 days.

3.2 Experimental procedure

At day old, 588 chicks were allocated into 4 groups of 147 chicks (7 replicates of 21 chicks each).

Chicks in group 1 were received basal diet and given tap water until the end of the experiment (CON group). In group 2, chicks were received basal diet and given tap water until the end of the experiment (SE group). They were administered with 0.3 ml

$1x10^8$ cfu/ml nal^r *S*.Enteritidis. Chicks in group 3 were received basal diet and given tap water supplemented with formic acid and propionic acid (pH 4) at 0 to 14 day and 36 to 42 day (SE+ORAs group). They were inoculated with *S*.Enteritidis as in group 3. In group 4, chicks received basal diet and given tap water supplemented with mixed organic acids (formic acid, propionic acid and HMTBa) (pH 4) at 0 to 14 day and 36 to 42 day. *S*.Enteritidis was administered as in groups 2 and 3. The protocol of the experiment is shown in Figure 3.1.

3.2.1 Protocol of Experiment

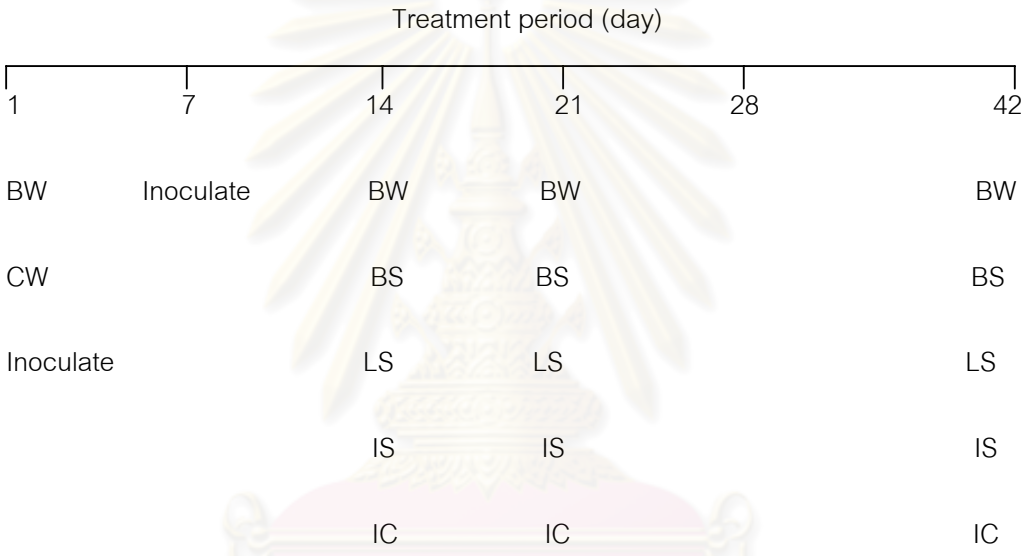


Figure 3.1 Diagram showing the whole period of experiment

BW = Weighing (body weight and feed)

CW = Cloacal swabs

BS = Blood sample collection

LS = Liver spleen pool sample collection

IS = Intestine sample collection (Duodenum, Jejunum, Ileum)

IC = collecting of ileal contents

3.3 Sample collection and tissue preparation

The chickens were weighed at days 1, 21, 35 and 42. The daily feed intake was recorded during days 0 to 21, days 22 to 35 and days 36 to 42. Mortality was recorded daily. Eleven chicks in each replicate at days 1 and 7 of age were inoculated with 0.3 ml and 1 ml, brain heart infusion broth (BHIB) culture of *S.*Enteritidis (nal^r) containing 10^8 colony forming units (cfu) by oral route using esophageal tube. Twenty eight chickens from each treatment group (4 chicks from one replicate) were randomly selected at days 14, 21 and 42 of age. They were sacrificed with intracardiac injection of pentobarbital sodium (120 mg/kg body weight) using 21G, 2.5 inch needle. The abdomen was exposed and the whole intestine from duodenum to cloaca was removed. The whole duodenal loop from pylorus to the entry of bile and pancreatic duct was excised. The intestinal section from the entry of pancreatic and bile duct to a section of Meckel's diverticulum was taken as the jejunum part. The ileal part was taken from Meckel's diverticulum to the ileo-caecal junction. The content in the ileum were collected into plastic bottles. The ileal contents from chickens in each replicate were pooled together and kept frozen at -20°C until analysis of nutrient digestibility. The liver and spleen sample were removed and placed in clean plastic bags for bacteriological study.

3.4 Bacteriological examination

Nalidixic acid resistant, field strain (nal^r) of *S.* Entetitidis, isolated from the chicken and obtained from the department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University was kept in stock agar at room temperature before use. It was transferred and streaked on Tryptic Soy Agar (TSA) plate and incubated at 37 °C for 18-20 h for examination of purity.

Cloacal swab samples using sterile cotton were placed into buffer peptone water (BPW) as pre-enrichment media were incubated at 37 °C for 18-20 h. The 0.1 ml of BPW was then dropped on to MSRV (Modified Semi-Rappaport Vassiliadis) and incubated at 42°C for 24-48 h. The suspected colonies from MSRV were transferred and incubated at 37°C for 24 h. The pink colonies with black spot on the XLT_4 (Xylose Lactose Tergitol™ 4) agar plate was suspected of *S.*Enteritidis. They were tested for biochemical

assays including glucose fermentation, hydrogen sulfide gas production from TSI (Triple sugar iron) agar, motility test, lysine decarboxylation and indole production were detected from MIL (Motility indole lysine medium) Serogrouping of *S.* Enteritidis (Serogroup D) was confirmed by a slide agglutination test using antiserum of *Salmonella* O polyvalent group A to 67, and specific serogroup D.

3.4.1. Qualitative and semi-quantitative culture of *S.*Enteritidis

Liver-spleen pool and ileo-cecal content was aseptically removed from each chick. The liver-spleen pool was weighed, chopped and put into BPW with 2 volumes of weight of liver-spleen pool, then blended in a stomacher. From the initial 10^{-1} dilution, 10-fold serial dilutions were made in BPW at dilutions of 1:100, 1:1000 and spread-plated onto XLT_4 agar plates plus 50 µg/ml of nalidixic acid. The plates were incubated for 24 h at 37°C *and S.*Enteritidis colonies were identified by biochemical test. (Chotikathum, 2005)

For all samples, a pre-enrichment incubation at 37°C for 18 h was performed on series of preparation comprising the primary mixture in BPW('0'), a separate 10-ml aliquot of the same ('1'), plus the decimal dilutions ('2' to '7'). After incubation, 0.1 ml of each of the preparation '0' and '1' were inoculated onto modified semisolid Rappaport-Vassiliadis (MSRV) agar with 0.01% novobiocin and incubated at 42°C for 16-24 h. Preparations '3' to '7' were refrigerated. Where spreading opaque growth was seen on MSRV, a 1 µl loop from the edge of the growth was inoculated onto XLT_4 agar plus50 µg/ml of nalidixic acids. XLT_4 plates were incubated at 37°C for 16-24 h. The plates were examined and any MSRV plates on which the growth had spread widely, but which were negative for Salmonella on XLT_4 plate plus 50µg/ml of nalidixic acids, were replated onto XLT_4.

If *Salmonella* was isolated from either of the preparations '0' or '1' from any sample, then each element of the corresponding dilution series '2' to '7" was cultured using the MSRV/ XLT_4 method.

The likely density of Salmonella in a sample was quantified in tenfold bands by reference to the QS 'score', this being +1 the designated number of the most dilute pre-enrichment broth that yielded a positive result.

For solid sample, the calculated relationship was

Salmonella density ($CFUg^{-1}$) = $10^{(QS\ score-2)}$ to $10^{(QS\ score-1)}$

Where there was no growth in any dilution, the QS score and *Salmonella* count were taken to be zero. (Wales et al., 2005)

3.5 Histomorphological study (Xia et al., 2004)

To determine the histomorphological changes of small intestine of chicks, the segments of duodenum, jejunum and ileum were sectioned and stained with hematoxylin and eosin (H&E) and then the villus height and the crypt depth were measured. Briefly, the tissues were placed in 10% buffer neutral formalin, dehydrated and embedded in paraffin. Transverse sections were cut at 4-6 μm thickness and stained with Harris' Alum Hematoxylin and counterstained with eosin following standard protocol. The photomicrographs were taken under light microscope at x 4 magnification. Height of villi and depth of crypts were measured using Scion Image Software (Scion image; Scion Corporation, Frederick, MD).

The measurement of villus height and crypt depth was followed the methods described by Martin-Rodrigues et al. (2007). The villus height was measured from the top of the villus to the villus-crypt junction and crypt depth was measured from the villus-crypt junction to the base of the crypt. Ten villi measurements were taken per section (6 sections on slide/treatment) from each part of intestine. Villus/Crypt ratio was calculated.

3.6 Determination of nutrient digestibility and acid-insoluble ash (Chotikatum, 2005)

Celite was added as a marker for the determination of apparent ileal digestibility of nutrients. Acid-insoluble ash in Celite was measured as described by Choct and

Annison (1992). Approximately 2 g of dried diet and 1 g of dried digesta samples from grounding were weighed into sintered glass crucibles (Pyrex®, England). These sintered glass crucibles were dried at 105°C for 8 h and weighed as dry matter sample. Later, the sample was ashed at 550 °C for 8 h. After ashing, the crucibles were cooled, and boiled slowly in 4 N HCl for 30 min on a hot plate in fume hood. The ash in crucibles was washed with distilled water using suction pump, and dried at 105°C for 6 h. The residue in crucibles was repeatedly ashed and boiled in the same way. Finally, the ash in crucibles was dried at 105 °C for 6 h, the crucibles were cooled in a desiccator and weighed while containing the ash. Percentage of acid-insoluble ash was calculated using the following equation:

$$AIA\ (\%) = \frac{Wf - We \times 100}{Ws}$$

Where Wf = weight of crucible with ash

We = weight of empty crucible

Ws = weight of sample (dry matter)

The percentage of ileal digestibility(ID) of nutrients eg. (protein and fat) was calculated using the following equation:

$$ID = 1 - \frac{(\text{Ileal nutrient (\%)} / \text{Ileal acid insoluble ash (\%)}) \times 100}{(\text{Diet nutrient (\%)} / \text{Diet acid insoluble ash (\%)})}$$

3.7 Calculation of the growth performance

Chicks were weighed at 0, 21, 42 days old. The feed intake was recorded during at 0-21, 22-35 and 36-42 days old chicks. Number and body weight of dead broilers were recorded for calculation of mortality rate and feed conversion ratio (FCR), respectively. Finally, FCR was calculated by total pen feed (gram) divided by total weight gain (gram).

Feed intake (g/b) = Total feed intake / final chick numbers

Daily feed intake (DFI, g/b/d) = Feed intake / days

Total weight gain (g/b) = Final body weight – Initial body weight

Average body gain (ADG, g/b/d) = Body weight gain / days

Mortality rate (%) = (Number of dead chicks / total chick numbers) x 100

Feed conversion ratio = $\frac{\text{Total pen feed intake}}{\text{Total body weight gain}}$
(FCR, Kg feed intake/Kg body weight gain)

3.8 Antioxidant enzyme and malondialdehyde analysis

3.8.1 Determination of glutathione peroxidase (Bolcal et al., 2007)

The amount of 0.3 grams of liver sample were homogenized with 4.5 ml of 50 mM potassium phosphate buffer by hand homogenizer. The liver homogenate were centrifuged at 1,500 g, 4°C for 30 minutes. Then, the supernatant and serum (0.3 ml) were diluted to 1:5 ratio. The enzyme activity in the resulting supernatant and protein concentrations were determined.

The amount of 43 µl of supernatant was incubated with start reagent (25 µl of 8.4 mM NADPH in 1% $NaHCO_3$ and 48 µl of 2.2 mM of H_2O_2). The optical density was read at wavelength 340 nm using UV-VIS spectrophotometer (Shimadzu UV 1201, 1 cm light path) as a first value. Then 625 µl of 5.8 mM reduced glutathione in 50 mM phosphate buffer and 25 µl of 10 U/ml glutathione reductase in 50 mM phosphate buffer in 0.5 mM EDTA and 1 mM NaN_3 were added.

The absorbance was read as the rate of disappearance of NADPH every 15 seconds for 1 minute. Reading was made against reagent blank.

One unit of glutathione peroxidase activity was defined as the amount of glutathione peroxidase that transformed 1 mmol NADPH to NADP per minute in

experimental condition. The specific activity was expressed in mUnit per mg of liver protein

The glutathione peroxidase activity was obtained by the following formula.

$$\text{GSH-px activity (mUnit)} = ((\Delta 340/\text{min/extinction coefficient}) \times D \times d \times (\underline{TV})) \times \frac{SV}{SW}$$

WhereExtinction coefficient = 0.0622

D = dilution factor

d = light path in cm

TV = total volume (ml)

SV = sample volume (ml)

SW = sample weight (mg)

3.8.2 Determination of glutathione (Beutler et al., 1963)

Liver (0.3 g) and serum (0.3 ml) were suspended in 1.8 ml of 100 mM KCl plus 0.003 M EDTA and homogenized as described above for glutathione peroxidase. The homogenates were centrifuged at 600 g for 10 minutes. The 1 ml of supernatant was added to 1.5 ml metaphospholic acid, and particulate debris was removed by the centrifugation at 3,000 g for 10 minutes. Reduced GSH was measured by adding 500 µl of supernatant to 2.0 ml of 0.2 M phosphate buffer and 0.25 ml 0.04% 5,5' dithiobis 2-nitrobenzoic acid. The absorbance was read at 412 nm. GSH (sigma) was used as the external standard. GSH content was expressed as nanomole of GSH per milligram homogenate protein.

3.8.3 Determination of malondialdehyde concentration (Ohkava et al., 1979)

The amount of 0.3 grams of liver sample and serum were homogenized with 1.5 ml of 1.15% of potassium chloride phosphate buffer by hand homogenizer. The liver

homogenate and serum were centrifuged at 3,000 g, 4°C for 30 minutes. Then, the malondialdehyde concentration and protein concentration were determined.

The amount of 300 µl of supernatant was put into 15 µl plastic tube with cover. 50 µl of 50 mM BHT, 50 µl of distilled water, 100 µl of 8.1% of SDS, 750 µl of 20% acetic acid, pH 3.5 and 750 µl of 0.5% TBA in 0.02 M NaOH were added. The solution was mixed and boiled at 95°C for 60 minutes. After that, it was cooled at room temperature for 10 minutes and 2 ml butanol:pyridine (15:1) was added and mixed for 1 minute. Then, solution was centrifuged at 5,000 rpm for 10 minutes and 1.7-1.8 ml of upper layer (pink) was removed into 2 ml micro tube, the micro tube was centrifuged at 12,000 rpm for 10 minutes and 1.2 ml sample was removed into a microcuvette. The optical density was read at wavelength 532 nm by using UV-VIS spectrophotometer (Shimadzu UV 1201, 1 cm light path). Reading was made against the blank.

The standard curve was plotted using the malondialdehyde at 0, 5,10,20,40 and 60 nmol/ml. The curve's slope was used to calculate the concentration of malondialdehyde.

The specific concentration was expressed in nmol per mg of liver protein.

3.9 Data analysis

All data were presented as Mean ± S.E.M. The effects of treatment were analyzed using One-way Analysis of Variance (ANOVA). Duncan's New Multiple Range test was used to compare the individual means. Data that were not meet the assumption on normal distribution or equal variance test were proceeded to appropriate non-parametric Kruskral Wallis statistical analysis. The qualitative Salmonella test was analyzed using Chi-square test. The level of statistical significance was $P<0.05$

CHAPTER IV

RESULTS

4.1 Effect of treatment on growth performance.

Growth performance of chicks during starter period (days 1-21 of age) is shown in Table 4.1, Figure 4.1 and 4.2. At day 21, ADG in SE+ (ORAs+HMTBa) group was higher than SE+ ORAs and SE groups. In addition, the lowest ADG was demonstrated in SE group and the highest found in control group. Feed and water intake were not different among all groups. Chicks in (ORAs+ HMTBa) group had slightly better FCR (P> 0.05) than SE+ ORAs and SE group. The mortality of chicks was not different among groups of birds with the lowest found in SE+ (ORAs+ HMTBa) group.

Table 4.1 Effect of various treatments on growth performance[1] of the broiler chickens during starter period (Days 1-21)

	Treatment[2]			
	CON	SE	SE+(ORAs)	SE+ (ORAs+ HMTBa)
Initial weight (g/b)	48.43± 0.17	48.43± 0.17	48.43± 0.17	48.43± 0.17
ADG (g/b/d)	34.61± 1.01[a]	32.13± 0.51[b]	32.25± 0.66[b]	33.22± 0.63[ab]
DFI (g/b/d)	43.41± 0.19	43.21± 0.24	43.49± 0.28	43.14± 0.17
WI (ml/b/d)	107.42 ± 16.06	106.89± 0.79	100.40± 3.31	101.37± 6.36
FCR	1.26± 0.03[b]	1.35± 0.02[a]	1.35± 0.02[a]	1.30± 0.02[ab]
Mortality (%)	2.04± 0.96	1.36± 0.87	2.04± 1.41	0.68± 0.68

[1]Mean± SE; n=7

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml; ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Means in the same row with unlike superscripts differed significantly (P<0.05).

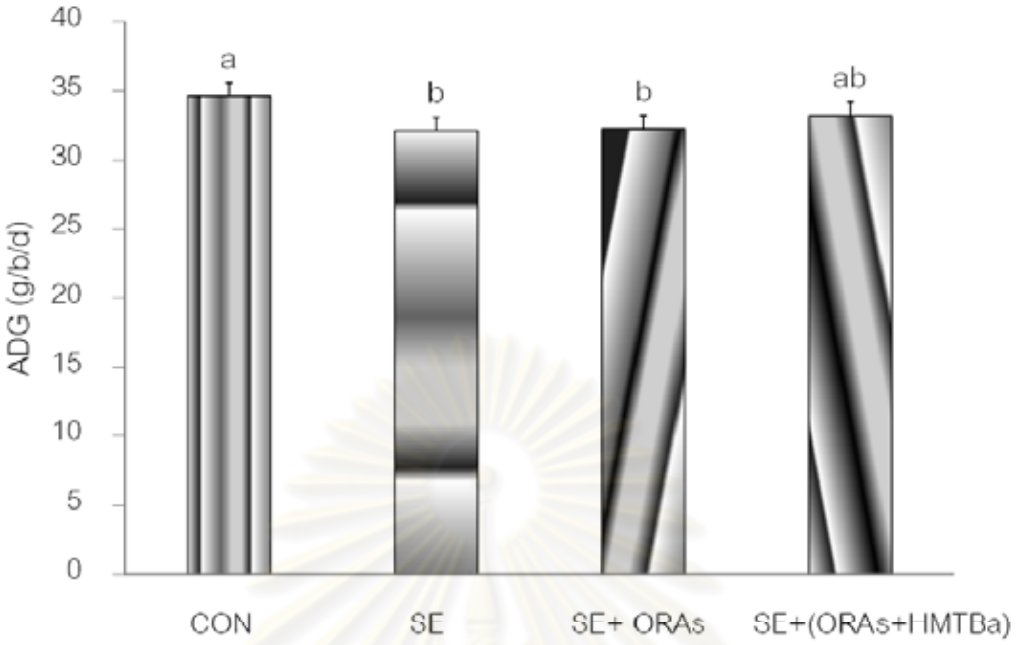


Figure 4.1 Effect of various treatments on average daily gain (g/b/d) of broiler chickens during days 1-21 of age.

[a,b] Different superscripts mean significantly different (P<0.05)

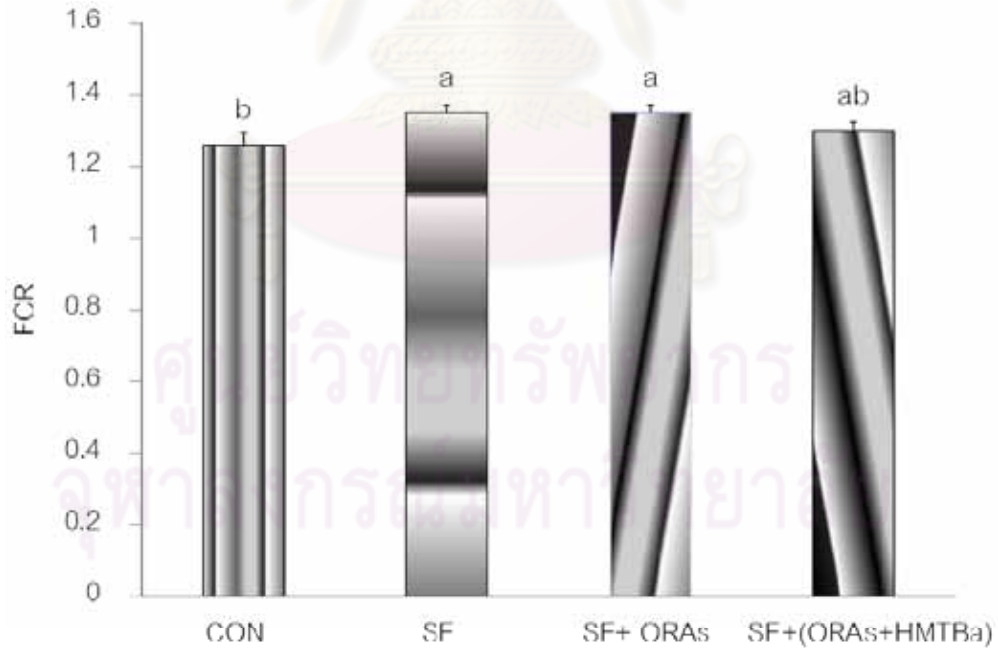


Figure 4.2 Effect of various treatments on feed conversion ratio of broiler chickens during days 1-21 of age.

[a,b] Different superscripts mean significantly different (P<0.05)

Table 4.2 depicted growth performance of chicks in grower-finisher period. It is showed that feed and water intake of all groups were not different. The ADG and FCR of chicks in SE group were also slightly better than other groups (P> 0.05)

Table 4.2 Effect of various treatments on growth performance[1] of the broiler chickens during grower-finisher period (Days 22-42)

	Treatment[2]			
	CON	SE	SE+(ORAs)	SE+ (ORAs+ HMTBa)
Initial weight (g/b)	874.7± 13.08	850.5± 16.26	852.7± 20.03	887.9±25.39
ADG (g/b/d)	77.41± 1.36	78.35± 1.25	76.29± 1.39	75.32± 1.02
DFI (g/b/d)	138.68± 1.54	134.35± 1.05	134.94± 2.35	136.51± 1.69
WI (ml/b/d)	350.10± 5.52	356.62± 6.34	348.07± 4.31	340.58± 4.94
FCR	1.79± 0.02	1.72± 0.03	1.77± 0.03	1.81± 0.03
Mortality (%)	0.84± 0.84	0	0	0.84± 0.84

[1]Mean± SE; n=7

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml; ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Mean in the same row with unlike superscripts differed significantly (P<0.05).

Growth performance of chicks in the total period (days 1-42 of age) of study is shown in Table 4.3, Figure 4.3. ADG of both organic acid groups were significantly lower compared with control group. Feed and water intake of all groups were not-significantly different. The highest FCR was found in SE +ORAs group when compared with negative control (P < 0.05).

Table 4.3 Effect of various treatments on growth performance[1] of the broiler chickens during total period of study (Days 1-42)

	Treatment[2]			
	CON	SE	SE+(ORAs)	SE+ (ORAs+HMTBa)
Initial weight (g/b)	48.4± 0.17	48.4± 0.17	48.4± 0.17	48.4± 0.17
Final weight (g/b)	2417.0± 58.87	2318.2± 47.34	2317.4± 82.75	2278.7± 44.15
ADG (g/b/d)	38.22± 0.40 [a]	37.44± 0.27 [ab]	36.79± 0.44 [b]	36.85± 0.31 [b]
DFI (g/b/d)	64.63± 0.54	63.19± 0.42	63.51± 0.83	63.83± 0.51
WI (ml/b/d)	162.08 ± 8.57	163.83± 2.22	157.93± 2.27	156.10± 3.08
FCR	1.69± 0.03	1.69± 0.01	1.74± 0.01	1.73± 0.01
Mortality (%)	2.72± 0.96	1.36± 0.88	2.72± 1.75	0.68± 0.68

[1] Mean± SE; n=7

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml; ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,c] Means in the same row with unlike superscripts differed significantly (P<0.05).

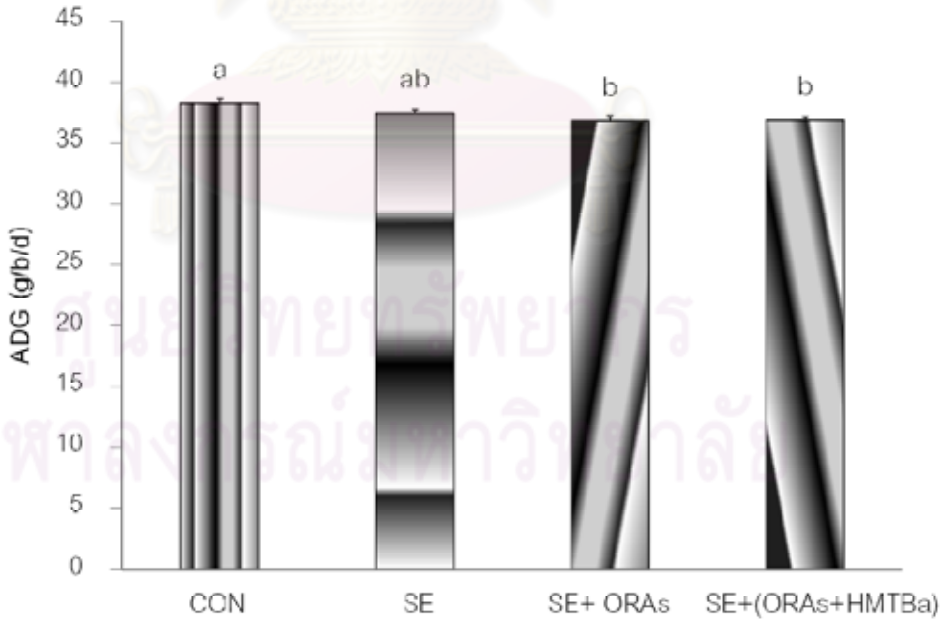


Figure 4.3 Effect of various treatments on average daily gain (g/b/d) of broiler chickens during 1-42 days of age.

[a,b] Different superscripts mean significantly different (P<0.05)

4.2 Qualitative and quantitative examination of SE

It is shown that 7 days after SE inoculation, chicks in SE groups and SE+ ORAs showed the highest positive samples and SE counts in liver and spleen pool (Table 4.4). For those SE inoculated chicks, the detection rate was higher than 50% in all groups. For non-inoculated chicks, the detection rate was lower especially those groups with organic acid supplement. The lowest SE positive sample was found in negative control group (3/28). The SE count of both inoculated and non-inoculated chick showed that SE+ (ORAs+ HMTBa) group significantly reduced the $\log_{10}$ SE count ($P<0.05$) compared to the positive control (SE group) but still higher ($P<0.05$) than negative control (CON group). At day 21 of age (14 days after SE inoculation), lower *Salmonella* counts to nil detection were demonstrated in all groups except the positive control group and those received ORAs in inoculated chicks. For those inoculated chicks at day 21, the qualitative detection rate was highest in SE + (ORAs) group. The background detection around 14.3% was found in negative control group due to some contamination during the study.

From Table 4.5, at day 14, SE counts in the gut (ileo-cecal contents) were highest in positive control groups in both inoculated and non-inoculated chicks. Organic acid supplementation in both groups 3 and 4 decreased SE count with significant ($P<0.05$) changes of non-inoculated chick in group 4. It is interesting to note that there was no SE count in inoculated chick but some counts were existed in non-inoculated chicks. The detection rate (number of positive sample) was highest in SE group for inoculated chicks. Chicks in group 4 (SE+ (ORAs+ HMTBa)) had numerically higher positive samples in non-inoculate than other group ($P>0.05$). At day 21, less *Salmonella* counts were depicted in non-inoculated chicks there were no significantly different ($P>0.05$) among groups. The qualitative detection rate was higher in chicks fed SE+ ORAs in water for inoculated chicks. The nil detection in non-inoculated chicks was also demonstrated.

Table 4.4 Effect of treatments on qualitative and quantitative SE analysis in liver-spleen pool[1] of broiler chicks

Group[2]	Day 14						Day 21					
	Non-inoculated			Inoculated			Non-inoculated			Inoculated		
	Positive/ Total sample	%[2]	*Salmonella* count (log_{10} cfu/g)	Positive/ Total sample	%	*Salmonella* count (log_{10} cfu/g)	Positive/ Total sample	%	*Salmonella* count (log_{10} cfu/g)	Positive/ Total sample	%	*Salmonella* count (log_{10} cfu/g)
CON	3/28	10.71^{b}	0.28 ±0.13^{b}				4/28	14.29	0.00			
SE	8/14	57.14^{a}	2.57 ± 0.37^{a}	9/14	64.29	3.43 ± 0.37^{a}	5/14	35.71	0.14 ±0.14	5/14	35.71	0.43 ±0.20
SE+(ORAs)	5/14	35.71^{ab}	2.57 ± 0.53^{a}	8/14	57.14	3.57 ± 0.20^{a}	2/14	14.29	0.00	10/14	71.43	0.29 ±0.18
SE+(ORAs+ HMTBa)	4/14	28.57^{ab}	1.29 ± 0.42^{b}	8/14	57.14	2.14 ± 0.14^{b}	1/14	7.14	0.00	5/14	35.7	0.00

[1]Mean± SE; n=7 (Salmonella count), n=14 (Qualitative analysis)

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^{8} cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Mean in the column row with unlike superscripts differed significantly ($P<0.05$).

Table 4.5 Effect of treatments on qualitative and quantitative SE analysis in ileo-cecal content[1] of broiler chicks

Group[2]	Day 14						Day 21					
	Non-inoculated			Inoculated			Non-inoculated			Inoculated		
	Positive/ Total sample	%[2]	*Salmonella* count ($\log_{10}$ cfu/g)	Positive/ Total sample	%	*Salmonella* count ($\log_{10}$ cfu/g)	Positive/ Total sample	%	*Salmonella* count ($\log_{10}$ cfu/g)	Positive/ Total sample	%	*Salmonella* count ($\log_{10}$ cfu/g)
CON	2/28	3.57^{b}	1.42 ± 0.43^{ab}				2/28	7.14	0.00			
SE	4/14	28.57^{ab}	2.71 ± 0.42^{a}	9/14	64.28	2.86 ± 0.83	1/14	7.14	0.14 ± 0.143	1/14	7.14^{b}	0.28 ± 0.184
SE+(ORAs)	6/14	42.86^{ab}	1.43 ± 0.37^{ab}	4/14	28.57	2.43 ± 0.65	0/14	0.00	0.00	0/14	0.00^{b}	0.14 ± 0.143
SE+(ORAs+HMTBa)	9/14	64.29^{a}	1.00 ± 0.53^{b}	4/14	28.57	1.14 ± 0.55	0/14	0.00	0.00	5/14	42.86^{a}	0.57 ± 0.429

[1]Mean± SE; n=7 (Salmonella count), n=14 (Qualitative analysis)

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^{8} cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,]Mean in the column row with unlike superscripts differed significantly (P<0.05).

4.3 Effect of treatment on apparent ileal digestibility of protein and fat.

Ileal digestibility of protein

The ileal digestibility of protein of the broiler chickens is demonstrated in Table 4.6. At day 21, it was found that the ileal digestibility of protein in SE+ ORAs and SE+ (ORAs+ HMTBa) groups were markedly higher ($P<0.05$) than positive control group. However, at day 42 it was found that the ileal digestibility of protein in broiler chickens were not different ($P>0.05$) among groups of broiler chickens

Ileal digestibility of fat

Similar finding was demonstrated in percentage of the ileal digestibility of fat (Table 4.6). At day 21, it was found that the ileal digestibility of fat in broiler chickens that received both organic acids were better than positive control group ($P<0.05$) and slightly better than those chicks that were not inoculated with SE (group 1).

Likewise, at day 42, the lieal digestibility of fat in broiler chickens were not different among groups of broiler chickens. However, positive control group (group 2) was slightly higher than other groups.

Table 4.6 Effect of treatments on apparent ileal digestibility of protein and fat [1] of the broiler chickens

		Treatment[2]			
		CON	SE	SE+ ORAs	SE+ (ORAs+HMTBa)
Protein (%)					
	Day 21	84.18±1.15^{ab}	81.40±1.28^{b}	87.23±0.19^{a}	87.15±1.28^{a}
	Day 42	76.90±1.12	80.21±1.89	78.59±1.37	79.95±0.52
Fat (%)					
	Day 21	85.17±1.10^{ab}	82.36±1.31^{b}	88.10±0.16^{a}	87.74±1.22^{a}
	Day 42	81.59±0.80	84.22±1.50	82.49±1.09	83.79±0.44

[1]Mean± SE; n=7

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml; ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,]Mean in the same row with unlike superscripts differed significantly (P<0.05).

4.4 Effect of various treatments on intestinal morphology.

The histomorphometry of each intestinal parts of broiler chickens at day 14 is demonstrated in Table 4.7. The villus height and villus/ crypt ratio in duodenum jejunum and ileum were not significantly different among groups of broilers. Furthermore, it is shown that the crypt depth in all parts of intestine were not different in all groups except ileal mucosa that CON group had the highest crypt depth (P<0.05).

At day 21, it is demonstrated that villus height in duodenum, jejunum and ileum of all groups were not different (Table 4.8). Crypt depths in duodenal and ileal parts in SE group were higher than other groups. For jejunum, crypt depth was not differed among groups. Villus/ crypt ratio of duodenal part of SE group was lower than other groups (P<0.05).

Table 4.7 Effect of mixed organic acids on the intestinal morphology[1] of broiler at day 14

	Duodenum			Jejunum			Ileum		
Treatment[2]	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt
CON	1148.97±57.76	76.85±3.76	15.15±1.13	898.49±54.44	69.11±3.63	13.23±1.14	764.38±40.30	87.09±5.74[a]	8.85±0.45
SE	1096.72±37.57	73.06±2.82	15.14±0.86	890.29±75.03	78.40±4.06	11.39±0.85	652.17±40.31	72.19±2.15[b]	9.12±0.76
SE+ ORAs	1223.05±66.31	79.79±2.72	15.36±0.76	869.11±69.89	76.23±2.25	11.33±0.64	693.36±56.99	73.97±1.61[b]	9.38±0.75
SE+ (ORAs+ HMTBa)	1212.91±53.45	79.48±2.10	15.26±0.48	948.79±65.23	78.82±3.28	12.06±0.77	680.96±74.08	69.14±2.28[b]	9.85±1.08

[1]Mean± SE; n=6

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Means in the same column with unlike superscripts differed significantly (P<0.05).

Table 4.8 Effect of mixed organic acids on the intestinal morphology[1] of broiler at day 21

	Duodenum			Jejunum			Ileum		
Treatment[2]	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt
CON	1351.40±44.64	96.25±2.94^{b}	14.13±0.70^{a}	978.66±37.65	85.48±4.35	11.61±0.72	681.00±60.09	71.80±4.17^{b}	9.71±1.07
SE	1232.02±41.07	121.78±13.55^{a}	10.70±1.15^{b}	940.78±56.02	94.10±11.33	11.69±3.03	702.96±33.03	88.47±4.19^{a}	8.01±0.42
SE+ ORAs	1283.22±39.19	98.59±5.98^{b}	13.29±0.98^{ab}	909.41±54.22	96.14±5.71	9.73±1.03	686.66±36.28	76.69±4.91^{ab}	9.11±0.68
SE+ (ORAs+ HMTBa)	1262.37±39.67	96.07±1.21^{b}	13.17±0.56^{ab}	1063.33±58.18	97.68±3.50	10.90±0.49	768.06±52.73	84.10±4.30^{ab}	9.16±0.58

[1]Mean± SE; n=6

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^{8} cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Means in the same column with unlike superscripts differed significantly ($P<0.05$).

Table 4.9 Effect of mixed organic acids on the intestine morphology[1] of broiler at day 42

	Duodenum			Jejunum			Ileum		
Treatment[2]	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt	Villus height (μm)	Crypt depth (μm)	Villus /Crypt
CON	1593.65±63.43^{ab}	101.90±9.28	16.25±1.52	1269.40±78.14	115.24±5.32^{a}	11.10±0.75	913.42±80.41	91.09±4.93^{a}	10.12±0.90
SE	1791.70±67.20^{a}	116.82±8.46	15.72±1.18	1310.54±56.07	98.64±4.37^{b}	13.39±0.70	851.82±13.78	76.91±3.57^{b}	11.18±0.47
SE+ ORAs	1500.26±36.84^{b}	100.64±1.24	14.93±0.51	1168.65±55.25	104.15±3.83^{ab}	11.27±0.60	775.45±37.82	83.08±2.49^{ab}	9.35±0.46
SE+ (ORAs+ HMTBa)	1587.35±89.51^{ab}	111.29±14.13	15.23±1.78	1046.51±87.36	92.89±3.62^{b}	11.15±1.97	849.34±66.40	82.74±3.08^{ab}	10.26±0.74

[1]Mean± SE; n=6

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^{8} cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

^{a,b,c} Means in the same column with unlike superscripts differed significantly ($P<0.05$).

Table 4.9 demonstrated the intestinal morphology of the broiler chickens at day 42. It is depicted that villus/crypt ratio in duodenum, jejunum and ileum of all groups were not different. Villus height of jejunum and ileum were not significant in all groups. In contrast, duodenal mucosa in SE group was higher than other groups ($P<0.05$). Crypt depths of jejunum and ileum in CON group were higher than other groups.

4.5 Effect of treatments on antioxidant enzyme activity and MDA concentrations

The antioxidant enzyme activities and MDA concentrations of broiler at day 14, 21 and 42 are shown in Tables 4.10, 4.11 and 4.12 respectively.

At day 14, GSH-px of serum and liver were not significantly ($P>0.05$) changed among groups. However serum GSH-px in SE group (group 2) tended to be the lowest ($P>0.05$) compared to other groups. Those organic acid supplement groups had higher GSH-px to the level found in negative control group.

Results of GSH of serum and liver were found to be similar to GSH-px ($P>0.05$). Additionally, there were a trend of increasing serum and liver GSH in SE+ ORAS and SE+ (ORAs+ HMTBa) groups. The lowest of GSH in serum and liver was demonstrated in SE and CON group, respectively.

Furthermore, MDA of serum and liver were not different among groups. It is found that SE group had the highest MDA ($P>0.05$) than other groups. Chicks in SE+ (ORAs+ HMTBa) group had the lowest MDA in serum while SE+ ORAs group had the lowest MDA levels in liver.

Table 4.10 Effect of treatments on antioxidant enzyme activity and MDA concentrations[1] of broilers at day 14

		Treatment[2]			
		CON	SE	SE+ ORAs	SE+ (ORAs+ HMTBa)
GSH-px	(mU/mg protein)				
	Serum	212.50±28.12	160.76±19.64	213.60±21.40	216.62±8.00
	Liver	233.97±19.08	230.30±22.96	253.16±11.42	266.42±26.93
GSH	(nmol/mg protein)				
	Serum	10.59±0.99	9.95±0.13	11.21±1.35	12.64±1.46
	Liver	56.43±7.76	59.03±7.02	78.61±16.94	76.52±16.31
MDA	(nmol/mg protein)				
	Serum	1.82±0.96	2.38±1.43	1.76±0.61	1.21±0.31
	Liver	1.67±0.25	1.86±0.17	1.67±0.20	1.71±0.50

[1]Mean± SE; n=4

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,] Mean in the same row with unlike superscripts differed significantly ($P<0.05$).

At day 21, GSH-px in serum and liver were not different among groups. It is depicted that CON group had the highest GSH-px than other groups ($P>0.05$). In addition SE+ (ORAs+ HMTBa) group had higher GSH-px than SE and SE+ ORAs group. The lowest GSH-px level was showed in SE group.

In addition, the highest GSH concentration was discovered in SE+ (ORAs+ HMTBa) group in both serum and liver. Furthermore, the lowest GSH in serum and liver was found in SE group.

Likewise, MDA in serum and liver was not significantly ($P>0.05$) different among groups. SE group had the highest MDA than other groups. In serum, the lowest MDA group was found in CON group while chicks in and SE+ ORAs group have the lowest of MDA in liver.

At day 42, GSH-px in serum of CON and SE+ ORAs groups was significantly higher ($P<0.05$) while the levels in liver were quite similar among groups.

GSH of serum and liver during this period was not different ($P>0.05$) among groups. The serum GSH in SE+ ORAs was highest compared to other groups. Chicks in SE+ (ORAs+ HMTBa) group had the highest GSH in the liver than other groups. The lowest of GSH in serum and liver was found in SE and CON groups.

Finally, serum and liver MDA were not significantly different among groups. SE group had the highest serum MDA than other groups. Moreover, the lowest MDA was depicted in SE+ (ORAs+ HMTBa) group in serum and CON group have the lowest in liver.

Table 4.11 Effect of treatments on antioxidant enzyme activity and MDA concentrations[1] of broilers at day 21

		Treatment[2]			
		CON	SE	SE+ ORAs	SE+ (ORAs+ HMTBa)
GSH-px	(mU/mg protein)				
	Serum	224.68±13.84	165.05±24.08	169.86±24.10	171.01±14.29
	Liver	270.50±20.14	264.45±28.90	266.42±24.92	267.74±24.65
GSH	(nmol /mg protein)				
	Serum	3.25±1.22	1.51±0.37	1.95±0.64	2.61±0.78
	Liver	48.05±13.64	45.09±15.55	46.67±16.00	49.49±13.21
MDA	(nmol/mg protein)				
	Serum	0.11±0.05	0.28±0.18	0.28±0.17	0.14±0.07
	Liver	1.67±0.25	1.86±0.17	1.67±0.20	1.71±0.50

[1]Mean± SE; n=4

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b,]Mean in the same row with unlike superscripts differed significantly (P<0.05).

Table 4.12 Effect of treatments on antioxidant enzyme activity and MDA concentrations[1] of broilers at day 42

		Treatment[2]			
		CON	SE	SE+ ORAs	SE+ (ORAs+ HMTBa)
GSH-px (mU/mg protein)					
	Serum	274.05±16.88[a]	145.52±27.07[b]	257.47±28.32[a]	169.18±29.70[b]
	Liver	298.29±16.04	242.33±18.96	247.32±22.97	247.23±21.67
GSH (nmol /mg protein)					
	Serum	4.03±0.70	4.38±0.32	4.81±1.33	4.25±1.22
	Liver	54.76±8.97	37.43±0.71	44.23±9.91	57.54±8.40
MDA (nmol/mg protein)					
	Serum	0.11±0.02	0.16±0.06	0.10±0.01	0.08±0.03
	Liver	0.69±0.34	1.02±0.20	1.19±0.42	0.84±0.25

[1]Mean± SE; n=4

[2] Treatments were CON: control; SE: *Salmonella Enterica* serovar Enteritidis 10^8 cfu/ml;

ORAs: Formic acid and Propionic acid; (ORAs+ HMTBa): Formic acid, Propionic acid and HMTBa (2-Hydroxy-4-Methylthio Butanoic acid)

[a,b]Mean in the same row with unlike superscripts differed significantly ($P<0.05$).

CHAPTER V

DISCUSSION

Starter period (1-21 days)

In this study, the horizontal transmission of SE was demonstrated. Half populations of chicks (11 chicks form 21 chicks) were inoculated with SE. (nal^r) at days 1 and 7. At day 14, it is discovered that SE was detected in both non-inoculated and inoculated groups in liver and spleen pool as well as ileo-cecal contents. The horizontal transmission occurs by the spread of SE into the bedding after each inoculation. The naive birds can acquire the SE directly from an environment. SE + (ORAs+ HMTBa) group had significantly lower number of SE count than SE and SE+ ORAs groups. The result was similar to the findings of Parker et al., (2007) that organic acids in water treatment can decrease *Salmonella* colonization and horizontal transmission to the environment. It is possible to decrease chicken carcass and egg contaminations by adding organic acids to the feed or drinking water at appropriate times. Medium-chain fatty acids had more antibacterial activity against *Salmonella* than short-chain fatty acids (van Immerseel et al., 2006). Organic acid can accumulate in cytoplasm of bacterial cell, dissociate into proton (H^+) and anion, resulting in the reduction in intracellular pH (Rick, 2003). The short-chain fatty acids (specifically butyrate) down-regulate expression of invasion genes in *Salmonella* spp (Meimandipour et al., 2010) at low doses. Moreover, medium-chain fatty acids and propionate decrease the ability of *Salmonella* spp. to invade epithelial cells, in contrast to acetic acid. (van Immerseel et al., 2006). Additionally, nil detection of SE was found in SE+ (ORAs+ HMTBa) group in liver and spleen pool. It is possible that HMTBa can luminally protect systemic infection of SE into liver and spleen pool. Dibner et al. (2004) found that HMTBa has activity against both acid intolerant species such as *Salmonella* spp. and *E.*coli. Chicks in pens often contaminate the litter with feces containing *Salmonella* and contaminated litter is an important source (Poppe, 2000). HMTBa can reduce the number of SE count in liver and

spleen pool to zero at day 21 of age as similar to the negative control group. The percent of positive samples were also the lowest in this mixed organic acids supplemented group. Although the sensitivity of SE detection used in this experiment is rather low (>10^1 cfu/ml), the qualitative determination is more specific to SE colonization in liver and spleen and can be used to compare the efficiency of organic acids. Mixed organic acids plus HMTBa seemed to ameliorate SE count in the ileo-cecal content of non-inoculated chicks than the inoculated chicks with the significant decrease in counts. Likewise at day 21, both qualitative and SE. count of chicks in SE+ mixed ORA plus HMTBa were zero. However results of inoculated chicks were controversial as higher number of SE. count and percentage of positive plate counts were found in chicks receiving HMTBa. The lack of efficiency may be due to the halt in acid supplementation according to the recommendation label at the third week of the trial. The re-infection from the environment may increase the appearance of SE in this group. Moreover, organic acids or short chain fatty acids can inhibit Salmonella growth when present in the dissociated form (Dunkley et al., 2008). Undissociated acids enhance permeability of the cell membrane and once inside the bacterial cell, the organic acids will dissociate and decrease the cytoplasmic pH, disrupting enzymatic reaction, cellular growth and inducing cell death (Nava et al., 2009). This causes bacteria to lose energy generating capacity in the form of ATP (Russell, 1992). It is proposed that the antimicrobial activity of organic acids was depended on the pKa of the acid, molecular weight and lipophillic/ hydrophilic character (Dierick et al., 2002). The pKa of SCFAs was < 4.8 (Chotikatum, 2005) and pKa of HMTBa was 3.86 (Dibner and Buttin, 2002). The organic acids will dissociate the environmental pH higher than the pKa and this will minimize their effects on bacteria. Consistent with liver-spleen pool was the nil detection of SE at day 21. From the overall results on SE count and qualitative SE determination, mixed organic acids with HMTBa helped to prevent SE infection better than conventional formic acid and propionic acid mixture.

Salmonella infection can lead to change in the intestinal mucosa (Suzuki, 1992). Borsoi et al. (2011) found that SE damaged intestinal mucosa and reduced enzyme production. Consequently, it is found that chicks in SE group had significantly

lower villus height and villus/crypt ratio when compared to SE+ mixed ORA group. It is proposed that *Salmonella* may damage the villi and microvilli en route to penetrating the intestinal mucosa and inhibit the secretion of brush-border digestive enzymes such as di-saccharidase and di-peptidase (Chotikatum, 2005). Lan (2004) found that SCFA can induce cell proliferation in the intestinal mucosa. Generally, shortening of the villi decreases the surface area for nutrient absorption, while increase crypt depth points to fast tissue turnover (Smulikowska et al., 2010). During migration of enterocytes from the crypt toward the villus tip, they acquire differentiated functions for digestion, including expression of enzymes such as disaccharidase and alkaline phosphatase (AP) (Uni et al., 1998). Moreover, brush border membrane of the small intestine especially jejunum is the major source of disaccharidase production (Chotinsky et al., 2001). SCFA specifically induce expression of the SGLT 1 protein (glucose transporter 1) in the brush border membrane, thus SCFA are involved in the intestinal fat-induced increase in the uptake of nutrients (Ferrer et al., 2003). In addition, intestinal villus and crypt morphology in chickens have been associated with intestinal function and chicken growth (Chotikatum, 2005). According to ileal digestibility at day 21, it is found that chicks in ORA group (SE+ ORAs and SE+ (ORAs + HMTBa groups) had markedly higher of protein and fat digestibility when compared to SE group. The terminal digestion of these products, as well as the hydrolysis of disaccharides (sucrose, trehalose and lactose) occurs at the brush border membrane by disaccharidase (Chotinsky et al., 2001). The digestion of carbohydrates occurs both within the lumen of the small intestine and brush border membrane of enterocytes. The maltase and peptide hydrolase, brush border hydrolytic and proteolytic enzymes were part of the cellular role in carbohydrate and protein digestion. Both improved intestinal morphology and may be possible to reflect the improvement in digestion of these substrates (Pongmanee, 2003). The growth performance of chicks receiving combination of HMTBa with formic acid and propionic acid were better than the positive control during the starter period and was not different from the negative control group. There was no difference on antioxidant enzyme activity and MDA concentrations in all treatments at day 21. One of the important host defense mechanisms to overcome by *Salmonella* is the production of microbicidal reactive oxygen species (ROS) during oxidative burst induced by invading bacteria (Choi et al.,

2010). The reactive oxygen intermediates, including superoxide anions (O_2^-), hydrogen peroxide(H_2O_2), and hydroxyl radicals(OH^-), are known to cause severe damage to DNA, RNA protein, and lipids (Janssen, et al., 2003). According to this study, it is found that GSH-px, GSH and MDA in SE group were slightly inferior when compared other groups ($P > 0.05$). Moreover, higher GSH-px and GSH in SE+ (ORAs+ HMTBa) group than SE+ ORAs group. Two-hydroxy-4-(methylthio) butanoic acid (HMTBa) is a source of L-methionine activity for poultry diets (Dibner et al., 1990). Met is a precursor of important molecules, for example, participates in methyl group metabolism and synthesis of other sulfur amino acid, notably cysteine. Cysteine is required for the synthesis of glutathione (GSH) and taurine, which are essential compounds for host defense against oxidative stress (Métayer et al., 2008).

This study showed that ADG and FCR of chicks in SE group during the starter period were worse when compared with other groups. According to Vandeplas et al. (2009), they found that *Salmonella* induce detrimental effect on nutrient digestibility and growth performance. This was consistent with the study of Vandeplas et al. (2009) that *Salmonella* colonization of the intestinal gut at high infection dose might consequently impede nutrient absorption and digestion. Furthermore, SE+ (ORAs+ HMTBa) group had higher ADG and lower FCR than positive control and SE+ ORAs groups. This is similar to the study of Liu et al. (2007) who showed that HMTBa supplement improved growth performance and carcass quality in broiler. However, Zhang and Guo (2008) found that addition of HMTBa in water was not affect growth performance of broiler.

Grower and Finisher period (22-42 days)

At the first two weeks (weeks 4 and 5) of this period, there was no organic acid supplementation in both groups 3 and 4 chicks. The acids were added in water only in the last week of the trial aiming to control *Salmonella* contamination during the slaughter process. Determinations of SE were not analyzed at day 42 as previous study showed zero SE count in both ileo-cecal content and liver-spleen pool. There was no difference of villus height and villus/crypt ratio in all groups in this study. Although the development

of intestinal mucosa still improves continuously, lacking of organic acid supplementation may influence on the insignificant changes of gut morphology in duodenum, jejunum and ileum. Cheminay and Hensel (2008) found that the inoculation of SE may induce mucosal as well as systemic immune responses. In natural infections, the bacteria enters the host by the oral route, invade specialized antigen-transporting membranous cell (M cell) within the follicle-associated epithelium, colonize the Peyer's patches of the small intestine, gain access to the gut-associated lymphoid tissue, migrate to the mesenteric lymph nodes (MLN) and disseminate to the liver and spleen (Shahabi et al., 2010). Intracellular survival and replication in host cells, including macrophages, is critical for bacteria pathogenesis and the development of serious systemic disease, since mutant strains that fail to replicate intracellular are avirulent (Fields et al., 1986). Likewise, *Salmonella* can trigger a wide range of innate immune responses (Shahabi et el., 2010). However, the route of SE infection can lead to disease or to an asymptomatic carrier state or stimulate the induction of mucosal, systemic and cellular immune responses. Infection of animals with virulent invasive *Salmonella* can result in suppression of the immune responses which in turn can facilitate the establishment of a carrier state. It is possible that SE can modify change in immune response and put positively stress on to the mucosal function. This resulted in the improvement of ileal digestibility of protein and fat of chicks in SE group during this period although the values were not different from other groups.

Farzan and Friendship (2010) found that *Salmonella* vaccination can improve growth performance in pig. Chicks in SE+ (ORAs + HMTBa) group had higher DFI and lower ADG when compared with SE and SE+ ORAs group. It is possible that increased feed intake may not affect an increase in BW gain because the added caloric intake might be converted to body fat to replace body water (Carew et al., 2003). During this period, antioxidant enzyme activity and MDA concentrations were not significantly changed among groups.

In the overall period, there were no significant different in final body weight, ADG, DFI and FCR between SE and SE+ (ORAs+ HMTBa) groups. Chicks in SE+ (ORAs

+HMTBa) had better ADG and FCR than SE+ ORAs group (P>0.05). It is similar to the study of Xi et al (2007) who found that HMTBa was better than DL-methionine on growth performance, carcass traits and N-retention in Chinese chicks.

In conclusion, the results of this study demonstrated that HMTBa and mixed organic acids supplemented in water helped to prevent *Salmonella* Enteritidis colonization and horizontal transmission in liver-spleen pool and ileo-cecal content. HMTBa combination with formic acid and propionic acid were more effective than formic and propionic acids in decreasing the levels of colonization in liver-spleen pool. HMTBa together with formic acid and propionic acid slightly improved the intestinal morphology by increasing the villus height and villus/crypt ratio while decreasing crypt depth compared to other groups. In addition, ileal digestibility of protein and fat were higher in SE+ (ORAs+ HMTBa) than SE group. Mixed ORAs (formic acid, propionic acid and HMTBa) improved growth performance during the starter period of the study. There was little effect of mixed ORAs on antioxidant enzyme activity and MDA concentrations.

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