# REDUCTION OF SALMONELLA-INDUCED ENTERIC AND SYSTEMIC INFLAMMATION BY MANNAN-OLIGOSACCHARIDE PREBIOTIC THROUGH IMPROVEMENT OF INNATE DEFENSE MECHANISMS

by

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## ABSTRACT

*Salmonella* Enteritidis, the principal cause of salmonellosis, has become a major burden in human health due to emergence of multiple-antibiotic resistant *S.* Enteritidis that has made antibiotic treatment difficult. Today, there is urgency to develop efficacious alternatives to the massive utilization of sub-therapeutic antibiotics in poultry. We, therefore, hypothesized that, in contrast to sub-therapeutic antibiotics, mannose-rich oligosaccharides **(MOS)**, a natural prebiotic, would improve intestinal innate defense mechanisms in healthy and *Salmonella*-infected chickens, and abrogate *S.* Enteritidis invasion of intestinal epithelium, thus mitigating *Salmonella*-induced intestinal and systemic inflammation. In contrast to virginiamycin **(VIRG)** and bacitracin **(BACT)** antibiotics, dietary MOS (0.2 %) significantly improved intestinal innate defensive mechanisms in chickens raised under good sanitary conditions, as demonstrated by increased development of mucins-secreting goblet cells that ultimately secrete higher amounts of mucins, and microflora enrichment with beneficial bacteria, especially bifidobacteria. Moreover, MOS was equally effective as VIRG and BACT in the control of intestinal *E. coli*. But, at higher (0.5 %) than the recommended (0.2 %) dosage, MOS conferred no additional intestinal health benefits.

To examine whether MOS may control *S.* Enteritidis through improvement of host's innate defense mechanisms, young chicks were deliberately infected with *S.* Enteritidis. Interestingly, whereas control (CTL) chicks suffered from drowsiness, diarrhea, starvation, exfoliation of epithelial cells and damaged villi integrity, such classic signs of *Salmonella*-induced enteric inflammation were abrogated by MOS and VIRG. However, the down-regulation of *IL 12* by MOS revealed that MOS terminated *S.* Enteritidis-induced enteric inflammation earlier than VIRG. Whereas VIRG relied mostly on its bactericidal properties, MOS conferred better natural protection against *S.* Enteritidis through increased secretions of neutral and acidic mucins that might have trapped *S.* Enteritidis thereby reducing its adhesion to and invasion of the intestinal epithelium. MOS's mucins-mediated defense mechanism was due to increased numbers of neutral and acidic goblet cells, rather than increased mucins-secreting capacity of goblet cells, as indicated by non-differential *MUC 2* expressions between infected chicks fed VIRG and MOS. The down-regulation of *MUC 1* by MOS indicated that MOS more

significantly reduced *S.* Enteritidis invasion and damage of epithelial cells than VIRG. Evidently, as revealed by electron microscopy analysis, intestinal villi were longer and healthier in *S.* Enteritidis-infected chicks fed MOS than VIRG.

*S.* Enteritidis invasion of the intestinal epithelium frequently causes extra-intestinal infections and systemic inflammation. A *Salmonella* LPS-induced systemic inflammation chicken model and microarray analysis approach was employed to determine whether MOS, in contrast to VIRG, may beneficially modulate innate immunity to mitigate systemic inflammation, and reduce glucose mobilization during late systemic inflammation. MOS inherently induced *IL 3* expression in non-challenged control hosts. However, consequent to LPS challenge, *IL 3* was induced in VIRG hosts but not differentially expressed in MOS hosts, therefore revealing that MOS counteracted LPS's detrimental inflammatory effects. Indeed, the lower energy demands of LPS-challenged birds fed MOS were sufficiently met through TCA citrate-derived energy, as indicated by *ATP citrate synthase* (*CS*) up-regulation. Contrastingly, VIRG host's elevated energy requirements increased gene expressions for intestinal gluconeogenesis (*PEPCK*) and liver glycolysis (*ENO2*). Although intriguing, intestinal gluconeogenesis occurred simultaneously with liver fatty acid synthesis, mediated by *CS* down-regulation, and *ATP citrate lyase* (*ACLY*), *malic enzyme* (*ME*) and *fatty acid synthetase* (*FAS*) up-regulations. In conclusion, this study revealed the mechanisms by which MOS, in contrast to VIRG, i) enhanced host's intestinal innate defense mechanisms against *Salmonella*; ii) terminated *Salmonella*-induced intestinal and systemic inflammation earlier; iii) and modulated innate immunity to markedly reduce glucose mobilization during late systemic inflammation. Therefore, MOS represents a biological strategy that can prevent or treat *S.* Enteritidis infections in poultry and humans, without posing the risk of developing antibiotic-resistance.

**RESUME**

*Salmonella* Enteritidis, la cause principale de la salmonellose, est devenue un problème majeur pour la santé humaine en raison du développement des souches de *S.* Enteritidis résistantes aux antibiotiques multiples, compromettant ainsi le traitement de la salmonellose avec des antibiotiques thérapeutiques. Donc, il y a maintenant urgence de développer des remplaçants efficaces aux antibiotiques de croissance très utilisés chez la volaille. Notre hypothèse de travail était que contrairement aux antibiotiques sous-thérapeutiques, l'utilisation d'oligosaccharides riches en mannose **(MOS)**, un prébiotique naturel, améliorerait les mécanismes de défense intestinale innés chez les poulets sains et infectés par des *Salmonelles*, prévenant ainsi l'invasion de l'épithélium intestinal par *S.* Enteritidis et atténuant de ce fait l'inflammation intestinale et systémique induite par la *Salmonelle*. Contrairement aux antibiotiques tels que la virginiamycine (**VIRG**) et la bacitracine (**BACT**), le MOS (0.2 %) a amélioré de manière significative les mécanismes de défense intestinale innés chez les poulets élevés dans de bonnes conditions sanitaires, tel que démontré par la prolifération des cellules à gobelet produisant et sécrétant ainsi plus de mucines, et l'enrichissement de la flore microbienne avec des bactéries bénéfiques, particulièrement bifidobacteria. Il est aussi important de noter que le MOS était aussi efficace que la VIRG et BACT dans le contrôle intestinal d'*E. coli*. Mais, à une dose plus élevée (0.5 %) que le dosage recommandé (0.2 %), le MOS n'a démontré aucun bénéfice additionnel dans la santé intestinale.

Afin d'examiner si le MOS pourrait être efficace dans le contrôle intestinale de *S.* Enteritidis tout en améliorant les mécanismes de défense innés, des poussins ont été infectés avec *S.* Enteritidis. Les poussins infectés alimentés avec la diète témoin ont souffert de somnolence, diarrhée, perte d'appétit, exfoliation des cellules épithéliales et endommagement des villosités, tandis que ces symptômes ont été abrogés par le MOS et la VIRG. Cependant, la réduction observée dans l'expression de *IL 12* par le MOS relatif à la VIRG démontra que le MOS a mis fin à l'inflammation induite par *S.* Enteritidis plus tôt. Le mode d'action de la VIRG est fondé sur ses propriétés bactéricides, tandis que lors de nos expériences le MOS augmenta la sécrétion des mucines neutres et acides. En attachant *S.* Enteritidis, ces mucines ont réduit l'adhésion et l'invasion de l'épithélium intestinale. Cette augmentation de la sécrétion des mucines fut possible par un plus grand

nombre de cellules à gobelet neutres et acides plutôt que par une sécrétion accrue de mucines par chaque cellule à gobelet, comme démontré par l'absence de changement d'expression de *MUC 2* entre les poussins infectés et alimentés avec la VIRG et le MOS. En revanche nous avons observé une diminution dans l'expression du *MUC 1* induite par le MOS. Ceci indique que le MOS a réduit de manière significative l'invasion et les dommages des cellules épithéliales causés par *S.* Enteritidis. Tel que démontré par nos analyses de microscopie électronique, les villosités intestinaux étaient plus longues et saines chez les poussins infectés et alimentés avec le MOS que la VIRG.

L'invasion de l'épithélium intestinal par *S.* Enteritidis cause fréquemment des infections extra-intestinales et une inflammation systémique. Pour déterminer si le MOS, contrairement à la VIRG, pouvait avantageusement moduler l'immunité innée afin d'atténuer l'inflammation systémique et réduire la mobilisation de glucose pendant la phase avancée de l'inflammation, nous avons induit une inflammation systémique par injection d'une dose d'endotoxine de *Salmonelle* (LPS) puis nous avons analysé les effets à l'aide de microréseaux d'ADN. Le MOS induit l'expression d'*IL 3* chez les poulets non soumis au LPS. En revanche, suite à l'injection de LPS, l'expression d*'IL 3* a été augmentée chez les poulets alimentés avec la VIRG mais pas chez ceux nourris avec le MOS, indiquant que le MOS a aboli les effets inflammatoires du LPS. En effet, les demandes énergétiques inférieures des poulets injectés avec du LPS et alimentés avec le MOS ont été suffisamment satisfaites par l'énergie dérivée par le cycle de l'acide citrique, comme indiqué par une augmentation dans l'expression de l'*ATP citrate de synthase* (*CS*). Par contre, les besoins énergétiques plus élevés chez les poulets alimentés avec la VIRG ont entrainé une augmentation de l'expression des gènes de la néoglucogenèse intestinale (*PEPCK*) et de la glycolyse du foie (*ENO2*). Bien qu'intriguant, la néoglucogenèse intestinale s'est produite simultanément avec la synthèse d'acide gras de foie, dû à une diminution dans l'expression de *CS*, et une augmentation de l'expression génique de l'ATP citrate lyase (*ACLY*), l'enzyme malique (*ME*) et la synthase d'acide gras (*FAS*). En conclusion, cette étude a démontré les mécanismes par lesquels le MOS, contrairement à la VIRG, i) a augmenté les mécanismes de défense intestinaux innés contre la *Salmonelle*; ii) a mis fin à l'inflammation intestinale et systémique causée par la salmonelle plus tôt; iii) et a réduit la mobilisation du glucose pendant une phase avancée

dans l'inflammation. Par conséquent, le MOS représente une stratégie biologique pour prévenir ou traiter les infections intestinales de *S.* Enteritidis chez la volaille et les humains sans favoriser le développement de résistance aux antibiotiques.

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## CONTRIBUTION TO KNOWLEDGE

Overall, this is a first-in-class research study, i.e. a full size doctoral research study unveiling the mechanisms by which a MOS prebiotic, in comparison with sub-therapeutic antibiotics, more significantly improved host's natural defense against *Salmonella*, mitigated both acute systemic and enteric inflammation, and beneficially modulated innate immunity to reduce glucose mobilization during late systemic inflammation. Whereas most studies have focussed mainly in evaluating MOS on growth performance, and identification and quantification of intestinal digesta-associated bacteria in chickens, this is the first study that has adopted a mechanistic approach to elucidate MOS's intestinal and general health benefits, as above-described. Most importantly, this study provides a rationale to substitute sub-therapeutic antibiotics with MOS in poultry production, thus addressing the *Salmonella* antibiotic-resistance problem. Although conducted in a chicken biological model, the promising findings of this study probe for further investigations in human health.

In **Chapter II**, MOS exhibited different mechanisms of intestinal defense than VIRG and BACT antibiotics, to equally and effectively control intestinal *E. coli* in healthy chickens. VIRG and BACT relied mostly on their powerful bactericidal properties. Interestingly, however, MOS markedly improved innate defense mechanisms, mediated by increased numbers of mucin-secreting goblet cells and microflora enrichment with probiotic bacteria, especially bifidobacteria. In addition, we demonstrated that MOS had equivalent effects to VIRG and BACT on growth and carcass part yields (breast, breast fillets, breast tenders, thighs, drumsticks and wings) when chickens are raised under good sanitary conditions. For the first time, we revealed that, at higher (0.5 %) than the recommended (0.2 %) dosage, MOS conferred no additional intestinal health benefits.

In **Chapter III**, we unveiled the mechanisms by which MOS and VIRG mediated intestinal defense against *S.* Enteritidis. In this novel study, we revealed that MOS more effectively mitigated intestinal *S.* Enteritidis infection than VIRG, through a complex mucin-mediated defense mechanism. For the first time, using a *S.* Enteritidis infection

model, we demonstrated that MOS, in contrast to VIRG: i) markedly increased neutral and acidic mucins secretions; ii) more significantly abrogated *S.* Enteritidis invasion and damage of intestinal epithelial cells; iii) caused earlier termination of *S.* Enteritidis-induced intestinal inflammation; iv) improved the health and development of intestinal villi. Moreover, we demonstrated that MOS's higher mucins-defense mechanism was mediated principally by increased neutral and acidic goblet cell numbers, but not due to increased mucins-secreting capacity of goblet cells.

In **Chapter IV**, using an in-depth gene expression microarray analysis, we elucidated the mechanisms underlying innate immunity and nutrient metabolism that occurred in the liver and intestines during late systemic inflammation, and as affected by MOS and VIRG. For the first time, we revealed that MOS inherently triggered immune-stimulation which caused 'arming' of host's innate immunity to more rapidly terminate *Salmonella* LPS-induced systemic inflammation than VIRG, and to profoundly reduce nutrient mobilization. Metabolic pathways have been built elucidating the mechanisms by which VIRG host's elevated energy requirements necessitated glucose mobilization through intestinal gluconeogenesis and increased liver glycolytic activities. This is the first study demonstrating that, although apparently conflicting, gluconeogenesis and fatty acid synthesis occurred simultaneously during late inflammation, however involving different body organs. For the first time, we have revealed occurrence of intestinal gluconeogenesis in chickens. Finally, this study evidenced cross-talks between intestinal mucosal immunity and systemic immunity, given that MOS and VIRG were administered via the diet.

## CONTRIBUTIONS OF AUTHORS

Three co-authored manuscripts are included in this thesis: i) published in Poultry Science, ii) published in PLoS ONE, and iii) ready to be submitted for publication.

In all manuscripts, **Bushansingh Baurhoo** conceived the original concepts, designed and conducted the research, analyzed the data and interpreted results, and wrote the manuscripts.

**Authors of Manuscript 1 (Chapter II): B. Baurhoo, P. R. Ferket, and X. Zhao.**

B. Baurhoo designed and carried out all experiments, performed data analysis, and wrote the manuscript. P. R. Ferket and X. Zhao reviewed the manuscript.

**Authors of Manuscript 2 (Chapter III): B. Baurhoo, A. Letellier, and X. Zhao.**

B. Baurhoo initiated the novel approach, designed and carried out experiments, analyzed data and interpreted results, and wrote the manuscript. A. Letellier assisted with *Salmonella* challenge study and microbiology analysis. X. Zhao reviewed the manuscript.

**Authors of Manuscript 3 (Chapter IV): B. Baurhoo, P. R. Ferket, C.M. Ashwell, J. de Oliviera and X. Zhao.**

B. Baurhoo designed and carried out experiments, analyzed data and interpreted results, deposited microarray data in the Gene Expression Omnibus (GEO) database, and wrote the manuscript. P. R. Ferket and X. Zhao assisted in experimental design. C. M. Ashwell assisted in microarray analysis, and J. de Oliveira assisted in microarray data analysis. P. R. Ferket and X. Zhao reviewed the manuscript.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AB …… | Alcian blue |
| AGP …….. | Antibiotic growth promoter |
| BACT …… | Bacitracin |
| BW …… | Bodyweight |
| Cal …….. | Calorie |
| CS ……. | Citrate synthase |
| d …….. | Day |
| ENO2 ……. | Phosphopyruvate hydratase |
| FCR …… | Feed conversion ratio |
| FI …… | Feed intake |
| GI …… | Gastrointestinal inflammation |
| HE …….. | Haematoxylin and Eosin |
| IL …… | Interleukin |
| LPS ……. | Lipopolysaccharides |
| ME …….. | Metabolizable energy |
| ME ……. | Malic enzyme |
| MOS …… | Mannanoligosaccharides |
| MUC ……... | Mucin |
| PAS ……. | Periodic acid Schiff |
| PEPCK …… | Phosphoenolpyruvate carboxykinase |
| SEM …… | Scanning electron microscopy |
| TCA …… | Tricarboxylic acid cycle |
| US …… | United states of America |
| TNF …… | Tumor necrosis factor |
| VIRG ……. | Virginiamycin |
| WHO …….. | World health organization |

# CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

## GENERAL INTRODUCTION

*Salmonella* is a leading food-borne pathogen of major public health and economic concerns, worldwide. In the U.S. only, *Salmonella* causes about 20,000 hospitalizations and 400 deaths annually (CDC, 2011). Of these, *Salmonella* serotype Enteritidis (*S.* Enteritidis) is the leading cause of human food-borne salmonellosis. The disease is characterized by symptoms ranging from headache, abdominal pain, nausea and vomiting to profuse diarrhoea and life-threatening gastrointestinal inflammation (CDC, 2011). *S.* Enteritidis invades the intestinal epithelium by using its Type III secretory system (T3SS) and disseminate through efferent lymphatics to the blood causing extra-intestinal infections and systemic inflammation (Griffin and McSorley, 2011).

Poultry is indisputably the largest reservoir of *S.* Enteritidis and an ecological amplifier that facilitate its dissemination into the food chain. *S.* Enteritidis is unique in that it is the only pathogen that can thrive and multiply within an egg (Terzolo et al., 1998; Gast and Holt, 2001; Sellier et al., 2007). Moreover, *S.* Enteritidis capability to reside asymptomatically in the intestines of mature chickens favors poultry meat contamination during processing (Sadeyen et al., 2004; Finstad et al., 2011). Not surprising, therefore, that *S.* Enteritidis-induced salmonellosis occurs principally due to consumption of undercooked contaminated retail poultry meat and eggs (Zhao et al., 2001; Kimura et al., 2004; Fearnley et al., 2011; Martelli and Davies, 2011). For instance, in the province of British Columbia (Canada), 33 % of retail poultry meat samples tested positive for *Salmonella* between 2006 to 2010, and, during that same time period and location, *S.* Enteritidis was predominantly identified among live chickens, chicken meat and human patients (Galanis et al., 2011). Similarly, from 1985 through 1999, *S.* Enteritidis (80 %) was the leading foodborne pathogen in *Salmonella* egg related food poisoning outbreaks in the U.S. (Patrick et al., 2004). Indeed, *S.* Enteritidis must be controlled at the farm level to ensure safety of poultry meat and eggs.

Sub-therapeutic antibiotics **(AGP)** have long been used in healthy poultry for growth promotion and to control intestinal pathogens. However, after much debate, it is now agreed among most, if not all, scientists that such practice has contributed to the development of multiple antibiotic-resistant *S.* Enteritidis strains that affect human health

(CDC, 2011; WHO, 2011). This conclusion is evidenced by presence of multiple antibiotic-resistant *S.* Enteritidis in contaminated retail poultry meat (Vaz et al., 2010; de Oliveira et al., 2011; Hur et al., 2011; Hyeon et al., 2011; Yildirim et al., 2011). Very recently, the World Health Organization (**WHO**) highlighted that lifesaving antibiotics are losing their healing power due to emergence of antibiotic-resistant bacteria and that release of new antibiotics is unlikely to occur in the near future (WHO, 2011). Member countries of the European Union well anticipated the link between AGP usage in livestock production and compromised human health, and hence banned usage of such antibiotics in January 2006. Evidence exists that in the absence of antibiotic pressure, antibiotic-resistant *S.* Enteritidis undergoes reversion mutation to considerably diminish or abrogate antibiotic resistance (O'Regan et al., 2010). Taken together, it is somewhat intriguing that AGP are still permitted for growth promotion purposes in Canada and the U.S. Nevertheless, considering that food safety remains the priority public health concern, a similar ban on AGP is indisputably inevitable in the near future.

The emergence of multiple-antibiotic resistant *S.* Enteritidis has not only made control of *S.* Enteritidis in poultry production more difficult, but has considerably worsened antibiotic treatment of *S.* Enteritidis-induced gastrointestinal infections in human medicine. Today, scientists are challenged with development of natural products that can: 1) successfully control *S.* Enteritidis in poultry production, thus providing safer poultry meat and eggs for human consumption; and 2) alleviate the public health burden of *S.* Enteritidis-induced salmonellosis. We previously demonstrated that mannose-rich fractions isolated from cell walls of *Saccharomyces cerevisiae* (**MOS**) conferred intestinal health benefits in chickens. However, research, so far, conducted with MOS has been limited to evaluating growth, microbiological analyses for identification and quantification of intestinal digesta-associated lactobacilli, bifidobacteria and *E. coli* (Baurhoo et al., 2007 a,b), and histological analysis of the intestines (Baurhoo et al., 2007 a,b; Solis de los Santos et al., 2009) in healthy chickens. However, the mechanisms of MOS and AGP actions on host's natural defense, inflammation and nutrient metabolism in a *S.* Enteritidis infection model still remain elusive.

Therefore, the main objective of this study was to investigate and compare the effects of an AGP (VIRG) and a MOS prebiotic on intestinal innate defensive

mechanisms in healthy and *S.* Enteritidis-infected chickens, and their capacity to mitigate *Salmonella*-induced enteric and systemic inflammation. We have integrated histological, microbiological and gene expression analyses to unveil the mechanisms by which VIRG and MOS differently modulated innate defensive mechanisms, innate immunity, nutrient metabolism and morphological development of intestinal structures. Specifically, our research study, conducted in healthy and infected chickens, focussed on: i) intestinal populations of *S.* Enteritidis and *E. coli*; ii) intestinal contents of probiotic bacteria, especially lactobacilli and bifidobacteria; iii) intestinal development of neutral- and acidic mucin-secreting goblet cells; iv) gene expressions of secretory and membrane-bound mucins; v) barrier integrity of intestinal epithelium; vi) health and development of intestinal villi; vii) gene expressions of pro-inflammatory cytokines; viii) microarray gene expression analysis of key players involved in innate immunity and metabolic pathways during late systemic inflammation.

# LITERATURE REVIEW

## 1.1 The Canadian broiler industry

The Canadian poultry industry produced over 1.2 million metric tons of meat and contributed $2.3 billion to the economy in 2010 (Statistics Canada, 2011). In Canada, the majority of chicken meat is produced in the provinces of Ontario (34.6 %) and Quebec (26.28 %). In comparison to other meat types, chicken meat, usually regarded as healthier, is the most preferred and consumed meat by Canadians. In 2008, per capita consumption of chicken meat (13.6 kg) was higher in comparison to 12.8 kg and 9.7 kg for beef and pork, respectively (Statistics Canada, 2011).

The success of the Canadian poultry industry achieved over the past 30 years is attributable to considerable scientific progress and modernization involving the use of genetically high yielding breeds, significant improvement in nutrition, disease control and animal husbandry. However, broiler chickens have genetically been selected for growth, feed efficiency and meat yield, rather than disease resistance (Cheema et al., 2003). Consequently, modern broilers are more susceptible to gastrointestinal infections by pathogens such as *Salmonella, E. coli, Campylobacter and Clostridium perfringens*, which cause enteric diseases in young chicks, increase mortality rates and economic losses, compromise animal welfare, and increase poultry meat contamination during processing. Indeed, humans suffer from food-borne diseases upon consumption of contaminated poultry meat.

## 1.2 The Avian Gastrointestinal Tract: The Good and Bad Residents

### 1.2.1 Establishment of intestinal microflora in chickens

The gastrointestinal tract (GIT) of poultry is inhabited by a complex and diverse microflora comprising several hundred species of bacteria (Gong et al., 2002). Given their beneficial modulating effects in improving host's health, indigenous or commensal bacteria have received much scientific attention over the years (van der Wielen et al., 2002). Much consideration has also been given to the development of feeding strategies that can modify the bacterial communities with objectives to enhance intestinal health in poultry. Nevertheless, detailed profiling of the chicken intestinal microflora still remains incomplete (Gong et al. 2007). But, modern molecular techniques such as 16S ribosomal

RNA (rRNA) gene-based pyrosequencing are powerful enough to reveal identity of the diverse intestinal microflora composition of broilers (Bjerrum et al., 2006; Gong et al., 2007).

The chick intestine is usually regarded as sterile at time of hatch, but bacterial colonization of the small intestine starts within the first hour of life; streptococci and enterobacteria (mostly *E. coli*) are usually detected first in the ceca and then throughout the intestinal tract within 24 hours (Smith, 1965). Generally, in healthy mature chickens, lactobacilli, *Enterococcus*, *Clostridium* and *E. coli* are predominant in the small intestine (Bjerrum et al., 2006; Gong et al., 2007) whereas the ceca is inhabited by a more diverse bacterial community, including lactobacilli, bifidobacteria, *Clostridium*, *E. coli*, *Streptococcus* and *Campylobacter* (Bjerrum et al., 2006; Gong et al., 2002, 2007). It is well recognized that the initial bacterial colonization of chick intestines is a major determinant in the proper establishment of a stable microflora in adult birds. The bacterial population increases in density and diversity along the chicken intestine. The duodenum contains approximately $10^3$ to $10^5$ bacterial cells per gram of digesta, the lower jejunum harbors $10^8$ to $10^9$ bacterial cells per gram of digesta, whereas bacterial density in the ceca can reach $10^{12}$ per gram of digesta (Gong et al., 2002). Establishment of a stable microbiota is a complex process that is influenced by multiple factors, including animal's age, diet formulation, rearing conditions, and the use of antibiotics, prebiotics or probiotics (Patterson and Burkholder, 2003; Gong et al., 2008).

Equilibrium between the harmless and harmful bacteria is a prerequisite for sound intestinal health. There is, however, little knowledge about the thin line that divides tolerance from pathogenicity to intestinal pathogens. What is clear is that once commensal bacteria have gained entry into the intestine, they establish a complex and dynamic ecosystem (Liévin-Le Moal and Servin, 2006). Toll-like receptors (TLRs) in the intestinal epithelial cell's membrane play a key role in discriminating pathogenic and harmless bacteria, and trigger appropriate actions that limit proliferation of the pathogens (Abreu et al., 2005).

### 1.3 *Salmonella* in poultry

#### 1.3.1 *Salmonella* infection in poultry

*Salmonella*, belonging to the family *Enterobacteriaceae*, are gram-negative, facultative anaerobes, non-sporing, and rod-shaped (1-2 μm) bacteria possessing long flagella structures on the outer membrane for motility. *Salmonella* have received significant attention over the last decade because of their ability to cause food-borne diseases in humans (Mead et al., 1999; Callaway et al., 2008). Intestinal colonization by these pathogens usually results in invasion of the intestinal epithelium and dissemination to extra-intestinal organs causing systemic infections (Brown et al., 1976). At the young age, within first week after hatch, chicks are more susceptible to *Salmonella* infections because of their immature innate immunity (Beal et al., 2004; Lowry et al., 2005) and poorly developed microflora (Gong et al., 2008). Young infected chicks suffer from *S.* Enteritidis-induced enteric diseases, causing fever, profuse diarrhea, ruffled feathers, bacteremia, gross hemorrhagic lesions, acute septicemia and gastroenteritidis (Goldberg and Rubin, 1988; Desmidth et al., 1997; Porter, 1998) that lead to varying mortality rates and economic losses (Suzuki, 1994; Duchet-Suchaux et al., 1995; Velge et al., 2005). On the other hand, adult birds have evolved to live with intestinal *Salmonella* serving as lifetime hosts for the pathogen without showing any clinical signs (Brown et al., 1976; Nakamura et al., 1985; Barrow et al., 1987; Sadeyen et al., 2004). Therefore, visual sickness appearance is not an effective indicator of *Salmonella* infection in chickens. Intestinal *Salmonella* from such carrier birds contaminate poultry meat upon accidental intestinal breakages during processing (Heyndrickx et al., 2002; Finstad et al., 2011).

#### 1.3.2 *Salmonella* transmission in poultry

Poultry is regarded as the largest reservoir of *S.* Enteritidis. In healthy appearing laying hens, *S.* Enteritidis invades the intestinal epithelium and silently infect the ovaries, leading to internal contamination of newly formed eggs (Guard-Petter, 2001; De Buck et al., 2004). *S.* Enteritidis is unique in that it is the only pathogen that can thrive and multiply within an egg (Gast and Holt, 2001); the bacteria can resist lysozyme and iron deficiency of egg albumen (Chart and Rowe, 1993; Sellier et al., 2007) and egg-yolk immunoglobulins (IgY) (Terzolo et al., 1998). Indeed, *S.* Enteritidis can vertically be

transmitted through contaminated fertilized eggs to hatched chicks (Gast and Holt, 2000). Egg fragments, belting rial, and chick paper pads are also major contaminants at the hatchery (Cox et al., 1990). Because the gastrointestinal tract of newly hatched chicks is essentially sterile, *Salmonella* can easily be transmitted from infected chicks or hatchery environment to uninfected birds through the common fecal-oral route within poultry houses (Cox et al., 1990; Rodriguez et al., 2006).

The method of rearing a large number of broiler chickens within confined environment facilitates *S.* Enteritidis transmission from infected to healthy chicks, such that the entire flock can rapidly become infected. Even when only 5% of day-old chicks were *Salmonella* positive, 72 to 95% of birds were tested positive after 3 weeks of the grow-out period (Byrd et al., 1998). *Salmonella* can also be disseminated through several other routes including feed, water, insects, rodents, infected birds and humans (Bailey et al., 2001; Ranta and Maijala, 2002); hence there is need for stringent biosecurity and pest control plans on commercial farms. Therefore, the transmission of *S.* Enteritidis vertically through trans-ovarian route in hens and horizontally via the fecal-oral route among infected chickens, amplifies dissemination of *S.* Enteritidis in the food chain and its eventual transmission to humans.

### 1.4 Salmonellosis: A major public health concern

#### 1.4.1 Incidence of salmonellosis in humans

Foodborne disease is a major public health concern, worldwide. Approximately, 48 million people suffer from foodborne illnesses in the U.S., with an average of 128,000 hospitalizations and 3,000 deaths annually (CDC, 2011). Of these, *Salmonella* causes about 20,000 hospitalizations and 400 deaths annually (CDC, 2011). Over 1 million people in the U.S. contract *Salmonella* annually, however the majority of cases go unreported. *S.* Enteritidis is the leading cause of human food-borne salmonellosis, which is characterized by diarrhoea, fever, headache, abdominal pain, nausea and vomiting. However, *Salmonella* invasion of the intestinal epithelium causes acute gastro-intestinal inflammation (CDC, 2011) and extra-intestinal infections (Griffin and McSorley, 2011).

### 1.4.2 Poultry meat and eggs: major causes of human *S.* Enteritidis infections

Poultry is indisputably the largest reservoir of *S.* Enteritidis. *S.* Enteritidis, frequently isolated from healthy, diseased or dead birds, is the leading cause of human salmonellosis, most commonly associated with poultry meat and eggs (Braden, 2006; CDC, 2011). *S.* Enteritidis have the ability to reside inside perfectly normal-appearing eggs and contaminate chicken meat during processing. Human infection with *S.* Enteritidis occurs mainly during direct contact with infected chickens (Hoelzer et al., 2011), and handling and consumption of undercooked contaminated retail poultry meat and eggs (Zhao et al., 2001; Kimura et al., 2004; Fearnley et al., 2011; Martelli and Davies, 2011). In Canada, according to an integrated surveillance of *Salmonella* along the food chain, 33% of poultry retail meat samples contained *Salmonella* between 2006 to 2010 and *S.* Enteritidis was predominantly identified among chicken, chicken meat and human patients (Galanis et al., 2011). Similarly, *S.* Enteritidis (80%) was the leading foodborne pathogen in *Salmonella* egg related food poisoning outbreaks reported to the Centers for Disease Control and Prevention from 1985 through 1999 (Patrick et al., 2004).

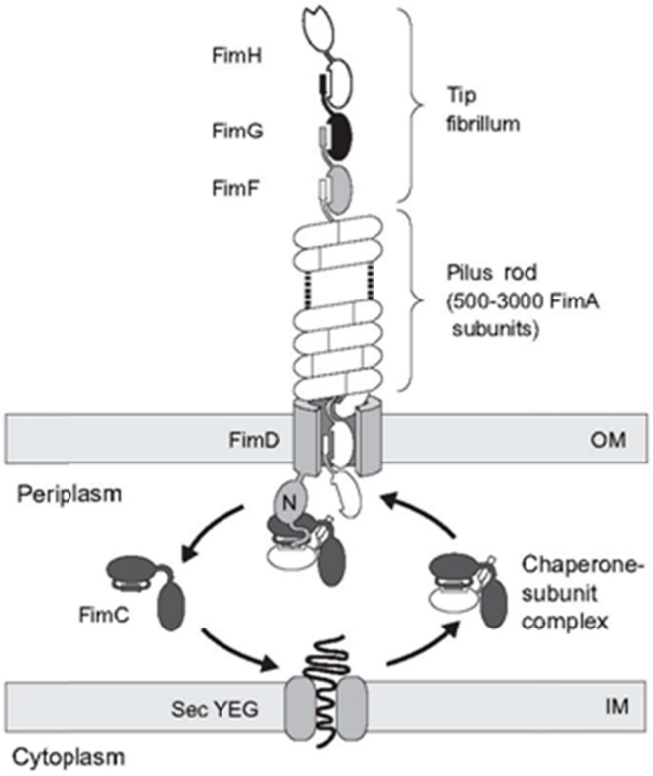
### 1.4.3 Mechanisms of *Salmonella* attachment to host's intestinal epithelium

Attachment of pathogens to the host's intestinal epithelium is a prerequisite for their successful invasion and pathogenicity. Fimbriae or pilus represents the binding sites of *Salmonella* to specific surfaces of host tissues. Fimbriae are extracellular heteropolymeric filamentous organelles anchored on the outer membrane of bacteria. Gram-negative bacteria, such as *Salmonella* and *E. coli*, express multiple types of fimbriae, including type 1 (Fim), plasmid-encoded (PE), long polar (LP), and thin aggregative (curli) (Darwin and Miller, 1999). But, most gram-negative bacteria (70 %) attach to mannose-containing glycoprotein receptors on epithelial cells via their type-1 fimbriae (Fernandez and Berenguer, 2000; Duncan et al., 2005). For this reason, type 1 fimbriae have been the most extensively studied fimbriae. Furthermore, type 1 fimbriae is unique in that it allows invasion of human and chicken epithelial cells; other fimbriae types failed to allow bacterial invasion (Martinez et al., 2000).

Type 1 fimbriae are approximately 1 μm long and 6 nm wide (Korhonen et al., 1980) filamentous organelles, essentially consisting of a FimH sub-unit, a mannose-binding lectin, and a FimA shaft (Figure 1.1). FimA, the major structural subunit, makes up more than 95% of the fimbrial shaft (Capitani et al., 2006) whereas FimH adhesin is the terminal subunit of type 1 fimbriae that specifically binds to α-D-mannose (Darwin and Miller, 1999; Madison et al., 2004; Thomas et al., 2004; Duncan et al., 2005; Capitani et al., 2006). Ofek et al. (1978) previously demonstrated that the FimH adhesion is responsible for adherence of gram-negative bacteria to mannose-containing glycoprotein receptors on human epithelial cells. There is compelling evidence that FimH contains mannose-binding lectin that mediate adhesion of gram-negative bacteria to mannose-containing receptors: i) deletion of the *fimH* gene abrogated the binding capacity of *E. coli* without any apparent effect on the morphological structure and antigenic properties of the fimbriae (Minion et al., 1986); and ii) purified FimH isolated from *E. coli* binds mannosylated glycoprotein (Krogfelt et al., 1990; Tewari et al., 1993).

FimH of different bacteria within the *Enterobacteriaceae* family contain proteins that are similar in size, antigenicity and functions, thus demonstrating similarity in compositions and mechanisms of binding amongst the type 1 fimbriae (Abraham et al., 1988). Nevertheless, the mannose-binding specificity of FimH appears to be heterogeneous. For instance, the heterogeneity of FimH within *E. coli* species (Sokurenko et al., 1997), or between *E. coli* and *Salmonella* (Firon et al., 1983) allows binding of type 1 fimbriae to different mannose receptors, such as those containing manomannose or trimannose (Sokurenko et al., 1997). Interspecies differences in FimH mannose specificity has been attributed to the fimbrial shaft, which may induce distinct conformational changes in the FimH subunit (Madison et al., 1994; Duncan et al., 2005), whereas intra-species differences can be attributed to differences in amino acid sequences of FimH (Sokurenko et al., 1997). These differences dictate minor differences in the way these bacteria bind to mannose residues on glycoprotein receptors of the intestinal epithelium.

**Figure 1.1:** Structure of type 1 fimbriae[1]




[1]*Adapted from Capitani et al. (2006)*

Based on the concept of mannose-sensitive adherence of type 1 fimbriae expressed by *Salmonella* and *E. coli*, strategies were developed to possibly exploit usage of purified mannose oligosaccharides to competitively attach to FimH adhesin of such pathogens, therefore blocking *Salmonella* and *E. coli* colonization of host's intestinal epithelial cells and their underlying pathogenicity effects. In this study, mannose-rich oligosaccharides (MOS), specifically extracted from cell walls of *Saccharomyces cerevisiae*, were investigated in its capacity to bind and eliminate *Salmonella* from the chicken intestines. In addition, as a new approach of this study, we investigated whether purified mannose-oligosaccharides may also influence host's innate defense mechanisms against *Salmonella*.

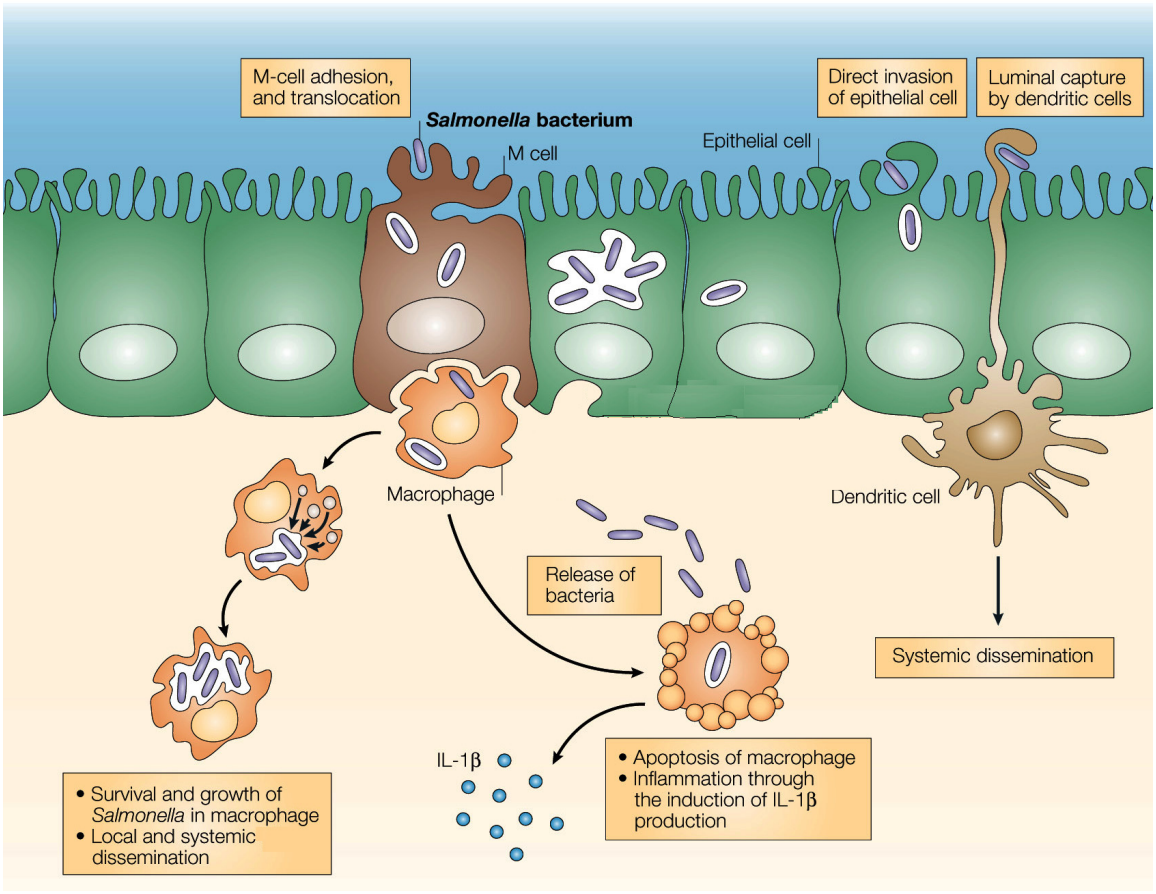
### 1.4.4 Mechanisms of *Salmonella* invasion of host's intestinal epithelium

*Salmonella* colonization of the intestinal epithelium is a prerequisite for its successful invasion and pathogenicity. Subsequent to successful attachment, *Salmonella* invades and crosses the intestinal epithelium by 3 main routes, involving direct invasion of epithelial cells, microfold (M) cells or dendritic cells (Figure 1.2). An essential feature of the pathogenicity of *Salmonella* is their ability to engage host's cells in a two-way biochemical interaction or cross-talk, which leads to responses from both the bacteria and host cells (Galan and Bliska, 1996). *Salmonella* invasion of intestinal epithelial cells triggers massive release of pro-inflammatory cytokines such as IL-1 and IL-8, in response to *Salmonella*-mediated activation of mitogen-activated protein kinase (MAPK) cascade and activation of transcription factors such as AP-1 (activator protein 1) and NF-kB (nuclear factor kappa B) (Hobbie et al., 1997). This is an important event in *Salmonella* pathogenesis. The mechanisms of *Salmonella* invasion of host's epithelium include:

***Direct invasion***: *Salmonella* adherence to epithelial cells via the FimH mannose binding lectin of type 1 fimbriae induces reorganization of actin skeleton and subsequent invasion of epithelial cells (Martinez et al., 2000).

***Bacterial invasion via M-cells:*** M cells, specialized cells of the gut-associated lymphoid tissues (GALT), are preferred sites for *Salmonella* invasion (Walker et al., 1988; Jones et al., 1994), because these cells possess shorter microvilli (brush border) and reduced mucosal layer thickness at the apical surface (Bye et al., 1984). Therefore, in addition to its involvement in the absorption and transportation of nutrients, M cells transfer pathogens from the intestinal lumen across the epithelial membrane to an underlying macrophage-, dendritic-, T-lymphocyte-, and B-lymphocyte rich dome area (Neutra, 1999; Owen, 1999). For successful invasiveness and survival, therefore, intestinal pathogens not only need to cross the epithelial lining but are also required to overcome killing by the numerous phagocytic cells of innate immunity.

**Figure 1.2:** Schematic illustration of the mechanisms of *Salmonella* invasion of epithelial cells[1]




[1] *Adapted and modified from: Sansonetti (2004)*

*Salmonella* first adheres to and then induces its own uptake into intestinal epithelial cells through its type III protein secretion system (T3SS). The T3SS is a specialized mechanism involving a sophisticated molecular needle-like apparatus encoded by *Salmonella* pathogenicity island 1 (SPI1), which injects bacterial effector proteins into the cytoplasm of host's M-cell that cause cytoskeleton rearrangement of epithelial cells, leading to bacterial uptake (Darwin and Miller, 1999; Ly and Casanova, 2007). This entry process is referred to as the Trigger entry mechanism. After invasion of epithelial cells, *Salmonella* is then rapidly engulfed by macrophages. By again making use of its effector

proteins from the T3SS encoded by SPI1, *Salmonella* trigger macrophage apoptosis, release of the pathogen, and release of the IL-1 pro-inflammatory cytokine causing inflammation (Sansonetti, 2004). Moreover, *Salmonella* induce secretions of the IL-8 chemoattractant that triggers neutrophil infiltration into the infected epithelial cell (Gewirtz et al., 1999). Alternatively, *Salmonella* use another T3SS encoded by *Salmonella* pathogenicity island 2 (SPI2) to inject effector protein molecules into the macrophage cytoplasm. These effector proteins allow *Salmonella* to develop and reside in a self-made compartment, known as the *Salmonella*-containing vacuole (SCV), which supports survival and multiplication of the pathogen (Steele-Mortimer et al., 1999; Holden, 2002). Ultimately, *Salmonella* are systemically disseminated to different body parts via the infected macrophages.

***Invasion via dendritic cells:*** Finally, dendritic cells open the tight junctions between adjacent epithelial cells to extend dendrites that sample *Salmonella* residing in the intestinal lumen (Rescigno et al., 2001). Interestingly, during this process, integrity of the epithelial barrier is preserved because dendritic cells express junctional proteins, such as claudins and occludins (Rescigno et al., 2001).

### 1.4.5 *Salmonella* lipopolysaccharide triggers inflammation

In order to combat invading *Salmonella*, it is critical that the host's innate immune system recognizes the pathogen and mounts an efficient inflammatory response. Of the different biologically active components present in the outer membrane of gram-negative bacteria such as *Salmonella*, the lipopolysaccharide (**LPS**) endotoxin is the most potent inducer of inflammation (Freudenberg et al., 2001). LPS is an important recognition marker which the innate immune system senses and reacts against *Salmonella*. Structurally, LPS consists of two parts: (i) a hydrophobic domain known as lipid A, (ii) a genetically variable hydrophilic polysaccharide portion, consisting of an O-specific chain and core region (Henderson et al., 1996). Lipid A is the biologically active centre of the LPS molecule and is responsible for the toxic and other biological effects of LPS (Schletter et al., 1995). The structure of lipid A is highly conserved among many Gram-negative bacteria, especially among *Enterobacteriaceae*.

It is well recognized that *Salmonella*-induced systemic inflammation is largely dependent on the release of LPS from the pathogen's outer membrane (Henderson et al., 1996). For this reason, the intraperitoneal administration of LPS has been commonly used to reproduce the typical features of acute systemic inflammation in experimental studies with chickens (Xie et al., 2000) and mice (Jeong et al., 2010; Hagiwara et al., 2011). A similar approach was adopted in this study.

## 1.5 The Gastrointestinal tract: Innate defense mechanisms against *Salmonella*

### 1.5.1 Physical barriers

#### 1.5.1.1 Epithelial cells

Intestinal epithelial cells form a highly dynamic physical barrier against invasion by luminal bacteria and their toxins. The protection is aided by two main structural components, including the microvilli and tight junctions; microvilli are multiple evaginations of a dense meshwork of actin filaments whereas tight junctions are characterized by the presence of junctional proteins such as claudins and occludins to maintain integrity and functions of epithelial barrier (Furuse et al., 1996, 1998). Therefore, the principal route by which bacteria or their toxins can cross the epithelium is via mechanisms that can injure or interfere with the normal functioning of regulatory proteins of the epithelial cells. For instance, *Salmonella* utilizes its type III secretory system and effector proteins to disrupt and invade the epithelial barrier (Darwin and Miller, 1999).

#### 1.5.1.2 Mucins secretions and the mucus blanket

The physical barrier that is maintained by epithelial cells is reinforced by a thick mucus layer essentially composed of mucins. The mucosal layer is the largest surface in contact with the external environment that plays an important role in lubrication of the intestinal tract, and transport of nutrients between luminal contents and epithelial lining (Corfield et al., 2000). Mucins are high molecular-weight glycoproteins which are synthesized and secreted by specialized goblet cells present both in the crypt and villus epithelium of the intestines.

Mucins represent the first line of host defense against invading pathogens or their associated toxins. Although the exact mechanism of mucin action remains elusive, mucins are reported to trap pathogenic bacteria and eliminate these from the intestine (Belley et al., 1999). Mucins possess specific mannosyl receptors that competitively attach to Type-I fimbriae of gram negative pathogens (Sajjan and Forstner, 1990). The continuous process of mucins secretions coupled with peristalsis movements allows excretion of trapped pathogens from the intestines. Mucins also protect the epithelium by binding toxins and chemical irritants commonly produced by pathogenic bacteria such as *Salmonella* (Mead, 2002). Interestingly, mucins favor proliferation of commensal bacteria capable of synthesizing mucinases, most particularly bifidobacteria (Katayama et al., 2005; Ryes-Gavilan et al., 2008).

### 1.5.2 Establishment of beneficial bacteria

One of the major benefits of commensal or beneficial bacteria to intestinal health is their ability to reduce intestinal colonization of pathogens by the competitive exclusion mechanism. Lactobacilli and bifidobacteria compete with pathogens for nutrients and binding sites at the brush border of intestinal epithelial cells (Rolfe, 2000; van der Wielen et al., 2002), and secrete antimicrobial substances such as bacteriocins and organic acids that suppress pathogen growth (Gibson and Wang, 1994; Jin et al., 1996). Nurmi and Rantala (1973) first demonstrated the possibility of controlling *Salmonella* infection in chicks by inoculating them with intestinal content from adult birds. This intestinal content is presumed to contain mostly commensal or beneficial bacteria that colonize the intestinal tract of the chicks and confer protection against harmful bacteria.

Additionally, beneficial bacteria are reported to increase mucins secreting capacity by goblet cells, therefore increasing protection against intestinal pathogens. Such observation has been evidenced by an *in vitro* study whereby *Lactobacillus plantarum* inhibited adherence of *E. coli* to intestinal epithelial cells by increasing mRNA expressions of mucins (MUC2 and MUC3) and subsequent mucus secretion (Mack et al., 1999). In the same study, *E. coli* did not alter expression of the mucin genes when cultured with the same line of mucus-secreting cells. Therefore, the establishment of an intestinal microflora consisting of beneficial bacteria is a desirable component of good

intestinal health, especially during opportunistic infections and invasions of intestinal epithelium by pathogens of health and economic importance, such as *Salmonella*.

### 1.5.3 Innate immunity

#### 1.5.3.1 Pathogen recognition

Mucosal innate immunity of intestinal epithelial cells provides the early line of host defense against microbes. The principal effector cells of innate immunity are neutrophils (heterophils are the avian equivalent of mammalian neutrophils), mononuclear phagocytes (monocytes and macrophages), and dentritic cells. Neutrophils, macrophages and dendritic cells possess different types of toll-like receptors (**TLR**s) called 'cellular pattern recognition receptors' to detect highly conserved features of a wide range of pathogens called 'pathogen-associated molecular patterns' (**PAMPs**) (Medzhitov, 2001; Janeway and Medzhitov, 2002). Examples of PAMPs are LPS and peptidoglycans from the cell walls of gram-negative and gram-positive bacteria, respectively (Janeway and Medzhitov, 2002; Barton, 2008). These structures are unique to bacteria and absent in host cells. There exist different types of TLRs in recognition to different PAMPs. For example, TLR-4, the first mammalian TLR to be discovered and characterized (Medzhitov et al., 1997), can recognize and bind to LPS from the outer wall of gram-negative pathogens (Chow et al., 1999; Hoshino et al., 1999). In addition to its presence on immune cells (neutrophils, macrophages and dendritic cells), TLR-4 has been identified at the apical end of human intestinal epithelial cells (Otte et al., 2004). The detection of gram-negative pathogens in the intestinal lumen is a critical factor that allows early innate immune response and avoidance of major inflammatory responses (Cario and Podolsky, 2000).

#### 1.5.3.2 Inflammation and pathogen clearance

Inflammation is a protective coordinated process of innate immunity that is induced by microbial infection or tissue injury to resolve the infection or repair the damage and return to a state of homeostasis (Barton, 2008). After breaching the epithelial barriers, LPS of gram-negative pathogens, including *Salmonella*, are recognized by TLR-4 of tissue-resident macrophages (Aderem, 2001; Barton, 2008). Following binding of

LPS to macrophage TLR-4, a series of signalling events occur that leads to the transcription of genes encoding for pro-inflammatory cytokines (TNF, IL-1 and IL-6), chemokines (IL-8), and molecules involved in immune responses such as adhesion molecules (E-selectin) and nitric oxide synthase (iNOS) (Ye et al., 2002; Janssens and Beyaert, 2003). Inflammatory cytokines and chemokines are critical factors of protection against bacterial infections, given that these alert infected hosts and regulate immune responses for pathogen clearance.

The released cytokines and chemokines induce a cascade of events at site of infection including: vasodilation and its resulting increased blood flow, increased expression of P-selectin and E-selectin surface proteins on endothelial cells of blood vessels, and rapid mobilization of neutrophils, monocytes and blood plasma into infected tissues (Dempsey et al., 2003; Barton, 2008). Increased expression of selectins allows immune cells to specifically be recruited into the infection site across blood vessels. In most cases, neutrophils first reach the infected tissues prior to an influx of monocytes which ultimately become macrophages (Nathan, 2006). Both neutrophils and macrophages are equipped with an array of antimicrobial weaponry, including proteolytic lysosomal enzymes such as elastase (serine proteases) and cathepsin, antimicrobial peptides (defensins and cathelicidins), and toxic metabolites derived from oxygen (termed as reactive oxygen species (ROS)) and nitrogen (termed as reactive nitrogen species (RNS)) that are responsible for destroying pathogens via the process of phagocytosis (Bogdan et al., 2000; Nathan et al., 2000, 2006). Heterophils are the avian equivalent of mammalian neutrophils in their activity to phagocytose pathogenic bacteria during host defense. The capacity of heterophils and macrophages to kill intestinal *S.* Enteritidis has been demonstrated in *in vitro* studies (Stabler et al., 1994). Because neutrophils have the shortest life span (6 to 10 h) among phagocytic cells, these undergo apoptosis and are eventually cleared by macrophages (Dempsey et al., 2003). But, *Salmonella* are capable of surviving and replicating within macrophages (Henderson et al., 1999), allowing their dissemination to different body parts.

In the event that neutrophils or macrophages can detect TNF-α, but cannot detect presence of intracellular pathogen, the immune cells release their antimicrobial granules into the extracellular space with objective of creating an inhospitable environment for

nearby pathogens (Nathan, 2006). However, in addition of being toxic to microbial organisms, these granules cause structural damage to host tissues or cells. For example proteinase 3, elastase and cathepsin G are broad spectrum proteases capable of destroying host cells leading to their liquefaction (Barton, 2008). Yet, such toxicity or havoc to pathogens and infected host cells are critical in clearing the infection in its early phase before the full immune response is mounted (Barton, 2008). An ideal inflammatory response would, therefore, be one that can clear the infection as rapid as possible while limiting damages to host tissues.

After successful clearance of pathogens, the inflammatory or innate immune response needs to be terminated (Levy et al., 2001). The exact mechanism involving resolution of the inflammation process is still being researched. There is evidence suggesting that IL-10, an anti-inflammatory cytokine, plays an integral role in this termination process by suppressing release and activity of pro-inflammatory cytokines (Fife et al., 2006; Taylor et al., 2006). There are also indications that lipid-derived mediators called lipoxins prevent the influx of neutrophils (Serhan and Savill, 2005; Serhan, 2007).

#### 1.5.3.3 Inflammation and nutrient mobilization

The acute phase response (APR) is one of the most critical sequelae to infection or inflammation that affects nutrient requirements and metabolism (Kushner, 1982). In avian species as well as mammals, acute phase response is mediated by a series of inflammatory cytokines that are synthesized and secreted by activated blood monocytes and tissue macrophages in response to pathogens or LPS (Argiles et al., 1992). TNF, IL-1 and IL-6 are the most common pro-inflammatory cytokines involved in acute phase response during an inflammatory reaction (Kaiser et al., 2000; Swaggerty et al., 2005).

Pro-inflammatory cytokines may act locally to amplify cellular immune responses or systematically to exert profound behavioural and metabolic effects (Hopkins and Rothwell, 1995; Johnson, 1997). Inflammation is generally characterized by fever (Dinarello et al., 1986), increased acute-phase protein syntheses by hepatocytes (Castell et al., 1989), anorexia (McCarthy and Daun, 1992), and nutrient mobilization (Richards et al., 1991; Johnson, 1997). Broilers subjected to challenge with LPS from *Salmonella* or *E.*

*coli* exhibited fever, anorexia, somnolence, ruffled feathers, increased liver weight, increased plasma neutrophil concentrations, and reduced plasma glucose levels (Klasing and Barnes, 1988; Xie et al., 2000; Mireles et al., 2005). These effects were accompanied by reduced growth and feed efficiency.

***Pro-inflammatory cytokines, fever and anorexia***

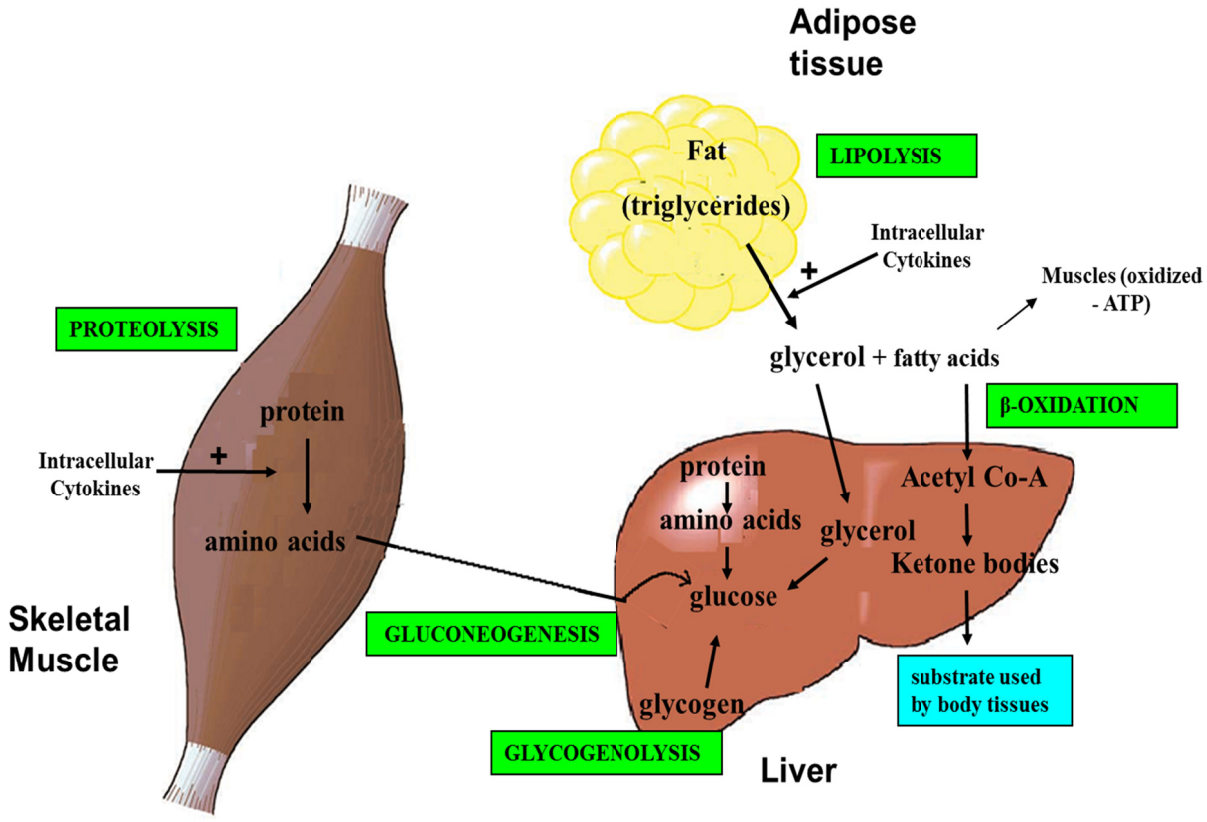
During infection, fever is mediated mainly by IL-1, IL-6 and TNF-α (Dinarello et al., 1986; Kozak et al., 1998; Harden et al., 2008). It is known that cytokines are large hydrophilic molecules which cannot diffuse across the blood-brain barrier (Dinarello, 1988). Alternatively, cytokines reach the CNS through cells that are devoid of the blood-brain barrier, namely microglia and astrocytes. These cells stimulate the hypothalamus to produce and secrete prostaglandins which readily diffuse throughout the brain, thereby eliciting fever and sickness behaviour (Katsuura et al., 1990; Breder and Saper, 1996). This theory is evidenced by studies in which injections of cyclooxygenase inhibitors, which prevent metabolism of arachadonic acid to prostaglandins, prevented sickness behaviour in pigs and chickens after challenge with LPS (Johnson et al., 1993a,b).

Cytokines also stimulate vagal afferent nerves of the intestines that transmit the stimulus to the CNS (Bluthe et al., 1994) causing synthesis of cytokines within the CNS (Dantzer, 1994; Laye et al., 1995). CNS cytokines profoundly reduce gastric acid secretions (McCarthy and Daun, 1992) and create conditions of satiety in the animals, thereby causing anorexia or reducing voluntary feed intake.

***Pro-inflammatory cytokines and reduced bodyweight***

During inflammation, anorexia and somnolence restrict normal feed intake by the host, therefore compelling the host to rely on its body reserves for energy to maintain proper functioning of cells, tissues and organs. Such effects are orchestrated by pro-inflammatory cytokines that work synergistically to profoundly alter carbohydrate, protein and fat metabolism, by acting on the liver, adipose tissue and skeletal muscle. The shift from anabolic to catabolic processes forms the basis of reduced growth and feed utilization in animals subjected to pathogenic agents. These are summarized in Figure 1.3 below.

**Figure 1.3:** Schematic illustration of nutrient mobilization activities occurring during inflammation[1]




[1]*Modified from Pedersen et al. (2001).*

***Liver metabolism*:** First, pro-inflammatory cytokines enhance uptake and oxidative utilization of blood glucose (Mizock, 1995). Then, to maintain blood glucose level, liver glycogen is depleted by the process of glycogenolysis. Glycogenolysis involves the breakdown of glycogen to glucose-6-phosphate and its subsequent hydrolysis by glucose-6-phosphatase to free glucose. Liver and skeletal muscles contain most of the body's glycogen reserves. However, because glucose-6-phosphatase is present in the liver only, the breakdown of hepatic glycogen leads to release of glucose, whereas the breakdown of muscle glycogen leads to the release of lactate. After depletion of liver glycogen, prolonged inflammation causes skeletal muscle catabolism and amino acid mobilization to the liver for gluconeogenesis (Holecek et al., 1995; Johnson, 1997).

Gluconeogenesis involves the formation of glucose-6-phosphate from precursors such as amino acids, glycerol and lactate with its subsequent hydrolysis by glucose-6-phosphatse to free glucose. The kidney also produces glucose by gluconeogenesis, but in smaller amounts than liver glycogenolysis and gluconeogenesis.

Acute phase proteins are synthesized and secreted by hepatocytes under the effect of pro-inflammatory cytokines, most particularly IL-6 (Castell et al., 1989; Richards et al., 1991). This occurs subsequent to increased amino acid uptake by hepatocytes due to cytokines (Warren et al., 1987). Serum amyloid A, an acute phase protein, acts as chemoattractant to recruit immune cells into inflammation sites for pathogen clearance or destruction (Badolato et al., 1994; Olsson et al., 1999). Additionally, ferritin binds to iron, ceruloplasmin oxidises iron and haptoglobin binds to haemoglobin; these inhibit uptake of iron by pathogens.

Moreover, in the liver, TNF-α caused increased synthesis of triglycerides (Feingold and Grunfeld, 1987). But, hepatic and circulating triglycerides are rapidly hydrolysed into free fatty acids and glycerol due to increased lipoprotein lipase activity in the liver (Grunfeld et al., 1989). The high influx of free fatty acids and glycerol from within the liver and adipose tissue are then used to generate energy. Fatty acids are converted into acetyl-coA via the β-oxidation cycle in the mitochondria. On the other hand, glycerol enter the glycolytic pathway to be converted into acetic acid which in the presence of co-enzyme A produce acetyl Co-A. Finally, acetyl-coA generates energy through the Krebs Cycle.

***Skeletal muscle degradation:*** TNF-α is the principal cytokine involved in skeletal muscle protein catabolism for amino acids mobilization (Argiles et al., 1992), occurring both in humans (Starnes et al., 1988) and rats (Flores et al., 1989). But, the effect of TNF-α on muscle catabolism seems to be dependent on glucocorticoid hormones (i.e corticosterone). Glucocorticoids are released by the adrenal cortex upon activity of circulatory cytokines on the CNS. In rats, muscle protein catabolism only occurred when TNF-α was injected with corticosterone, but not by TNF-α alone or in rats that had undergone adrenalectomy (Hall-Angeras et al., 1990). On the other hand, IL-1 and IL-6 have been shown to decrease protein synthesis in skeletal muscle, thereby preventing its

regeneration (Ferrucci and Guralnik, 2003). Amino acids released during muscle proteolysis, due to pro-inflammatory cytokines, are ultimately utilized in acute phase protein synthesis in the liver (Johnson, 1997).

***Adipose tissue catabolism*:** Adipose tissue catabolism occurs by lipolysis, thereby increasing blood triglyceride levels that eventually generate energy in the liver (Feingold et al., 1992). TNF-α causes adipose tissue catabolism or lipolysis by phosphorylating and activating hormone-sensitive lipases, thereby increasing circulating free fatty acids and glycerol to the liver (Greenberg et al., 2001). TNF-α, IL-1β and IL-6 have also been shown to inhibit or reduce lipoprotein lipase activity in the adipose tissue (Feingold and Grunfeld, 1992; Feingold et al., 1992). Lipoprotein lipase is known to hydrolyse circulating lipoprotein-rich triglycerides, such as very low density lipoprotein (VLDL), into free fatty acids; only free fatty acids can be absorbed by adipose tissue, esterified and stored as fats cells. It is evidenced that TNF-α decreased activity of lipoprotein lipase in cultured fat cells, but stimulated lipolysis (Memon et al., 1994). During acute phase response, therefore, mobilization of fatty acids is achieved by pro-inflammatory cytokines that cause lipolysis and prevent biosynthesis of the adipose tissue.

## 1.6 Antibiotic Growth Promoters (AGP) in poultry production

### 1.6.1 AGP usage in poultry production

Antibiotics are compounds that can either be naturally produced by specific micro-organisms or synthesized, which inhibit the growth of or destroy bacteria of different types (NRC, 1980). The era of antibiotic growth promotion began when Moore et al. (1946) reported that sub-therapeutic usage of the antibiotic streptomycin in chickens resulted in substantial improvement in growth performance. Since then, the term Antibiotic Growth Promoters (**AGP**) has been utilized, given the associated growth and feed efficiency improvements when added to the diets. At a time when global food demand was high, such a discovery was received with great interests. Since 1950's, sub-therapeutic usage of antibiotics which had no application in human health, have been used in the poultry industry to improve growth, feed efficiency and profitability.

Antibiotics are either therapeutically used to treat sick animals or added at sub-therapeutic levels to the feed to improve growth performance, feed efficiency and control intestinal pathogens. The control of intestinal pathogens by antibiotics restricts their contamination of poultry meat during processing, thereby ensuring food safety. In comparison to therapeutic antibiotics, such as erythromycin, lincomycin, oxytetracycline, enrofloxacin and penicillin, AGP, such as virginiamycin, bacitracin, bambermycin and chlortetracycline hydrochloride, are macro-molecules that are not absorbed by the gut and elicit their effects on the microflora and morphological structures of the intestines to improve nutrient digestion and absorption, and safety of poultry products. In Canada, virginiamycin, bacitracin, bambermycin, lincomycin, and chlortetracycline hydrochloride are still permitted AGP for use in poultry production (Canadian Food Inspection Agency, 2011). It is estimated that 70 % of all antibiotics used in the U.S. are meant for non-therapeutic purposes (Union of Concerned Scientists, 2009). The study also revealed tremendous increase in the yearly usage of AGP in livestock production from 16.1 million pounds in 1980 to approximately 24.6 million pounds in 2001. In 2001, about 10.5 million pounds were used in poultry production, 10.3 million pounds in swine production and 3.7 million pounds in cattle production.

### 1.6.2 Biological effects of AGP on growth enhancement

It is clearly evidenced that orally ingested AGP through the diet promote growth and improve feed efficiency (Belay and Teeter, 1996; Engberg et al., 2000; Waldroup et al., 2003; Miles et al., 2006; Ashayerizadeh et al., 2011), decrease flock variability (Miles and Harms, 1984). The exact mechanism by which AGP promote growth is not clearly understood, but findings that AGP have no growth-promoting effects in germ-free birds (Coates et al., 1955; Coates et al., 1963) suggest that AGP mainly act on the intestinal microflora. Most of the AGP used in poultry production have a broad spectrum of activity and act mainly on gram-positive bacteria, such as *Clostridium*, lactobacilli, bifidobacteria and *Streptococcus* (Engberg et al., 2000; Gaskins et al., 2002), the predominant bacteria in healthy chickens (Bjerrum et al., 2006; Gong et al., 2007). Moreover, AGP reduces intestinal loads of harmful bacteria such as *Salmonella*, *E. coli* and *Campylobacter* which frequently cause subclinical diseases in mature chickens (Hughes and Heritage, 2004). A

reduction in the intestinal microbial load reduces competition for vital nutrients between the bird and the microbial flora. Additionally, AGP causes thinning of intestinal villi wall (Coates et al., 1955), and reduction in intestinal tract length (Stutz et al., 1983; Miles et al., 2006) and weight (Henry et al., 1986). A reduction in gut mass due to dietary addition of AGP is similar to those observed in germ-free birds (Murakami et al., 1994), thereby reducing required nutrients for maintenance of the intestines and increasing available nutrients for growth.

### 1.6.3 The future of AGP in poultry production

It has long been argued among scientists and poultry producers that AGP withdrawal would cause a dramatic increase in pathogenic bacteria load in broilers. However, after the voluntary AGP ban in Denmark (15$^{th}$ February 1998), *Salmonella* prevalence in broilers was considerably reduced between 1995 to 2001 (Evans and Wegener, 2003), without causing any loss in productivity or livability, except that FCR marginally increased from 1.74 (1995-1997) to 1.78 (1998-2000) (WHO, 2002). The report also highlighted that 60 – 80 % of chickens had bacteria resistant to three widely used antibiotics, namely avilamycin, avoparcin and streptogamins, before the ban in Denmark, and that, after the AGP ban, antibiotic-resistant bacteria among broilers dropped to 5 - 35 % only. Indeed, Denmark has proved to the world that AGP is not necessary in efficient poultry production. Based on Denmark's experience, the WHO firmly condemns the use of antibiotics in healthy animals meant for growth promotion or disease prevention, and the irresponsible promotions engaged by commercial companies (WHO 2011). In too many countries, AGP use in animals seems to outweigh antibiotic use in human medicine. The occurrence of resistant infections caused by *Salmonella* and *Campylobacter* spp., common foodborne bacteria, has been linked with AGP usage in food animals (WHO, 2011). Not surprising that, in many countries where massive AGP utilization is still permitted, multiple-antibiotic resistant *S.* Enteritidis strains have emerged and are frequently detected in poultry carcasses and poultry retail meat (de Oliveira et al., 2011; Hyeon et al., 2011; Yildirim et al., 2011). Recently, the WHO launched the slogan “No action today, no cure tomorrow” on the occasion of World Health Day 2011 to raise awareness that lifesaving antibiotics are losing their healing

power due to emergence of antibiotic-resistant bacteria (WHO, 2011). Today, scientists fear that the reckless use of AGP may lead to a pre-antibiotic era where simple infections do not respond to treatment, and routine operations and interventions may become life-threatening.

The European Commission, the World Health Organisation, the Centers for Disease Control and the American Public Health Association are entirely in support of the immediate prohibition of AGP that are the same as, or closely related to, antibiotics used in humans, by legislation if necessary, because antibiotic-resistant pathogens are transmitted to humans via the food-chain (WHO, 2011). It is forecasted that a ban on AGP is most inevitable in the US and Canada due to increasing public health concerns and international trade pressures rather than a legislative decision. Consumer demands for safer meat products have compelled the McDonald's Corporation and Kentucky Fried Chicken to refuse chicken meat produced with AGP (KFC, 2002; McDonald's Corporation, 2003). Moreover, the World Trade Organization favours the trade of meat products from animals that are not fed with AGP. The European Union has not only banned AGP in its local livestock production, but has also banned importation of meat products from the US. Very recently, on March 21 2011, Russia also banned importation of chicken, pork and beef meat from the US for the very simple reason that these contain excessive amounts of antibiotics (Politicol News, 2011). No matter how hard the lobbyists, politicians and presidents push made in US meat products, today these are being banned in many countries (Politicol News, 2011). Contrastingly, after its ban on AGP, Denmark has seen significant increase in poultry meat production, particularly driven by increased market demands for safer poultry meat. But, Canada still imports chicken, pork and beef from the US, since there are no regulations or standards on antibiotic containing foods.

### 1.7 Mannanoligosaccharide: An alternative prebiotic to AGP in broiler production

To help alleviate some of the penitential problems that might possibly occur upon removal of AGP, poultry researchers and producers emphasize the necessity of developing effective biological alternatives that can sustain or improve intestinal health in broilers. Prebiotics are 'non-digestible feed ingredients that beneficially affect the host by

selectively stimulating the growth and/or activity of one or a limited number of intestinal microflora, such as lactobacilli and bifidobacteria, and thus improve host health (Gibson and Roberfroid, 1995). Prebiotics are non-digestible oligosaccharides, especially those containing fructose, xylose, galactose, glucose and mannose (Gibson and Roberfroid, 1995; Gibson, 1998); the most common and successful ones are those based on fructose and mannose (Gibson, 1998). Although prebiotics have a similar mode of action to probiotics, these do not contain live microbes, but stimulate the growth of commensal or beneficial bacteria in the gut of the host (Vijaya Kumar et al., 2005).

MOS is a complex mixture of mannoproteins, mannose and glucose derived from cell wall fragments of the yeast *Saccharomyces cerevisiae*, which is neither digested by endogenous digestive enzymes nor absorbed by the host. Despite its classification as a prebiotic, MOS is not used as a substrate in microbial fermentation to uniquely and selectively enrich beneficial bacteria of the intestines. Instead, MOS functions to improve intestinal health in chickens by selectively adsorbing and eliminating gram-negative pathogenic bacteria from the intestine, enhancing intestinal development of mucus-secreting goblet cells, and increasing colonization by beneficial bacteria.

### 1.7.1 Effects of MOS on growth performance

Published reports show highly variable production performance responses in chickens fed MOS supplemented diets. In a meta-analysis of 26 published studies with broilers, Hooge (2004) reported that MOS and AGP were similarly effective in improving BW and feed efficiency in comparison to an AGP-free diet. Body weight and FCR improvements due to dietary MOS are also reported in other studies with broilers (Chee et al., 2010; Ghosh et al., 2011) and turkeys (Fairchild et al., 2001; Sims et al., 2004). In other studies, however, MOS failed to improve BW and feed efficiency when compared to broilers fed AGP-free diets (Waldroup et al., 2003; Baurhoo et al., 2007b; Brummer et al., 2010; Moreles-Lopez et al., 2010; Khalaji et al., 2011). Despite the lack of improvements in FCR, Kim et al. (2011) observed better growth among MOS-fed broilers. Interestingly, however, in a coccidial-infection study with broiler chickens, MOS caused improvements in growth and FCR (Gomez-Verduzco et al., 2009).

### 1.7.2 MOS reduces intestinal colonization by gram-negative bacteria

Ofek et al. (1977) incubated human epithelial cells with *E. coli* K12 in the presence of D-mannose and observed that the sugar inhibited adherence of the pathogen to epithelial cells. After further investigation, the authors concluded that mannose-binding adhesin present in type 1 fimbriae of *E. coli* mediated adherence of the pathogen to mannose-containing glycoprotein receptors of human epithelial cells (Ofek et al., 1978). Obviously, D-mannose significantly reduced attachment of *S.* Typhimurium to the small intestine of 1-day-old chicks (Oyofo et al., 1989). It was later revealed that FimH adhesin is the terminal subunit of type 1 fimbriae that specifically binds D-mannose and mediates attachment of gram-negative bacteria to target cells (Thomas et al., 2004).

Since then, mannose rich fractions present in the outer cell wall of *S. cerevisiae* has been exploited in the improvement of intestinal health in livestock species, and in particular poultry. The efficacy of MOS to agglutinate gram-negative pathogens expressing type 1 fimbriae, including *E. coli*, *S.* Typhimurium and *S.* Enteritidis, has been demonstrated in an *in vitro* study (Spring et al., 2000). By adhering to mannose-specific adhesin (FimH) of gram-negative bacteria, MOS prevents colonization of the pathogens to mannose-containing glycoprotein receptors of intestinal epithelial cells (Ofek et al., 1978; Thomas et al., 2004). The pathogens are, hence, excreted through the gut instead of colonizing the intestinal epithelium. Evidently, in challenge studies with broilers, MOS reduced intestinal concentrations of *Salmonella* (Spring et al., 2000; Fernandez et al., 2002; Ghosh et al., 2011) and *E. coli* (Baurhoo et al., 2007b; Ghosh et al., 2011; Kim et al., 2011) in the absence of AGP.

### 1.7.3 MOS increases intestinal development of mucins-secreting goblet cells

Goblet cells are specialized mucins-secreting cells, which are produced from stem cells at the base of the intestinal crypts. Thereafter, goblet cells migrate along the villi membrane over a period of approximately three days to reach the villi tip where they are eventually sloughed and released into the intestinal lumen (Uni et al., 2003). During that life-span time period, goblet cells secrete mucins as a natural defense mechanism against intestinal pathogens. We previously discussed the role of the mucus gel layer and mucins in trapping and eliminating gram-negative bacteria from the intestines. Interestingly, the

literature reveals that MOS favors proliferation of mucin-secreting goblet cells in the intestinal villi membrane of both chickens and turkeys. For instance, the number of goblet cells was significantly increased when broilers (Baurhoo et al., 2007a) and turkeys (Solis de los Santos et al., 2007) were fed MOS (0.1 % or 0.2 %) supplemented diets. Furthermore, both the neutral and acidic types of goblet cells were increased in MOS-fed turkeys (Solis de los Santos et al., 2007). But, in MOS-fed broilers, Chee et al. (2010) observed increases in the number of acidic goblet cells due to MOS, whereas the neutral type of goblet cells was unchanged. In addition to increasing goblet cell number per villus, MOS also increased the size of goblet cells (Brummer et al., 2010). Indeed, both the number and size of goblet cells are major determinants of the amounts of mucins secreted into the intestinal lumen.

### 1.7.4 MOS modifies the intestinal microflora compositions

MOS has been shown to beneficially modify microflora composition of the gastrointestinal tract, and improving intestinal health in chickens. For instance, dietary inclusion of MOS significantly increased the populations of intestinal digesta-associated lactobacilli and bifidobacteria in chickens (Fernandez et al., 2002; Baurhoo et al., 2007a,b; Biggs et al., 2007). Increased intestinal lactobacilli loads due to MOS have also been reported in MOS-fed broilers (Chee et al., 2010). In MOS-fed turkeys, Sims et al. (2004) also observed increased intestinal concentration of bifidobacteria. Microflora enrichment with lactobacilli and bifidobacteria is highly desirable given that these probiotic bacteria exert gut health benefits by competitively excluding pathogenic bacteria for binding sites and competing for nutrients (Rolfe, 2000), and by producing toxic compounds that inhibit pathogens (lactobacilli secrete bacteriocins and bifidobacteria secrete organic acids and other bactericidal substances) (Gibson and Wang, 1994; Jin et al., 1996). In other studies, however, MOS did not alter intestinal lactobacilli loads in broilers (Spring et al., 2000; Moreles-Lopez et al., 2010; Khalaji et al., 2011) nor in turkeys (Fairchild et al., 2001). Therefore, the literature reveals inconsistency in the effects of MOS on intestinal lactobacilli and bifidobacteria in broilers and turkeys.

## Working Hypotheses and Research Objectives

### 1. Working Hypotheses

- In comparison with VIRG and BACT, MOS (0.2 %) will increase the number of mucins-secreting goblet cells, and increase lactobacilli and bifidobacteria concentrations in the intestines, such that enhancement of these intestinal defense mechanisms will favor villi development, and reduce the intestinal digesta-associated and litter *Salmonella* and *E. coli* concentrations. As a result of improved intestinal health conditions, MOS (0.2 %) fed broilers will be heaviest and with increased breast meat yield. Finally, all of these beneficial effects of MOS (0.2 %) will be further increased at a higher dietary level of MOS (0.5 %).
- In the event of enteric *S.* Entertidis infection, in comparison with VIRG, MOS will increase the number of neutral and acidic mucins-secreting goblet cells, and increase membrane-bound mucins barrier overlying the intestinal epithelium. Enhancement of these intestinal mucins-mediated defense mechansisms will significantly eliminate intestinal digesta-associated *S.* Entertidis, mitigate *S.* Entertidis invasion and damage of intestinal epithelium, reduce *S.* Entertidis-induced inflammation, and improve the health and development of intestinal villi.
- During late systemic inflammation, MOS will cause activation of innate immune system that will more significantly reduce inflammatory reactions than VIRG, thereby reducing nutrient mobilization from catabolism of body reserves to meet the lower energy requirements of MOS-fed hosts.

### 2. Research Objectives

- To evaluate the effects of MOS (0.2 % and 0.5 %) and VIRG on growth and feed efficiency; intestinal development of villi and mucins-secreting goblet cells; intestinal digesta-associated populations of beneficial (lactobacilli and bifidobacteria) and pathogenic (*E. coli*) bacteria; and carcass part yields in broilers raised under good sanitary conditions.

- To study the effects of MOS and VIRG on epithelial barrier integrity; villi morphology; development of neutral and acidic mucins-secreting goblet cells; secretions of membrane-bound and secretory mucins; and pro-inflammatory cytokine gene expressions in the intestines of young chicks infected with *S.* Enteritidis.

- To determine the effects of MOS and VIRG on expressions of key genes involved in innate immunity and metabolic pathways in the liver and intestines of chickens during late LPS-induced systemic inflammation by using in-depth microarray analysis.

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# CHAPTER II. EFFECTS OF DIETS CONTAINING DIFFERENT CONCENTRATIONS OF MANNANOLIGOSACCHARIDE OR ANTIBIOTICS ON GROWTH PERFORMANCE, INTESTINAL DEVELOPMENT, CECAL AND LITTER MICROBIAL POPULATIONS, AND CARCASS PARAMETERS OF BROILERS[1]

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## 2.1 ABSTRACT

The effects of two levels of mannanoligosaccharides (MOS) in feed were compared with antibiotic growth promoters (AGP) on growth performance, intestinal morphology, cecal and litter microbial populations, and carcass parameters in broilers raised in a sanitary environment. Dietary treatments included: 1) AGP-free diet (CTL); 2) VIRG (diet 1 + 16.5 mg / kg virginiamycin); 3) BACT (diet 1 + 55 mg / kg bacitracin); 4) LMOS (diet 1 + 0.2 % MOS); 5) HMOS (diet 1 + 0.5 % MOS). Birds were randomly assigned to 3 replicate pens / treatment (n = 55 / pen). BW and feed intake were recorded weekly throughout 38 d. At d 14, 24 and 34, a 1-cm segment of duodenum, jejunum and ileum was used in morphological analysis (n = 9 birds / d / treatment). At same bird ages, cecal contents were assayed for lactobacilli, bifidobacteria, *Salmonella*, and *E. coli*, whereas litter was analyzed for *Salmonella* and *E. coli*. Carcass yields (breast fillet and tenders, thigh, drumstick and wing) were determined at d 38. BW, feed conversion and carcass yields did not differ among treatments. In contrast to birds fed VIRG or BACT, LMOS and HMOS consistently increased ($P < 0.05$) villi height and goblet cell number per villus in all intestinal segments at d 24 and 34. Bifidobacteria concentrations were higher ($P < 0.05$) in LMOS and HMOS fed birds at all time points. Birds and litter from all treatments were free of *Salmonella*. At d 14 and 24, cecal *E. coli* counts were not different among treatments. In comparison to birds fed CTL, at d 34, BACT, LMOS and HMOS significantly reduced ($P < 0.05$) cecal *E. coli* concentrations. Litter bacterial counts were not altered by dietary treatments. In conclusion, under conditions of this study, MOS conferred intestinal health benefits to chickens by improving its morphological development and microbial ecology. But, there were no additional benefits of the higher MOS dosage.

(**Key words**: antibiotic, prebiotic, mannanoligosaccharides, intestinal health, broilers)

## 2.2 INTRODUCTION

The gastrointestinal tract plays a vital role in the digestion and absorption of nutrients required for maintenance and growth. The proliferation of pathogens in the intestines often results in inflammatory responses that cause productivity losses, increased mortality and increased contamination of poultry products. Sub-therapeutic antibiotics have long been used in broiler diets for growth improvement and the control of intestinal pathogens. However, issues regarding the development of antibiotic-resistant bacteria and intensive use of sub-therapeutic antibiotics have led to public demand to limit the use of antibiotics in animal agriculture (Smith et al., 2003). Consequently, there is growing demand for natural alternatives to sub-therapeutic antibiotics that can sustain or improve farm performance and safety of broiler products.

The broiler intestine harbors a diverse microflora, consisting of both beneficial and pathogenic microorganisms. Attachment to mucosal surfaces is a prerequisite for successful colonization of enteric bacteria; otherwise, bacteria are rapidly excreted *via* the hydrokinetic properties of the intestine. Gram-negative pathogens that express Type 1 fimbriae, such as *Salmonella* and *Escherichia coli*, recognize D-mannose receptor sites on the intestinal epithelium. The adhesive properties of Type 1 fimbriae are determined by the fimbrial tip containing a mannose-specific lectin, FimH (Thomas et al., 2004). Mannose, either in the pure form (Oyofo et al., 1989a) or yeast cell wall (Spring et al., 2000), competitively binds to the FimH lectin of gram-negative pathogens. This concept was demonstrated by reduced intestinal colonization of *Salmonella* and *E. coli* after the addition of a mannan-oligosaccharide (**MOS**) to chicken diets (Oyofo et al., 1989b,c; Spring et al., 2000; Baurhoo et al., 2007b).

The addition of a commercially available MOS product (BioMos, Alltech Inc., Nicholasville, KY), rich in mannoproteins, mannose and glucose, and derived from cell wall of the yeast *Saccharomyces cerevisiae*, to broiler diets significantly increased goblet cell number of the intestinal villi (Baurhoo et al., 2007a). Goblet cells are specialized cells that secrete mucins, glycoprotein compounds, which bind pathogenic microorganisms and reduce their adherence to the intestinal mucosa (Blomberg et al., 1993). In turkeys, increasing dietary inclusion levels of a different MOS product from 0.5 kg / tonne to 1 kg / tonne caused significant increase in goblet cell number per villus

(Solis de los Santos et al., 2007). Similarly, live BW was significantly higher in groups with medium (0.4 and 0.2 % in the first and second 8-wk periods, respectively) and high (1.0 and 0.4 % in the first and second 8-wk periods, respectively) levels of MOS compared with turkeys fed the low levels of MOS (0.1% MOS during the entire 16 weeks) (Zdunczyk et al., 2005).

Data regarding the use of MOS on intestinal health parameters in broilers raised under conditions of good sanitation are lacking. Moreover, MOS effects on intestinal health at dietary levels greater than 0.2 % are still not reported in broiler chickens. In the absence of sub-therapeutic antibiotics, dietary inclusion of MOS above the recommended dosage (0.2 %) might improve the microbial ecology and morphological development of broiler intestines, thereby conferring greater protection against enteric pathogens. Therefore, the objectives of this study were to evaluate the effects of a recommended (0.2 %) and a higher (0.5 %) dietary MOS dosage on growth performance, microbial populations in the ceca and litter, intestinal morphology, and carcass yield parameters in broilers raised under good sanitary conditions. In addition, MOS effects were compared to those of an antibiotic-free diet and ones containing commonly used antibiotics, namely virginiamycin and bacitracin.

## 2.3 MATERIALS AND METHODS

### *Bird Husbandry*

Eight-hundred-twenty-five 1-day-old male Cobb 500 broilers were obtained from a local commercial hatchery (Couvoir Simetin, Mirabel, Québec, Canada) and randomly assigned to 1 of 5 dietary treatments (3 replicate pens; 55 birds per pen). The study was conducted in a clean or sanitary environment (strict biosecurity, properly disinfected experimental facility, clean pine wood shavings and good management). Birds in respective pens were housed in an environmentally controlled room following a standard temperature regimen that gradually decreased from 32 to 24 °C by 0.5 °C daily, tunnel ventilation and under a 20L:4D cycle. A higher bird density was used during the initial period from 1 to 14 d (16.9 birds per $m^2$) to accommodate birds sacrificed for microbiological analysis at d 14. Bird densities on d 21 and d 35 were 12.5 birds per $m^2$ and 11.5 birds per $m^2$ respectively. Concrete floor pens were covered with 8 cm of clean

pine wood shavings, and equipped with one tube feeder and one automatic waterer. All experimental conditions and animal care protocols were approved by the McGill University Animal Care Committee. Birds were group weighed by pen and feed intake (**FI**) determined at weekly intervals.

***Experimental Diets***

Birds were provided *ad libitum* access to water and a standard corn-soybean meal diet that met or exceeded NRC (1994) nutrient requirements (Table 2.1). Diets, in crumble form, included a starter diet from 1 to 21 d and a grower diet from 22 to 38 d. The five dietary treatments, individually prepared by mixing exact amounts of the additives and filler to the basal diet, included: 1) control diet (**CTL**, AGP-free); 2) **VIRG** (diet 1 + 16.5 mg / kg virginiamycin); 3) **BACT** (diet 1 + 55 mg / kg bacitracin); 4) **LMOS** (diet 1 + 0.2 % BioMos, Alltech Inc., Nicholasville, KY); 5) **HMOS** (diet 1 + 0.5 % BioMos).

***Histological Analysis***

At 14, 24 and 34 d of age, 3 birds per treatment pen (n = 9 / treatment) were randomly selected, weighed and euthanized by electrical stunning and bleeding of the carotid artery. Within 5 minutes of euthanization, segments (1 cm) of duodenum (2 cm from gizzard), jejunum (adjacent to Meckel's diverticulum) and ileum (adjacent to cecal tonsils) were dissected, washed in physiological saline solution, and fixed in 10 % buffered formalin. The fixed samples were embedded in paraffin. For each intestinal segment, a 2-µm section was placed onto a glass slide and stained with haematoxylin and eosin for histological analysis. Measured variables included villus height, goblet cell number per villus, crypt depth, and muscularis layer thickness as described previously (Baurhoo et al., 2007a). Histological sections were examined using a phase contrast microscope with integrated image analysis NIS-Element BR v. 2.3 software (Nikon DXM 1200c, Nikon Corporation, Tokyo, Japan). For each parameter, 10 replicate measurements were taken per bird and the average of these values was used in statistical analysis.

***Microbiological Analysis***

At 24 and 34 d, ceca of the euthanized birds (n = 3 / replicate pen) were aseptically removed, placed into sterile stomacher bags (Spiral Biotech Inc., Norwood, MA), and kept on ice. But only at d 14, were cecal digesta from 3 birds pooled per sample (n = 9 / replicate pen) to obtain enough working sample for bacterial analysis. Fresh cecal contents were diluted 10-fold by weight in buffered peptone water (Fisher Scientific, Ottawa, Ontario, Canada) and mechanically homogenized using a stomacher (Model 400 Lab Blender, Seward Medical, London, UK) for 30 s. The samples were then serially diluted in 0.85 % sterile saline solution. All microbiological analyses were performed in duplicates and the average values were used for statistical analysis.

Lactobacilli concentrations were determined using Lactobacilli MRS Agar (Fisher Scientific) and incubated at 37 ºC for 48 h. Bifidobacteria concentrations were measured using Wilkins-Chalgren agar (Oxoid, Nepean, Ontario, Canada), glacial acetic acid (1 mL/L) and mupirocin (100 mg/L; Oxoid), and incubated at 37 ºC for 3 days (Rada et al., 1999). *Salmonella* counts were assayed using Brilliant Green Agar (Oxoid) for 24 h at 37 ºC as described by van Immerseel et al. (2005). Rapid *E. coli* 2 Agar (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and *E. coli* supplement (Bio-Rad Laboratories) were used for quantification of *E. coli* after 24 h culture at 37 ºC as recommended by the supplier. After the incubation periods, colonies of the respective bacteria were counted.

Litter was sampled using a modified method as described by Rybolt et al. (2005). Briefly, a handful amount of litter was collected in the middle of each pen and equidistant from each other at each side end of the pen to the floor pen surface and away from the drinker region using examination gloves. Litter samples were, then, throughly mixed, placed into respective sterile Whirl-Pak microbiological bags (Nasco, Fort Atkinson, WI) and kept at -20 °C until microbiological analysis. A 10 g sample was then used for subsequent enumeration of *Salmonella* and *E. coli* as previously described.

***Carcass Yields***

At d 38, 3 birds per pen (n = 9 / treatment) were randomly selected, individually weighed, and euthanized for determination of carcass characteristics. Feed was removed

4 h prior to processing. After bleeding, birds were scalded for 30 s and deplumed in a rotary plucker. Carcasses were eviscerated by removal of the head, feet, abdominal fat pad, and viscera. The eviscerated hot carcasses were placed in a cool chamber at 4 ºC for 4 h and then cut into their component parts. Whole breast, breast fillet, breast tenders, thigh, drumstick, and wing weights were expressed in absolute weights and as percentages of respective eviscerated carcass weight.

***Statistical Analysis***

Data were analyzed as a one-way ANOVA using the MIXED procedure of SAS (SAS Institute, 2003). A completely randomized design was used for performance parameters with pen as experimental unit. For microbiology, histology and carcass parameters, a Nested Model Design was used with pens nested within treatments and birds as the sub-samples. Treatment means were separated using the least square means option of SAS. Differences among treatment means were tested using Scheffe's Multiple Comparison test and statistical significance was declared at $P < 0.05$. All microbiological concentrations were subjected to $\log_{10}$ transformation prior to statistical analysis.

## 2.4 RESULTS

***Bird performance and Carcass Yields***

The effects of virginiamycin, bacitracin and both MOS dosages on BW, FI or feed conversion ratio (**FCR**) were similar throughout the study (Table 2.2). Moreover, none of the treatment diets altered relative carcass weights (whole breast, breast fillet, breast tenders, thigh, drumstick and wing) and carcass yields at d 38 (Table 2.3). No major incidence of mortality was observed during the entire experimental period.

***Histological Parameters***

At d 24 and 34, villi were consistently longer in the duodenum in both LMOS and HMOS fed birds than those fed the VIRG or BACT diet (Table 2.4). HMOS significantly increased duodenum villi height when compared to birds fed the CTL diet at d 14. In comparison to birds fed the BACT diet, both LMOS and HMOS increased goblet cell

number at d 24 (Table 2.5). At d 34, goblet cell number was greater in LMOS fed birds than in those fed the CTL, VIRG or BACT diet.

Neither villi height nor goblet cell number was affected by dietary treatments in the jejunum at d 14. However, at d 24 and 34, goblet cell numbers were consistently larger in LMOS and HMOS fed birds than those fed the remaining dietary treatments. At d 24, the LMOS diet significantly increased villi height when compared to birds fed CTL or BACT. The same comparisons were made between HMOS fed birds and birds fed CTL or BACT, but no significant differences were detected. Similarly, villi tended to be longer among LMOS and HMOS fed birds than those fed the CTL, VIRG or BACT diet at d 34.

In the ileum, histological parameters were not affected by dietary treatments at d 14. However, at both d 24 and 34, increases in villi height and goblet cell numbers were observed when birds were fed LMOS and HMOS in comparison to VIRG or BACT fed birds.

In all intestinal segments (duodenum, jejunum and ileum), increasing MOS supplementation from 0.2 to 0.5 % did not have additional effects on villi length and goblet cell numbers. In addition, crypt depth, and muscularis layer thickness were not altered by dietary treatments at all times (data not shown).

***Enumeration of bifidobacteria and lactobacilli in the ceca***

In comparison to VIRG and BACT diets, LMOS and HMOS significantly increased bifidobacteria concentrations at d 14, 24 and 34 (Figure 2.1). Moreover, at all times, birds in the HMOS group harbored larger bifidobacteria populations than those fed the CTL diet. But, bifidobacteria concentration was higher in LMOS fed birds than those fed CTL at d 34 only. On the other hand, the HMOS diet significantly increased concentrations of bifidobacteria than the LMOS diet at d 24.

At d 24 and 34, the LMOS and HMOS diets respectively increased the cecal concentrations of lactobacilli when compared to CTL or BACT fed birds (Figure 2.2). But, lactobacilli concentrations were similar among birds fed the different dietary treatments at d 14. When the comparison was made between birds fed LMOS or HMOS,

lactobacilli concentrations were not different at all times. Similar observations were observed between virginiamycin and bacitracin fed birds.

***Enumeration of E. coli in the ceca and litter***

Birds were free of *Salmonella*, confirming absence of the pathogens on our experimental facilities. VIRG, BACT, LMOS and HMOS had similar effects on intestinal concentrations of *E. coli* (Figure 2.3). Additionally, at d 14 and 24, none of these dietary additives significantly altered *E. coli* concentrations when compared to the CTL diet. But, at d 34 only, *E. coli* concentrations were reduced in birds fed BACT, LMOS and HMOS than those fed CTL.

However, at d 14 and 24, *E. coli* concentrations in the litter were not altered by any dietary treatment (Figure 2.4). At d 34, litter *E. coli* count was only reduced by the LMOS diet when compared to litter *E. coli* levels of the control bird group. Finally, *E. coli* concentrations in both the intestines and litter were not different between LMOS and HMOS or VIRG and BACT.

## 2.5 DISCUSSION

The present study indicates that neither MOS (0.2 or 0.5 %) nor antibiotics improved live performance characteristics (BW, FI and FCR) of broilers. Such observations occurred despite overcrowding in the initial part of the study that affected bird development and feed efficiency at 21 d of bird age. But, considering all published reports by a meta-analysis, Hooge (2004) reported that MOS (0.1 % or 0.2 %) and antibiotics were similarly effective in improving BW and feed efficiency, supported by Rosen (2007) using a holo-analysis. The lack of growth improvement due to MOS and antibiotics observed in this study could be attributed to the clean or sanitary conditions under which birds were raised, thereby agreeing with reports by Roura et al. (1992). Moreover, in their classic findings, Coates et al. (1963) reported that chicks housed in a germ-free environment grew faster than those raised in a conventional environment. Our findings, therefore, demonstrate that feed additives were not needed for growth maximization under conditions of this study. The lack of differences in the economically

important carcass part yields, including whole breast, breast fillet, breast tenders, thigh, drumstick and wing, might be the consequence of similarity in BW responses among treatment groups. Similar observations have previously been reported (Fritts and Waldroup, 2003; Waldroup et al., 2003; Parks et al., 2005).

As an important finding of this study, MOS improved the development of morphological structures of the intestine, as indicated by increase in villi length and numbers of goblet cells. Such observations were consistent with previous studies with broilers and turkeys (Baurhoo et al., 2007a; Solis de los Santos et al., 2007). An increase in villi length due to MOS has been associated with increased lactobacilli and bifidobacteria colonization of broiler intestines (Baurhoo et al., 2007a); but the exact mechanism underlying this effect is still not clear. Similar results were observed in the present study, but increasing MOS level from 0.2 % to 0.5 % neither consistently increased cecal lactobacilli and bifidobacteria concentrations nor morphological development of the intestines.

The number of goblet cells in the villi membrane was greater in MOS fed broilers, thereby agreeing with previous studies conducted with broilers and turkeys fed MOS (0.1 % or 0.2 %) supplemented diets (Baurhoo et al., 2007a; Solis de los Santos et al., 2007). Goblet cells synthesize and secrete glycoprotein compounds known as mucins, key components of the first line of host defense against intestinal pathogens. Although the exact mechanism of mucin action remains elusive, mucins are reported to trap pathogenic bacteria and eliminate these from the intestine (Belley et al., 1999). Additionally, specific mannosyl receptors in the oligosaccharide units of mucins competitively bind to Type-I fimbriae of gram negative pathogens (Sajjan and Forstner, 1990). At the same time, indigenous bacteria are reported to stimulate mucin secretion that would increase elimination of intestinal pathogens. Such observation is evidenced by an *in vitro* study whereby the probiotic *Lactobacillus plantarum* inhibited adherence of *E. coli* to intestinal epithelial cells by increasing mRNA expression of mucin genes (MUC2 and MUC3) (Mack et al., 1999). An increase in mucin secretion due to MOS, therefore, could represent important defensive mechanisms against intestinal pathogens. In addition, the uniqueness of bifidobacteria to secrete 1,2-α-*L*-fucosidase and endo-α-*N*-acetylgalactosaminidase allow themselves to feed and proliferate on mucin glycoproteins

(Katayama et al., 2005; Ruas-Madeido et al., 2008); this may, therefore, explain the pronounced increase in bifidobacteria concentrations due to dietary MOS.

Establishment of a population of intestinal lactobacilli and bifidobacteria has been associated with competitive exclusion of pathogens (Rolfe, 2000; van der Wielen et al., 2002), and secretion of antimicrobial substances against pathogens (Gibson and Wang, 1994; Jin et al., 1996). Dietary MOS supplementation, therefore, represents a nutritional strategy that could favor intestinal colonization of beneficial bacteria, thereby conferring intestinal health benefits to the host. However, reports indicate inconsistency of MOS effects on intestinal lactobacilli and bifidobacteria in broilers (Spring et al., 2000; Fernandez et al., 2002; Biggs et al., 2007) and turkeys (Fairchild et al., 2001; Sims et al., 2004).

MOS and antibiotics successfully reduced cecal concentrations of *E. coli*, but, this response was only observed at d 34. A delay in MOS action was also observed when broilers were orally challenged with *Salmonella* Enteritidis (PT4) (Fernandez et al., 2002). It is well documented that MOS competitively adsorbs to the mannose-specific Type-1 fimbriae of *E. coli*, thereby limiting their colonization of the intestinal epithelium; the pathogens are ultimately excreted from the intestines (Newman, 1994; Spring et al., 2000). We previously discussed that increased mucin secretion and increased growth of beneficial bacteria represent additional key mechanisms underlying MOS actions against intestinal pathogens. However, increasing dietary MOS inclusion level from 0.2 % to 0.5 % did not have additional effect in modulating the intestinal microflora. Additionally, the present study reveals that decreasing the intestinal concentrations of *E. coli* due to dietary MOS or antibiotics may not necessarily be accompanied by significant reduction of these bacteria in the litter. Such observation is not surprising, considering broiler litter is a favorable medium containing superfluous nutrients from feces, feathers, skin, dust and wasted feed for microbial growth (Kelleher et al., 2002).

Based on findings of this study, that MOS increased villi height at d 24, and decreased concentrations of intestinal pathogens at d 34, we may hypothesize that improvement in growth may occur beyond 34 d of bird age. An increase in efficiency of nutrient absorption and partitioning towards growth is most likely to occur in view of the fact that an increase in villi height has been associated with increased absorptive area of

the intestine (Stappenbeck et al., 2002), and a decrease in intestinal pathogens has been linked with reduced immune response, and reduced competition for vital nutrients between the bacteria and host (Bedford et al., 2000). But, considering that broilers would be close to market liveweight at this age, any possible improvement in growth due to MOS would be marginal when broilers are raised in a sanitary environment.

In conclusion, under good sanitary conditions of this study, MOS (0.2 % and 0.5 %) imparted better intestinal health benefits than antibiotics as measured by increased morphological development (villi height and goblet cell number), increased colonization by beneficial bacteria, and decreased pathogenic bacterial counts. However, there were no additional intestinal health benefits when feeding broilers a higher MOS dosage (0.5 %) than the recommended level (0.2 %). There was also no improvement in growth due to MOS or antibiotics.

## 2.6 ACKNOWLEDGEMENTS

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**Table 2.1**. Composition (g / kg) of basal diets and calculated analysis (g / kg) of finalized dietary treatments.

| **Ingredients** | **Starter[1] (1 to 21 d)** | **Grower[1] (22 to 38 d)** |
|---|---|---|
| Corn | 544.66 | 612.32 |
| Soybean meal[2] | 394.02 | 324.37 |
| Soybean oil | 9.95 | 14.93 |
| Starter vitamin-mineral premix[3] | 40.08 | 0 |
| Grower vitamin-mineral premix[4] | 0 | 37.41 |
| L-Lys | 3.48 | 3.88 |
| DL-Met | 2.09 | 2.09 |
| Calculated analysis | | |
| ME (Kcal/kg) | 3147 | 3199 |
| CP | 224.9 | 200.0 |
| Lys | 13.5 | 12.0 |
| Met | 5.5 | 5.2 |
| Ca | 9.5 | 9.0 |
| Available P | 4.9 | 4.5 |

[1]Basal diets were completed to 1000 by adding feed additives or an inert filler, or both, for a total of 5 g / kg (CTL: 5 g filler; VIRG: 4.63 g filler + 0.37 g virginiamycin (active ingredient + carrier); BACT: 4.5 g filler + 0.5 g bacitracin methylene disalicylate (active ingredient + carrier); LMOS: 3 g filler + 2 g MOS; HMOS: 5 g MOS).

[2]Partially defatted extruded soybean meal, composition per kg: ME, 3100 Kcal; CP, 475 g; Lys, 27.8 g; Met, 6.3 g; Arg, 32.6 g; Ca, 1.9 g; available P, 7 g.

[3]Supplied the following per kilogram of feed: vitamin A, 9,107 IU; vitamin D, 2,277 IU; vitamin E, 36.4 IU; biotin, 0.1 mg; choline, 547 mg; folic acid, 0.9 mg; niacin, 36.4 mg; pantothenic acid, 9.1 mg; riboflavin, 6.4 mg; thiamin, 1.8 mg; pyridoxine, 3.6 mg; vitamin B12, 18.2 mg; vitamin K, 2.7 mg; Cu, 18.2 mg; Fe, 138.2 mg; Mn, 74.4 mg; Zn, 45.5 mg; Co, 0.4 mg; I, 0.9 mg; and Se, 0.3 mg.

[4]Supplied the following per kilogram of feed: vitamin A, 9,353 IU; vitamin D, 2,238 IU; vitamin E, 37.4 IU; biotin, 0.1 mg; choline, 467.6 mg; folic acid, 0.9 mg; niacin, 37.4 mg; pantothenic acid, 9.4 mg; riboflavin, 6.5 mg; thiamin, 1.9 mg; pyridoxine, 3.7 mg; vitamin B12, 18.7 mg; vitamin K, 2.8 mg; Cu, 18.7 mg; Fe, 136.5 mg; Mn, 76.3 mg; Zn, 46.8 mg; Co, 0.4 mg; I, 0.9 mg; and Se, 0.3 mg.

**Table 2.2**. Effects of antibiotics and mannanoligosaccharide on growth performance in broiler chickens.

| Treatments[1] | d 1 to 21 | | | d 1 to 35 | | |
|---|---|---|---|---|---|---|
| | BW (g/bird) | FI[2] (g/bird) | FCR[3] | BW (g/bird) | FI[2] (g/bird) | FCR[3] |
| CTL | 561.0 | 1077.7 | 1.92 | 1683.7 | 2721.5 | 1.62 |
| VIRG | 576.0 | 1057.5 | 1.84 | 1727.8 | 2781.8 | 1.61 |
| BACT | 563.5 | 1061.0 | 1.88 | 1692.9 | 2688.4 | 1.59 |
| LMOS | 561.6 | 1081.6 | 1.93 | 1703.4 | 2703.5 | 1.58 |
| HMOS | 545.0 | 1030.1 | 1.89 | 1634.3 | 2537.3 | 1.55 |
| SEM | 9.2 | 19.5 | 0.05 | 21.2 | 62.7 | 0.03 |

[1]CTL: antibiotic free diet; VIRG: CTL + 16.5 mg / kg virginiamycin; BACT: CTL + 55 mg / kg bacitracin; LMOS: CTL + 0.2 % mannanoligosaccharide; HMOS: CTL + 0.5 % mannanoligosaccharide.

[2]FI: Feed intake

[3]FCR: Feed conversion ratio

**Table 2.3**. Effects of antibiotics and mannanoligosaccharides on weights and yields of broiler carcass parts at 38 d of age

| Treatments[1] | Eviscerated carcass | Whole breast | | Fillet | | Tenders | | Thigh | | Drumstick | | Wing | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Weight (g) | Weight (g) | Yield[2] (%) | Weight (g) | Yield[2] (%) | Weight (g) | Yield[2] (%) | Weight (g) | Yield[2] (%) | Weight (g) | Yield[2] (%) | Weight (g) | Yield[2] (%) |
| CTL | 1391.19 | 670.98 | 48.46 | 315.90 | 22.65 | 67.10 | 4.83 | 288.68 | 20.79 | 228.64 | 16.46 | 129.91 | 9.36 |
| VIRG | 1366.69 | 614.37 | 44.99 | 292.24 | 21.39 | 65.41 | 4.79 | 312.40 | 22.83 | 224.02 | 16.36 | 128.22 | 9.39 |
| BACT | 1379.12 | 641.73 | 46.51 | 302.74 | 21.85 | 68.38 | 4.97 | 303.92 | 21.94 | 228.66 | 16.64 | 127.56 | 9.27 |
| LMOS | 1347.77 | 636.80 | 47.26 | 291.32 | 21.66 | 62.64 | 4.65 | 288.77 | 21.41 | 218.04 | 16.17 | 129.72 | 9.62 |
| HMOS | 1289.43 | 609.41 | 47.47 | 283.26 | 22.04 | 67.12 | 5.23 | 275.13 | 21.39 | 224.84 | 17.51 | 123.12 | 9.61 |
| SEM | 44.34 | 21.36 | 0.69 | 13.67 | 0.64 | 2.50 | 0.16 | 11.95 | 0.44 | 8.20 | 0.44 | 3.86 | 0.22 |

[1]CTL: antibiotic free diet; VIRG: CTL + 16.5 mg / kg virginiamycin; BACT: CTL + 55 mg / kg bacitracin; LMOS: CTL + 0.2 % mannanoligosaccharide; HMOS: CTL + 0.5 % mannanoligosaccharide.

[2]Calculated as percentage of eviscerated carcass weight.

**Table 2.4**. Effects of antibiotics and mannanoligosaccharides on villi height (μm) in the duodenum, jejunum and ileum of broiler chickens

| Treatments[1] | Duodenum | | | Jejunum | | | Ileum | | |
|---|---|---|---|---|---|---|---|---|---|
| | d 14 | d 24 | d 34 | d 14 | d 24 | d 34 | d 14 | d 24 | d 34 |
| CTL | 1192.1[b] | 1397.4[ab] | 1583.4[ab] | 1069.7 | 1099.3[b] | 1187.6 | 503.4 | 580.7[ab] | 741.94[abc] |
| VIRG | 1297.2[ab] | 1329.0[b] | 1462.8[b] | 1120.7 | 1201.2[ab] | 1166.9 | 508.4 | 609.7[ab] | 594.54[c] |
| BACT | 1213.2[ab] | 1359.0[b] | 1510.9[b] | 1107.9 | 1115.5[b] | 1199.4 | 541.6 | 518.2[b] | 645.96[b] |
| LMOS | 1327.7[ab] | 1624.9[a] | 1716.4[a] | 1155.2 | 1371.9[a] | 1393.2 | 546.7 | 640.5[a] | 852.34[a] |
| HMOS | 1382.5[a] | 1602.2[a] | 1652.1[ab] | 1128.7 | 1222.8[ab] | 1375.1 | 594.8 | 680.2[a] | 771.93[ab] |
| SEM | 35.7 | 44.4 | 39.6 | 37.8 | 44.8 | 44.9 | 32.7 | 23.4 | 32.95 |

[1]CTL: antibiotic free diet; VIRG: CTL + 16.5 mg / kg virginiamycin; BACT: CTL + 55 mg / kg bacitracin; LMOS: CTL + 0.2 % mannanoligosaccharide; HMOS: CTL + 0.5 % mannanoligosaccharide.

[a,b,c] Values with different superscript within same column are different (Scheffe's t-test, $P \leq 0.05$)

**Table 2.5**. Effects of antibiotics and mannanoligosaccharides on goblet cell number per villus in the duodenum, jejunum and ileum of broiler chickens

| Treatments[1] | Duodenum | | | Jejunum | | | Ileum | | |
|---|---|---|---|---|---|---|---|---|---|
| | d 14 | d 24 | d 34 | d 14 | d 24 | d 34 | d 14 | d 24 | d 34 |
| CTL | 141.5[ab] | 151.2[ab] | 146.0[b] | 131.6 | 135.6[b] | 171.3[b] | 113.8 | 115.9[b] | 208.84[a] |
| VIRG | 140.9[ab] | 162.3[ab] | 135.8[b] | 133.6 | 127.5[b] | 140.1[b] | 108.4 | 110.4[b] | 130.37[b] |
| BACT | 135.3[b] | 128.9[b] | 144.8[b] | 157.9 | 144.6[b] | 156.0[b] | 122.7 | 91.8[c] | 123.04[b] |
| LMOS | 163.8[ab] | 199.4[a] | 186.3[a] | 159.9 | 240.1[a] | 236.6[a] | 143.2 | 152.8[a] | 192.73[a] |
| HMOS | 186.6[a] | 195.5[a] | 160.4[ab] | 177.7 | 217.4[a] | 228.0[a] | 138.9 | 134.1[ab] | 169.41[a] |
| SEM | 11.8 | 11.3 | 7.3 | 10.6 | 14.8 | 10.6 | 8.6 | 7.1 | 8.91 |

[1]CTL: antibiotic free diet; VIRG: CTL + 16.5 mg / kg virginiamycin; BACT: CTL + 55 mg / kg bacitracin; LMOS: CTL + 0.2 % mannanoligosaccharide; HMOS: CTL + 0.5 % mannanoligosaccharide.

[a,b,c] Values with different superscript within same column are different (Scheffe's t-test, $P \leq 0.05$)

**Figure 2.1.** Concentrations ($log_{10}$ CFU / g) of bifidobacteria in the ceca of broiler chickens fed CTL (antibiotic free diet); VIRG (CTL + 16.5 mg / kg virginiamycin); BACT (55 mg / kg bacitracin); LMOS: (CTL + 0.2 % mannanoligosaccharide); HMOS: (CTL + 0.5 % mannanoligosaccharide). [a,b,c] Values with different superscript within a group are different (Scheffe t-test, $P < 0.05$).

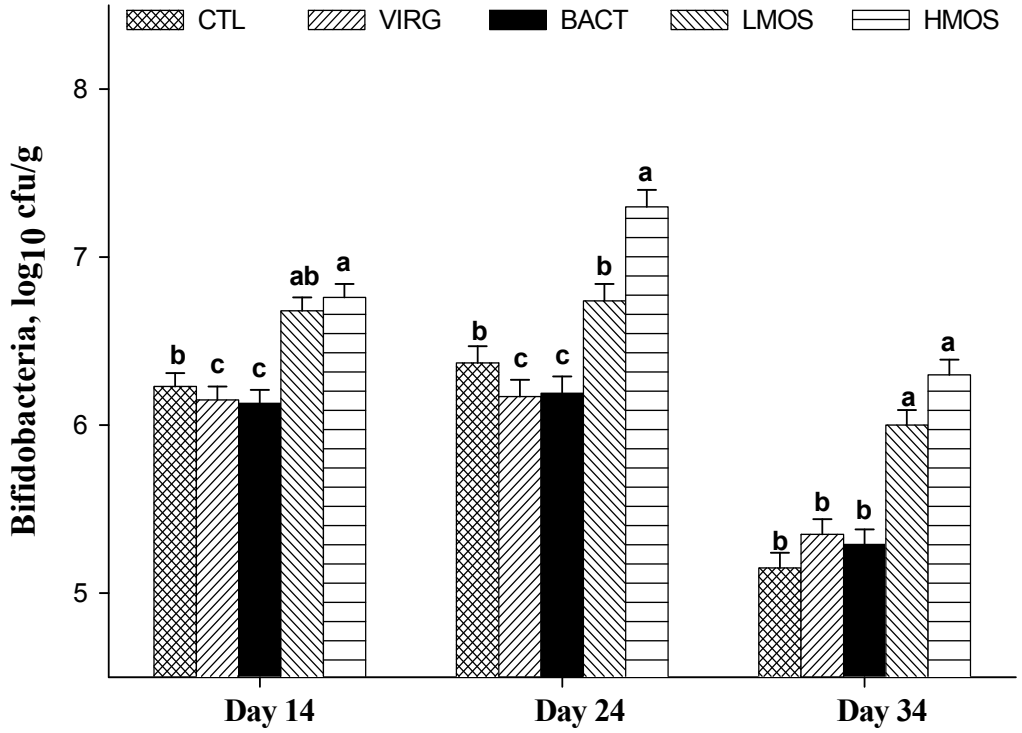


**Figure 2.2.** Concentrations ($log_{10}$ CFU / g) of lactobacilli in the ceca of broiler chickens fed CTL (antibiotic free diet); VIRG (CTL + 16.5 mg / kg virginiamycin); BACT (55 mg / kg bacitracin); LMOS: (CTL + 0.2 % mannanoligosaccharide); HMOS: (CTL + 0.5 % mannanoligosaccharide). [a,b] Values with different superscript within a group are different (Scheffe t-test, $P < 0.05$).

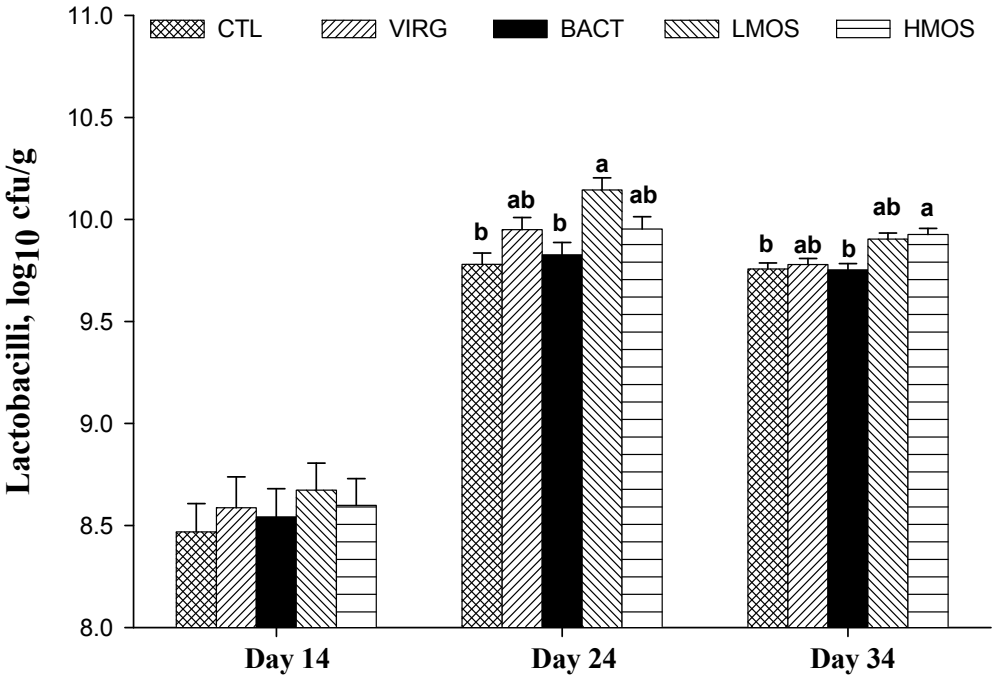


**Figure 2.3.** Concentrations ($log_{10}$ CFU / g) of *E. coli* in the ceca of broiler chickens fed CTL (antibiotic free diet); VIRG (CTL + 16.5 mg / kg virginiamycin); BACT (55 mg / kg bacitracin); LMOS: (CTL + 0.2 % mannanoligosaccharide); HMOS: (CTL + 0.5 % mannanoligosaccharide). [a,b] Values with different superscript within a group are different (Scheffe t-test, $P < 0.05$).

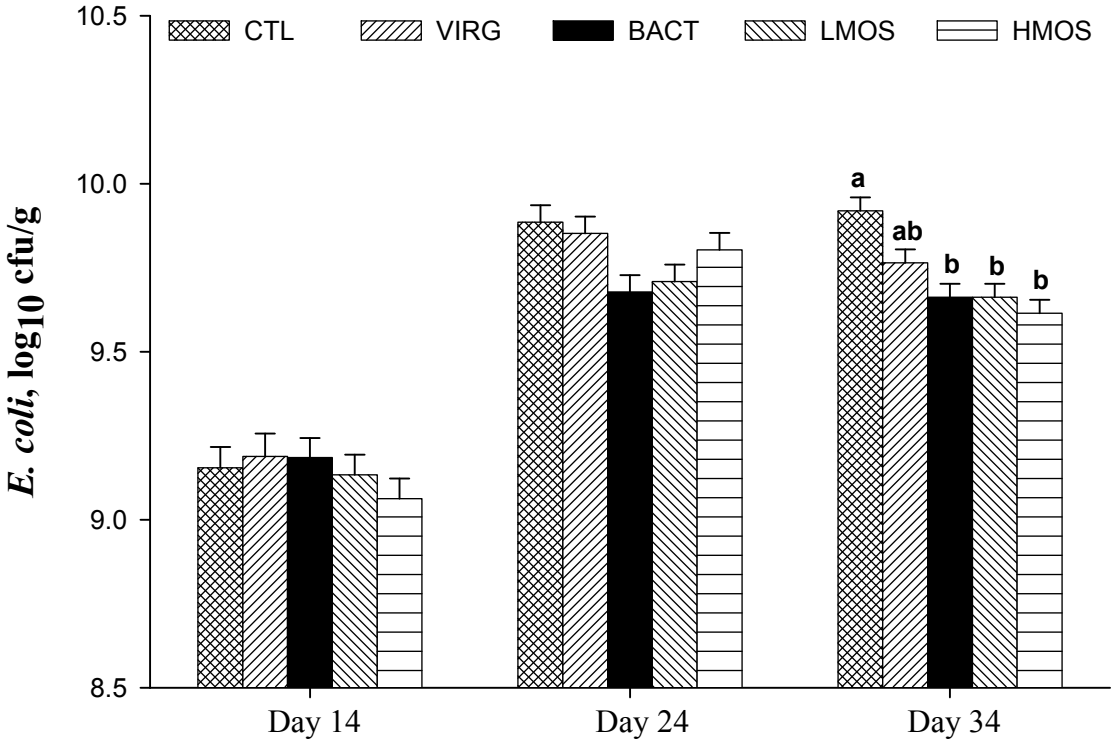
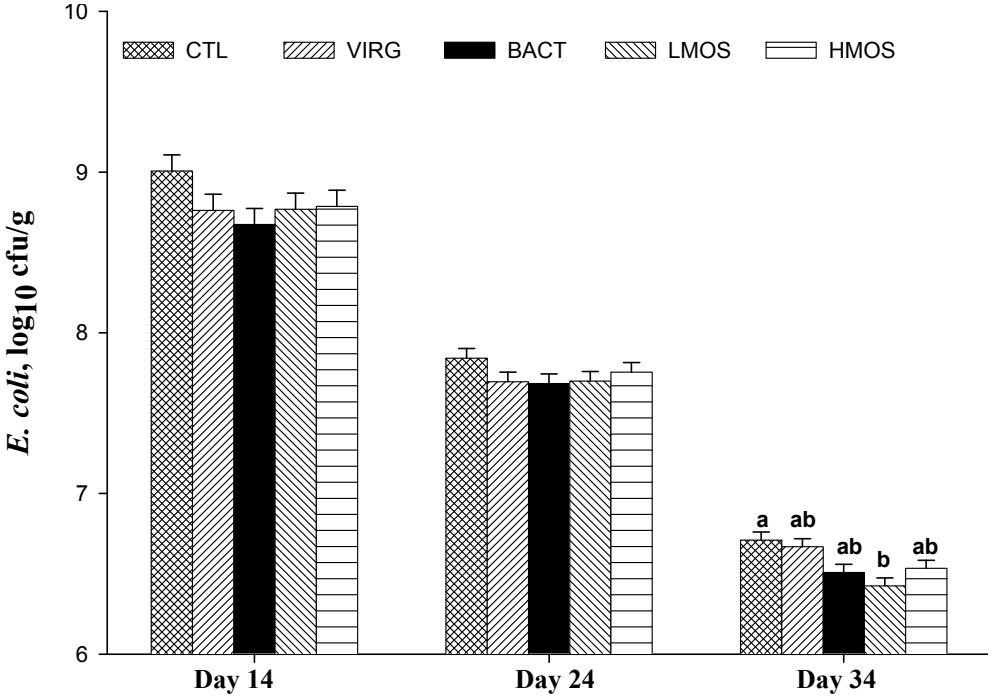


**Figure 2.4.** Concentrations ($log_{10}$ CFU / g) of *E. coli* in the litter of broiler chickens fed CTL (antibiotic free diet); VIRG (CTL + 16.5 mg / kg virginiamycin); BACT (55 mg / kg bacitracin); LMOS: (CTL + 0.2 % mannanoligosaccharide); HMOS: (CTL + 0.5 % mannanoligosaccharide). [a,b] Values with different superscript within a group are different (Scheffe t-test, $P < 0.05$).



## CONNECTING STATEMENT 1

In Chapter II, in comparison to VIRG and BACT, MOS (0.2 %) significantly improved innate defensive mechanisms against intestinal pathogens in healthy chickens raised under good sanitary conditions, as demonstrated by increased development of mucin-secreting goblet cells and enrichment of the intestinal microflora with probiotic bacteria, especially bifidobacteria. Improvements in intestinal health due to MOS than antibiotics reflected in longer and healthier villi. Moreover, we revealed that MOS conferred no additional intestinal health benefits at higher dietary inclusion rate than 0.2 %.

In Chapter III, we have used a *S.* Enteritidis infection chick model to determine whether MOS's capacity to improve innate defense mechanisms, in contrast to VIRG, may abrogate the detrimental effects of *S.* Enteritidis on epithelial barrier integrity and mitigate *S.* Enteritidis-induced enteric inflammation. A systematic approach was adopted to elucidate the mechanisms of MOS and VIRG actions on key players of intestinal innate defense. We were particularly interested in making a clear distinction between the different types of mucins.

# CHAPTER III. MANNANOLIGOSACCHARIDE PREBIOTIC TERMINATED *SALMONELLA* ENTERITIDIS-INDUCED ENTERIC INFLAMMATION EARLIER THAN ANTIBIOTIC: REPROGRAMMING THE INTESTINAL INNATE DEFENSE MECHANISMS[1]

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[1]Ready to be submitted for publication

## 3.1 ABSTRACT

***Background*:** *Salmonella* Enteritidis, a global threat to public health causing foodborne diseases, triggers acute intestinal inflammation by using its virulence factors to invade intestinal epithelial cells. Treatment of *S.* Enteritidis infections with antibiotics has become more problematic with emergence of multiple-antibiotic resistant *S.* Enteritidis. Various types of alternatives to antibiotics are being investigated. Among these, mannose-rich oligosaccharides (MOS) from cells walls of *Saccharomyces cerevisiae* are implicated in binding mannose-specific lectin of *Salmonella*, and reducing its adherence to intestinal epithelial cells. However, whether MOS can exert reciprocal intestinal health benefits to maintain epithelial barrier functions, improve innate defense mechanisms through mucin secretions, and abrogate inflammation during *S.* Enteritidis infection, are unknown.

***Objective:*** To investigate MOS's capacity, in contrast to virginiamycin (VIRG) antibiotic, to prevent or treat *S.* Enteritidis-induced intestinal inflammation, and elucidate potential mechanisms of action.

***Methods:*** To induce acute enteric inflammation, young chicks were infected orally with *S.* Enteritidis. Non-infected chicks received sterile saline. Ileal epithelial barrier integrity and development of neutral and acidic mucins-containing goblet cells were analyzed. Inflammatory cytokine, mucins secreting capacity of goblet cells, and secretions of membrane-bound mucins by epithelial cells were determined by gene expression analyses.

***Results:*** MOS considerably improved host's innate defensive mechanisms, as indicated by increased development of neutral and acidic mucins-secreting goblet cells, and healthier villi morphology in both non-infected and *S.* Enteritidis -infected chicks. *MUC 1* down-regulation by MOS, when compared to VIRG, suggested that MOS more significantly reduced *S.* Enteritidis adhesion and invasion of the intestinal epithelium, thus preserving epithelial barrier functions. Interestingly, MOS terminated *S.* Enteritidis-

induced enteric inflammation earlier than VIRG, as demonstrated by *IL 12* down-regulation.

***Conclusions:*** This study provides a rationale to explore MOS as a novel bio-therapeutic strategy to alleviate *Salmonella*-induced enteric inflammation in human and animal health.

## 3.2 INTRODUCTION

*Salmonella enterica* serotype Enteritidis (*S.* Enteritidis), a leading cause of foodborne diseases and global threat to public health, is an invasive enteric pathogen that causes profuse diarrhea and acute intestinal inflammation (CDC, 2011), and in many cases lead to extra-intestinal infections such as septicemia, arthritis, endocarditis, meningitis and urinary tract infections (Katsenos et al., 2008; Kobayashi et al., 2009; Mutlu et al., 2009; Tena et al., 2007). *S.* Enteritidis-induced salmonellosis is majorly caused by consumption of undercooked contaminated retail poultry meat (Galanis et al., 2011) and eggs (Patrick et al., 2004).

To initiate diseases, *S.* Enteritidis first adheres to and then invades intestinal epithelial cells through a specialized type III secretion system (T3SS-1) that injects effector proteins into host's epithelial cells, triggering actin cytoskeleton rearrangements and bacteria internalization (Clark et al., 1998; Galan and Collmet, 1999; Ly and Casanova, 2007). Invaded *Salmonella* is then rapidly engulfed by resident macrophages. *Salmonella* uses its type III secretion system (T3SS-2) to reside and multiply within a *Salmonella*-containing vacuole of macrophages (Steele-Mortimer et al., 1999; Holden, 2002). Ultimately, *Salmonella* induces acute enteric inflammation through release of pro-inflammatory cytokines, and activation of mitogen-activated protein kinase (MAPK) cascade, and activation of transcription factors such as AP-1 (activator protein 1) and NF-kB (nuclear factor kappa B) (Hobbie et al., 1997). Therefore, the adherence and invasion of *S.* Enteritidis of intestinal epithelial cells are critical virulence steps in its pathogenicity.

Antibiotic is the only currently available treatment for salmonellosis in human and veterinary medicine. However, in recent years, emergence of multiple-antibiotic resistant

*S.* Enteritidis (Vaz et al., 2010; de Oliveira et al., 2011; Hur et al., 2011; Yildirim et al., 2011) has profoundly compromised salmonellosis treatment. Today, there are increasing interests to develop natural immuno-modulators that can prevent and treat salmonellosis. In mice infection studies, the probiotic *Saccharomyces cerevisiae* successfully prevented *Salmonella* invasion of the intestinal epithelium, increased serum immunoglobulin (IgA) levels, prevented *Salmonella* translocation to the liver, and avoided *Salmonella*-induced death (Martins et al., 2007, 2010). In addition, *S. cerevisiae* totally abolished *Salmonella* invasion of human colorectal cancer cells *in vitro*, maintained integrity of epithelial cells and reduced inflammation (Martins et al., 2010).

*S. cerevisiae* exerts health benefits principally through mannose-rich oligosaccharides **(MOS)** from its cell walls. Indeed, MOS binds mannose-specific lectin (FimH) of gram negative pathogens that express Type 1 fimbriae (Ofek and Beachey, 1978; Thomas et al., 2004), inhibiting *Salmonella* colonization of the intestinal epithelium (Spring et al., 2000). In healthy hosts, MOS improved intestinal protection through increased goblet cell development (Baurhoo et al., 2007, 2009; Solis de los Santos et al., 2007), increased expressions of mucin genes (Chee et al., 2011) and commensal microflora enrichment with lactobacilli and bifidobacteria (Baurhoo et al., 2007, 2009). However, whether MOS can improve innate defensive mechanisms through increased goblet cells development and mucin secretions, and terminate inflammation earlier than antibiotics during *S.* Enteritidis infection, are unknown. Therefore, as a novel approach of this study, the experiment reported herein, was designed to investigate MOS's capacity to potentially prevent or treat intestinal *S.* Enteritidis-induced inflammation. We specifically focused on host's intestinal innate defense mechanisms against *S.* Enteritidis using a *S.* Enteritidis-infected chicken model. Our results suggest MOS as a novel bio-therapeutic strategy to alleviate *Salmonella*-induced enteric inflammation in human and animal health.

## 3.3 MATERIALS AND METHODS

### *Birds and Experimental Diets*

Hatched Cobb 500 broiler chicks (La Coop Fédérée Couvoir Provincial, Victoriaville, Quebec, Canada) were raised in 2 groups (n=144/group). Each group of

chicks was further divided into 3 sub-groups (n = 48 chicks/sub-group) and fed 1 of 3 experimental diets, including a control diet (**CTL**), **VIRG** (diet 1 + 16.5mg / kg virginiamycin) or **MOS** (diet 1 + 0.2 % Bio-Mos, Alltech Inc., Nicholasville, KY), as described (Baurhoo et al., 2007). To induce enteric inflammation, group 1 chicks (4 cages/diet; 12 birds/diet) were orally gavaged with 0.5 ml of 2.4 x $10^9$ CFU/ml of selected rifamycin-resistant *S.* Enteritidis strain whereas group 2 non-infected controls were gavaged with 0.5 ml of sterile saline at 3 d of age.

Chicks were housed in cages and raised in temperature- (32 to 24 °C with 0.5 °C decrease daily), light- (20 h light:4 h darkness) and ventilation-controlled rooms. Chicks were kept in a containment unit of the animal facility, and had free access to feed and water. All experimental procedures were approved by the ethics committee of the University of Montreal.

***Selection of S. Enteritidis***

A chicken specific rifamycin-resistant *S.* Enteritidis PT4 strain (SHY04-1540) was provided by the Department of Pathology and Microbiology (University of Montreal, St Hyacinthe, Quebec, Canada). The bacteria, originally isolated from diseased chicken carcasses, were grown overnight at 37 ºC under aerobic conditions in 5ml nutrient broth. After serial dilution in sterile saline, the bacteria were grown on Trypsic Soy Agar (TSA) containing 20µg/ml rifamycin (Sigma-Aldrich, St Louis, MO) to induce and select for rifamycin resistance. Thereafter, a single colony of the selected rifamycin-resistant *S.* Enteritidis was grown overnight at 37 ºC in nutrient broth and *Salmonella* concentrations were verified by serial dilutions and plating onto Sheep Blood Agar (Oxoid) at 37 ºC for 24 h.

***Salmonella Detection in Chicks and Feed***

Prior to this study, both the diets and chicks (feces and cecal contents) were screened for *Salmonella* by using specific *Salmonella* culture technique, to confirm whether these were free from the introduced rifamycin-resistant *S.* Enteritidis strain. First, each sample was diluted 10-folds by weight in nutrient broth. Thereafter, a 1 ml of diluted samples was incubated at 37 $^{o}$C for 24 h in 9 ml of Tetrathionate Brilliant Green Bile

Broth (Oxoid, Nepean, Ontario, Canada) and 200 µL iodine. Following this *Salmonella*-specific enrichment step, a 100 µl volume was grown at 37 $^{o}$C for 24 h on Brilliant Green Sulphapyridine Agar (**BGSA**, Oxoid) containing 20 µg/ml of novobiocin (Sigma-Aldrich). Presence of *Salmonella* was confirmed by agglutination tests using polyvalent O-antisera (Poly A1, B, C2 and D; Difco, NJ). All diets tested negative for *Salmonella*. But, *Salmonella* serotype Kentucky (Group C2) was detected in feces from chick carton boxes, indicating that chicks were contaminated with the pathogen, probably at the hatchery. Among all tested birds (n=180) in this study, only 4.2% were positive for *Salmonella*. However, none of these *Salmonella* strains were rifamycin-resistant *S.* Enteritidis given that these grew on novobiocin-supplemented, but not rifamycin-supplemented BGSA. When serotyped (LEAQ de Saint Hyacinthe, MAPAQ, St Hyacinthe, Quebec), the *Salmonella* isolates were identified as serotype Kentucky or Heidelberg (Group B), thus revealing that the chick's commensal microflora was free from *S.* Enteritidis, and that *Salmonella* contamination was sporadic only.

***Quantification of Intestinal S. Enteritidis***

Chicks (n=6/diet/sub-group) were euthanized by carbon dioxide asphyxiation at 1, 4, 7, 10 and 13 d after *S.* Enteritidis infection. From each euthanized bird, 1g of ceca was serially diluted in nutrient broth and grown at 37 ºC for 24 h on BGSA containing 20 µg/ml of rifamycin. Thereafter, colonies of rifamycin-resistant *S.* Enteritidis were counted. Non-infected control chicks were screened to detect the presence of rifamycin-resistant *S.* Enteritidis, as above-described. All microbiological analyses were performed in duplicates, and the average values were used in statistical analysis.

***Mucin and Pro-Inflammatory Cytokine Gene Expressions***

The ileum (2-cm segment adjacent to the cecal tonsils) from each euthanized bird (n=6/diet/sub-group) was immediately excised, snap-frozen in liquid nitrogen, and stored at -80 ºC for later RNA extraction. Total RNA from ileal tissues was isolated using Trizol reagent and Purelink RNA Mini Kit (Invitrogen). One µg of total RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, ON, Canada), following the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR)

analysis was performed using the Bio-Rad CFX384 RT-qPCR Detection System, SsoFast Evagreen Supermix (Bio-Rad) and primer-set sequences (Table 3.1). RT-qPCR reactions were performed at 95 $^{o}$C for 5 min, followed by 39 cycles of 95 $^{o}$C for 15 s and 60 $^{o}$C for 30 s. A melting curve program was included at the end of each qPCR reaction to verify the presence of a unique product. Samples were analyzed in technical duplicates. Relative expression levels for mucins (MUC 1 and MUC 2) and interleukin 12 (IL 12) genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and differential gene expressions were determined using the comparative standard curve method.

***Histomorphological Analysis***

Ileal sections (1-cm) were excised from the same euthanized birds (n=6/diet/sub-group), washed in physiological saline solution, fixed in 10% neutral-buffered formalin, washed thrice in 0.1 M phosphate buffered saline (pH 7.4) for 15 min each time, dehydrated using increasing ethanol concentrations at 70% (overnight), 80% (1 hr), 90% (1 hr) and 100% (1 hr over 3 consecutive times), and then embedded in paraffin. Using a microtome, 5-µm tissue samples were cross-sectioned and stained with hematoxylin and eosin (**HE**). Villus height was measured from the tip of the villus to the top of the lamina propria under light-field microscopy with an integrated image analysis NIS-Element BR v. 2.3 software (Nikon DXM 1200c, Nikon Corporation, Tokyo, Japan). Measurements of 10 villi per chick were averaged and used in statistical analysis.

***Differentiation and Quantification of Goblet Cells***

Goblet cells containing neutral mucins were detected with periodic acid Schiff staining (**PAS**), as described previously (Solis de los Santos et al., 2007). Briefly, following deparaffinization and dehydration, slides were incubated in 0.5% periodic acid for 5 min, washed and incubated with Schiff's reagent (Sigma-Aldrich) for 30 min. Thereafter, slides were counterstained with hematoxylin for 5 min. The number of PAS positive cells (red) along the villi membrane was counted by light microscopy. For differentiation of goblet cells containing acidic mucins, high iron diamine-Alcian blue (**AB**) stain was used. Another set of deparaffinization and dehydration slides were incubated in high iron diamine solution overnight, rinsed in water, and then incubated in

Alcian blue solution for 5 min (1% in 3% acetic acid, pH 2.5). After rinsing with water, slides were dehydrated and mounted. The number of Alcian blue-positive cells (black) along the villi was counted by light microscopy.

***Scanning Electron Microscopy***

Ileum segments (1-cm) were carefully dissected on dental wax into four tissue blocks (~3x3mm each), submerged in 0.1M sodium phosphate buffer (**SPB**, pH 7.2), and fixed with ice-cold 3% glutaraldehyde in SPB for at least 72 h. The tissue blocks were then washed 3 times in SPB, each time for 20 min at 4 °C. After tissue blocks were post-fixed with 1% osmium tetroxide (OsO4) for 1 h at 4°C, these were rinsed in SPB and dehydrated through a graded series of ethanol (30%, 50%, 70%, 95% and 100% each time for 30 minutes). Scanning electron microscopy (**SEM**) samples were critical point dried in a Samdri PVT-2 using liquid carbon dioxide. Specimens were mounted on aluminum stubs with silver paint and coated with a total of 50-60kÅ of gold/palladium in a Hummer 6.2 Sputtering Device. SEM was performed in a JEOL 5900LV at 20 kV accelerating voltage.

***Statistical Analysis***

A one-way ANOVA was used for cecal *S.* Enteritidis and gene expression analyses at each sampling time. Histological data were analyzed as a two-way ANOVA and a 2 x 3 factorial arrangement to determine the main effects of diet and inoculation, and their interaction effects by using the MIXED procedure of SAS (SAS Institute, 2003). The statistical model used for histological data analysis was as follows: $Y_{ijk} = \mu + Diet_i + Inoculation_j + Diet_i*Inoculation_j + e_{ijk,}$ where $\mu$ represents the overall mean value, $Diet_i$ is the fixed effect of diet (i = CTL, VIRG or MOS), $Inoculation_j$ is the fixed effect of inoculation (j = saline or *S.* Enteritidis), $Diet_i * Inoculation_j$ is the interaction effect of diet and inoculation, and $e_{ijk}$ is the random error. Differences among treatment means were tested using Scheffe's Multiple Comparison test and statistical significance was declared at $P < 0.05$. *S.* Enteritidis concentrations were $\log_{10}$ transformed before statistical analysis.

## 3.4 RESULTS

### *Antibiotic Reduced Intestinal Concentrations of S. Enteritidis at 1 d After Oral Infection Only*

Rifamycin-resistant *S.* Enteritidis was detected in chicks deliberately infected with the bacteria only, thus demonstrating success of our challenge model. VIRG reduced rifamycin-resistant *S.* Enteritidis counts when compared with CTL- and MOS-fed chicks, but this effect was significant at 1 d post *S.* Enteritidis infection only (figure 3.1).

### *S. Enteritidis Failed to Induce Clinical Symptoms among Antibiotic- and Prebiotic-Fed Chicks*

To confirm that *S.* Enteritidis successfully induced enteric inflammation among infected chicks, clinical symptoms were monitored. *S.* Enteritidis-infected chicks fed CTL suffered from drowsiness, lethargy, ruffled feathers, moderate diarrhea, starvation and withdrawal from water. These clinical signs manifestly occurred among CTL-fed chicks at 7 d post-infection, but were abolished by VIRG and MOS. As expected, pathological symptoms were absent among non-infected control chicks

### *Prebiotic Markedly Reduced Pro-Inflammatory Cytokine Expressions in S. Enteritidis-Infected Chicks*

We next investigated MOS and VIRG capacity to potentially reduce *S.* Enteritidis-induced enteric inflammation. In contrast to CTL, MOS significantly reduced the *S.* Enteritidis-mediated induction of *IL 12* (figure 3.2B), revealing that MOS counteracted *S.* Enteritidis-induced enteric inflammation (Walter et al., 2001). This anti-inflammatory effect of MOS occurred at all times of this study, except at d 1. VIRG also suppressed intestinal inflammation, as indicated by *IL 12* down-regulation; however, the antibiotic effect occurred at 4 and 7 d post-*S.* Enteritidis infection only. Interestingly, in contrast to VIRG, MOS caused *IL 12* down-regulation at 10 and 13 d after *S.* Enteritidis infection, revealing that MOS more powerfully mitigated *S.* Enteritidis-induced inflammation than VIRG. On the other hand, in the absence of *S.* Enteritidis infection, *IL 12* was not majorly differentially expressed among CTL-, VIRG- and MOS-fed hosts (figure 3.2A).

Intriguingly, however, *IL 12* was repressed by MOS at d 7 whereas VIRG dramatically induced *IL 12* at d 13.

### *Prebiotic and Antibiotic Mitigated S. Enteritidis-Induced Damage of Intestinal Epithelial Cells*

To determine whether MOS and VIRG may limit *S.* Enteritidis invasion of intestinal epithelial cells, we assessed morphological damage to the epithelium. Histopathology examination of the villus by light microscopy revealed that *S.* Enteritidis caused villus histologic lesions, exfoliation of epithelial cells at villus tips, and infiltration of immune cells, such as neutrophils and macrophages, in villus lamina propria in CTL hosts only (figure 3.6B). Epithelial damage by *S.* Enteritidis pronouncedly occurred at 7 d after infection but was absent at d 13 (data not shown), indicating that *S.* Enteritidis invasion of epithelial cells was time sensitive.

### *Prebiotic Improved Villi Morphological Characteristics in Non-Infected and S. Enteritidis-Infected Hosts*

We next verified the effects of dietary treatments and *S.* Enteritidis on villi morphology. Light microscopy analysis showed that *S.* Enteritidis had no detrimental effects in reducing villi height across dietary treatments (figures 3.3A, B). But, in non-infected hosts, VIRG profoundly reduced villi height than CTL or MOS at d 10 and 13, comparable to our previous studies (Baurhoo et al., 2007, 2009). But, here, this VIRG effect occurred consistently in non-infected and infected chicks (figure 3.3C). In depth examination of villi integrity by SEM revealed that villi were healthier and longer with a characteristic leaf-shape structure when both non-infected and infected chicks were fed MOS (figure 3.4 and figure 3.5). *S.* Enteritidis affected villi morphological characteristics, causing villi to be shorter, pointed at its tips and more densely populated. However, such detrimental effects on villi integrity which aberrantly occurred among CTL-fed hosts were markedly counter-regulated by MOS more than VIRG.

***Prebiotic Increased Development of Neutral and Acidic Goblet Cells in Non-Infected and S. Enteritidis-Infected Chicks***

Previously, we reported that MOS increased goblet cell counts in HE-stained intestinal villus membrane compared with CTL and VIRG (Baurhoo et al., 2007, 2009). But, here, to make a clear distinction between neutral and acidic mucin-containing goblet cell types, morphological tissues were stained with PAS and AB, respectively. In contrast to non-infected hosts fed CTL or VIRG, MOS markedly increased both neutral (figure 3.6A and figure 3.8A) and acidic (figure 3.7A and figure 3.9A) goblet cell counts, revealing MOS's inherent capacity to stimulate development of intestinal neutral and acidic goblet cells.

Considering that goblet cell-secreted mucins are capable of trapping and eliminating intestinal pathogens (Sajjan and Forstner, 1990; Fasina et al., 2010), we further investigated MOS's effects on goblet cell development during *S.* Enteritidis infection. Subsequent to *S.* Enteritidis infection, MOS significantly increased the number of neutral (figure 3.6B and figure 3.8A) and acidic (figure 3.7B and figure 3.9A) goblet cells when compared with hosts fed CTL or VIRG, thus confirming MOS's ameliorating effects of intestinal defense mechanism against *S.* Enteritidis. In this study, MOS consistently increased acidic goblet cell numbers. However, VIRG majorly reduced goblet cell numbers in both non-infected and *S.* Enteritidis infected chicks. When considering non-infected control and *S.* Enteritidis-infected chicks together, goblet cell numbers were higher among hosts fed MOS than CTL or VIRG (figure 3.8C and figure 3.9C). Strikingly, whether hosts were fed CTL, VIRG or MOS, neutral (figure 3.8A) and acidic (figure 3.9A) goblet cell counts were not influenced by *S.* Enteritidis respective to their non-infected controls. Additionally, *S.* Enteritidis effects on goblet cell development were highly variable irrespective of dietary treatments (figure 3.8B and figure 3.9B).

***Prebiotic Reduced Gene Expressions of Secretory Mucins in Non-Infected Chicks Only***

Goblet cells are thought to secrete abundance of secretory mucins in the intestinal lumen during infections. To assess the mucins secreting capacity by goblet cells as influenced by MOS and VIRG, we measured gene expression for secretory mucins (MUC 2) in non-infected and *S.* Enteritidis-infected chicks. Surprisingly, *MUC 2* was not

differentially expressed between infected chicks fed CTL, VIRG and MOS (figure 3.10B). This observation occurred despite the higher neutral and acidic goblet cell numbers among MOS-fed hosts. However, MOS reduced *MUC 2* expression in non-infected hosts when compared with CTL- or VIRG-fed hosts at d 10 and 13 (figure 3.10A). These findings suggest complete disagreement between increased goblet cell numbers and higher mucins secreting capacity by goblet cells.

***Prebiotic Reduced Gene Expressions of Membrane-Bound Mucins in Non-Infected and S. Enteritidis-Infected Chicks***

Membrane-bound mucins are another mucin type secreted by enterocytes of epithelial cells (Derrien et al., 2010). To assess the effects of MOS and VIRG on secretions of membrane-bound mucins in non-infected and *S.* Enteritidis-infected chicks, we measured gene expression for membrane-bound mucins (MUC 1). Subsequent to *S.* Enteritidis infection, MOS significantly reduced *MUC 1* expression when compared with CTL-fed hosts at all times of this study, except at d 1 (figure 3.11B). VIRG also reduced *MUC 1* expressions, but this effect occurred at 4 and 7 d after *S.* Enteritidis infection only. Interestingly, in comparison to infected hosts fed VIRG, MOS also reduced *MUC 1* expressions at d 10 and 13. All of these *MUC 1* expression results were in concordance with our *IL 12* expression results, revealing that abundance of membrane bound mucins were not needed responsive to MOS's capacity in reducing *S.* Enteritidis-induced inflammation than CTL and VIRG. But, *MUC 1* was not differentially expressed between non-infected hosts fed CTL, VIRG and MOS (figure 3.11A), except that *MUC 1* was surprisingly repressed by MOS at d 7 and induced by VIRG at d 10 and 13.

## 3.5 DISCUSSION

Antibiotic is the only medical and veterinary option for the treatment of enteric *S.* Enteritidis infections. But, in this study, for the first time, we have demonstrated that a natural MOS prebiotic can equally, if not better, mitigate enteric *S.* Enteritidis infections through enhancement of host's intestinal innate defense, principally through a complex mucins-mediated defense mechanism. The mechanisms of MOS's actions against intestinal *S.* Enteritidis are herein described.

In this study, we used a *S.* Enteritidis-infected chick model, which has important applications in fundamental and nutrigenomic scientific investigations (Stern et al., 2005; Baurhoo et al., 2011). Although there might be differences from human enteric *S.* Enteritidis infection, the chick model shares many clinical and pathological features with regards to loss of intestinal barrier function, goblet cell development, mucin secretions, and inflammatory responses. Moreover, to provide better assessment of host's innate defensive mechanisms, we specifically used young chicks based on their susceptibility to *Salmonella* invasion of the intestinal epithelium, because these are biologically devoid of a mature protective intestinal microflora (Gong et al., 2008) and fully functional adaptive immunity (Beal et al., 2004; Lowry et al., 2005).

*S.* Enteritidis induced enteric inflammation in deliberately infected CTL-fed hosts, as evidenced by marked clinical symptoms, which are mediated principally by IL 1 and IL 6 pro-inflammatory cytokines (Breder and Saper, 1996; Martins et al., 2011), together with exfoliation of intestinal epithelial cells (Clark et al., 1998) and massive influx of immune cells such as neutrophils and macrophages into the lamina propria of *S.* Enteritidis-invaded villi (Gewirtz et al., 1999), thus revealing success of our *S.* Enteritidis enteric inflammation model. However, MOS and VIRG beneficially counter-regulated *S.* Enteritidis-induced enteric inflammation, given that these prevented *S.* Enteritidis invasion and exfoliation of epithelial cells. But, subsequent to infection, VIRG more rapidly cleared intestinal *S.* Enteritidis than MOS, probably by virtue of its different mode of action: VIRG exhibits strong bactericidal properties (Cocito, 1969) whereas MOS binds mannose-specific lectin of *S.* Enteritidis (Ofek and Beachey, 1978; Thomas et al., 2004) reducing its adherence to mannose-containing glycoprotein receptors on intestinal

epithelial cells. Indeed, excretion of mucins-trapped *S.* Enteritidis through peristalsis movements of the intestines is timely.

We also reveal that MOS terminated *S.* Enteritidis-induced enteric inflammation earlier than VIRG, given that MOS markedly down-regulated the gene for IL 12, a key pro-inflammatory cytokine of protective innate immunity profoundly secreted by intracellular macrophages of epithelial cells during inflammation (Walter et al., 2001; Trinchieri, 2003). This finding is in line with our previous report demonstrating MOS's capacity to terminate *Salmonella* LPS-induced systemic inflammation earlier than VIRG, and to reduce glucose mobilization activities (Baurhoo et al., 2011). MOS may be thought as an immuno-suppressor, but we previously demonstrated MOS's inherent immuno-stimulatory properties (Baurhoo et al., 2011) and associated this effect with MOS's capacity to terminate inflammation earlier. In this study, all chicks suffered from additional stress during the night of d 6 due to accidental power failure (about 6 h) that caused darkness, uncomfortable low temperatures and restricted access to feed and water. Nevertheless, in contrast to CTL and VIRG, MOS abrogated this additional stress, as indicated by the abrupt *IL 12* repression which unexpectedly occurred at d 7.

Mucins are high molecular weight glycoproteins that display specific mannosyl receptors in its oligosaccharide units, similar to glycoprotein attachment sites of epithelial cells, which competitively bind Type 1 fimbriae of gram-negative pathogens (Sajjan and Forstner, 1990; Vimal et al., 2000; Edelman et al., 2003), limiting *Salmonella* adhesion to and invasion of the underlying epithelial cells. Intestinal mucins exist as membrane-bound (not gel-forming) and secretory (gel-forming) types (Montagne and Lalles, 2004), and these are secreted by enterocytes of epithelial cells and villi-residing goblet cells, respectively (Derrien et al., 2010). However, to our knowledge, this is the first *in vivo* study that clearly demonstrates a role for membrane-bound and secretory mucins in host's first line of intestinal defense against *Salmonella*, and as affected by dietary antibiotic and prebiotic.

Secretory mucins that form the overlying-viscoelastic mucus gel layer of the intestinal epithelium are histologically divided into 2 broad categories: neutral and acidic, with the latter further subdivided into sulfated ('sulfomucins': rich in sulfate residues) and sialylated ('sialomucins': rich in sialic acid) mucin types (Montagne et al. 2004), based

on the density and types of neutral or acidic groups present in their oligosaccharide side chain (Roberton and Wright, 1997). Despite our knowledge about mucin's structural components, the physiological relevance of distinct mucin subtypes is yet not clear. In this study, whereas sialomucin (blue) goblet cells were practically absent, the sulfomucin (black) type of acidic goblet cells was predominantly observed in both the non-infected and infected hosts (figure 7), which is characteristically indicative of immature goblet cells found in young hosts (Turck et al., 1993; Forder et al., 2007). The fact that higher degree of sulfation in sulfomucins limits their degradation by bacterial mucin-degrading glycosidases (Roberton and Wright, 1997), sulfomucins confers extra barrier protection of the underlying epithelium against intestinal pathogens especially in young hosts.

In agreement with Solis de los Santos et al. (2007), we show that neutral and acidic goblet cell numbers were significantly increased by MOS, but dramatically reduced by VIRG in non-infected hosts. However, of greater biological relevance to *Salmonella* infection, we demonstrated that, in addition to preventing loss of intestinal barrier integrity and reduced *IL 12* expression, MOS significantly increased both the neutral and acidic mucin-containing goblet cell types in an enteric *S.* Enteritidis-infection model, whereas VIRG considerably reduced development of these mucins-secreting goblet cells. Therefore, in addition to its capacity to directly bind *S.* Enteritidis, MOS reduced *S.* Enteritidis adhesion and invasion of intestinal epithelial cells, despite higher *S.* Enteritidis load in the intestinal lumen, through barrier protection conferred by increased mucins (neutral and acidic) secretions, whereas VIRG relied principally on its strong bactericidal properties. Both of these defense mechanisms exhibited by MOS involved *S.* Enteritidis adherence at its mannose-specific lectin. The exact mechanism of MOS action on goblet cell development is still not clear, but we believe it is linked with the antigenic properties of yeast cell walls which inherently triggers immune activation (Baurhoo et al., 2011).

The mucins-secreting capacity of goblet cells during *S.* Enteritidis infection was, nevertheless, not different whether hosts were fed the CTL, VIRG or MOS diet, as demonstrated by *MUC 2* non-differential regulation. But, our findings might be expected given that aberrant *MUC 2* induction occurs during apoptosis and loss of goblet cells (Boshuizen et al., 2005) to sustain mucins secretions from the fewer remaining functional goblet cells, and that goblet cell integrity was not compromised in this study despite *S.*

Enteritidis invasion of epithelial cells in CTL-fed hosts. However, in the event of higher neutral and acidic goblet cell numbers, *MUC 2* down-regulation occurred in non-infected hosts fed MOS. Taken together, these apparently conflicting findings indicate that MOS's higher mucin-mediated defense mechanism is governed principally by higher goblet cell numbers rather than increased mucin-secreting capacity of goblet cells. Unfortunately, to the best of our knowledge, no reliable method exists yet to accurately quantify intestinal mucins in chickens. It is worth highlighting that the highly hydrated (~95 % water) mucus gel comprises of 3 % mucins only (Allen, 1981).

Increased secretions of membrane-bound mucins, as mediated by *MUC 1* overexpression, is a well-recognized defense mechanism of damaged epithelial cells during enteric infections in humans (Furr et al., 2010) and mice (McAuley et al., 2007). Here, we demonstrate that, in comparison to CTL, MOS markedly reduced *MUC 1* expressions, revealing that MOS counteracted *S.* Enteritidis invasion of intestinal epithelial cells. Interestingly, our *MUC 1* down-regulation results demonstrate that MOS more significantly reduced epithelial cells damage by *S.* Enteritidis than VIRG. These findings are in full support of our histological and *IL 12* expressions results, and reveal adaptability of intestinal epithelial cells to secrete higher membrane-bound mucins during *S.* Enteritidis invasion. We associated the abrupt *MUC 1* repression at d 7 to MOS's capacity to abolish stress accidentally induced to hosts, similar to our *IL 12* expression results.

All intestinal health benefits exerted by MOS discussed so far are clearly supported by its capacity to improve villi morphological characteristics. Histologically, long villi correlate with good intestinal health that favors maximization of nutrient digestion, absorption and utilization (Tappenden, 2006). *S.* Enteritidis caused villi to be shorter, pointed at the tips and more densely populated, thus reducing villi absorptive capacity of nutrients. Evidently, such detrimental morphological changes pronouncedly occurred in *S.* Enteritidis infected chicks fed CTL, which suffered from *S.* Enteritidis invasion and exfoliation of villi epithelium, and enteric inflammation. In agreement with our previous results (Baurhoo et al., 2007, 2009), we show that villi were longer when non-infected hosts were fed MOS, but shorter when fed VIRG. However, we extended our previous results obtained in adult chickens to young chicks, which contrastingly

possess a poorly developed microflora (Gong et al., 2008). The exact mechanisms of MOS and VIRG actions on villi height are still unknown. But, we previously associated increased villi height due to MOS with increased intestinal lactobacilli and bifidobacteria (Baurhoo et al., 2007) that stimulate villi angionesis, vascularization and development (Stappenbeck et al., 2002).

In conclusion, our results demonstrate that MOS inherently improved host's innate defense mechanisms. In contrast to antibiotics like VIRG, MOS supplementation: 1) remarkably improved host's mucins-mediated defense mechanisms against intestinal *S.* Enteritidis, as demonstrated by increased neutral and acidic mucin goblet cell numbers that ultimately secrete higher neutral and acidic mucins; 2) reduced *S.* Enteritidis invasion of intestinal epithelium as evidenced by *MUC 1* down-regulations; and 3) terminated *S.* Enteritidis-induced enteric inflammation earlier, as revealed by *IL 12* down-regulations. Therefore, by virtue of its capacity in reprogramming host's innate defensive mechanisms, MOS may represent a novel biological strategy to remedy *Salmonella*-induced enteric inflammation in human and animals, without posing the risk of developing antibiotic-resistance.

## 3.6 ACKNOWLEDGEMENTS

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**Table 3.1.** Primers-set sequences used in gene expression analysis by quantitative RT-qPCR.

| Gene | Forward (5′ - 3′) | Reverse (5′ - 3′) | Amplicon length (bp) | PubMed Accession No. |
|---|---|---|---|---|
| IL 12 | CTGGAAACTGCCCCGTACTG | GGGCTTGCATCATGTCATCA | 150 | NM_213588 |
| MUC 1 | TCTCTCAGTGCCATCTACCC | GGACCGCAGTGATGTTGG | 127 | XM_430395 |
| MUC 2 | TCACCCTGCATGGATACTTGCTCA | TGTCCATCTGCCTGAATCACAGGT | 228 | XM_421035.2 |
| GAPDH | TGCCATCACAGCCACACAGAAG | ACTTTCCCCACAGCCTTAGCAG | 123 | NM_204305 |

**Figure 3.1.** Intestinal rifamycin-resistant *S.* Enteritidis concentrations of chicks at different time points after oral infection. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). *, $P < 0.05$ by Scheffe's $t$ test.

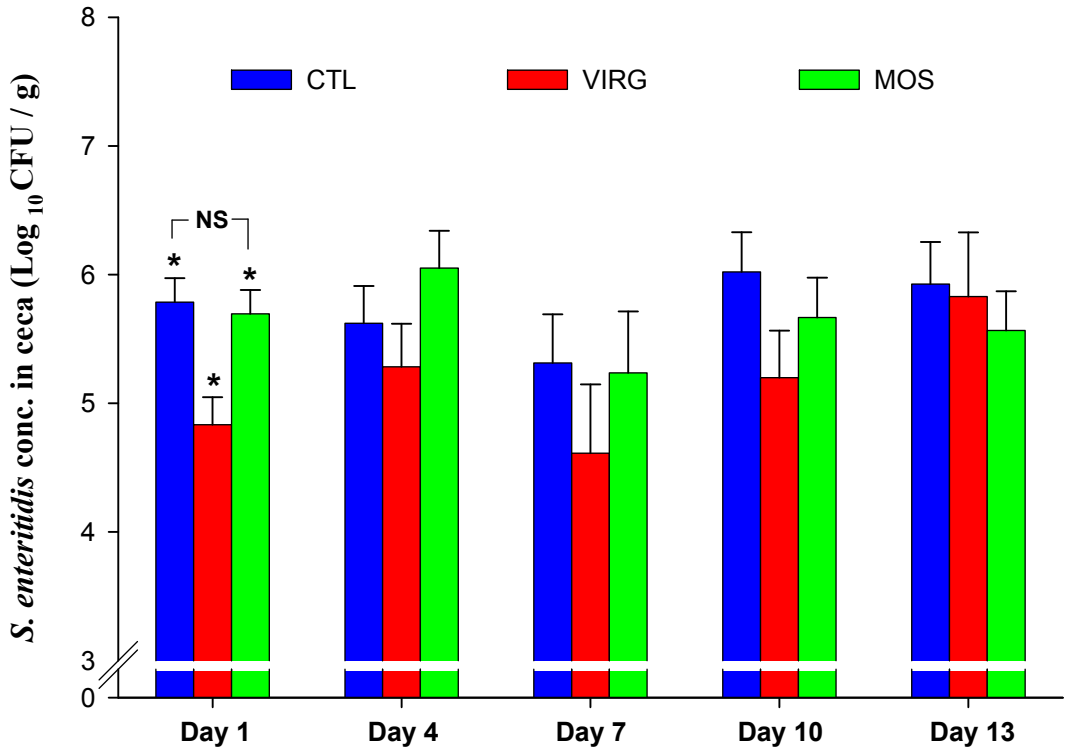
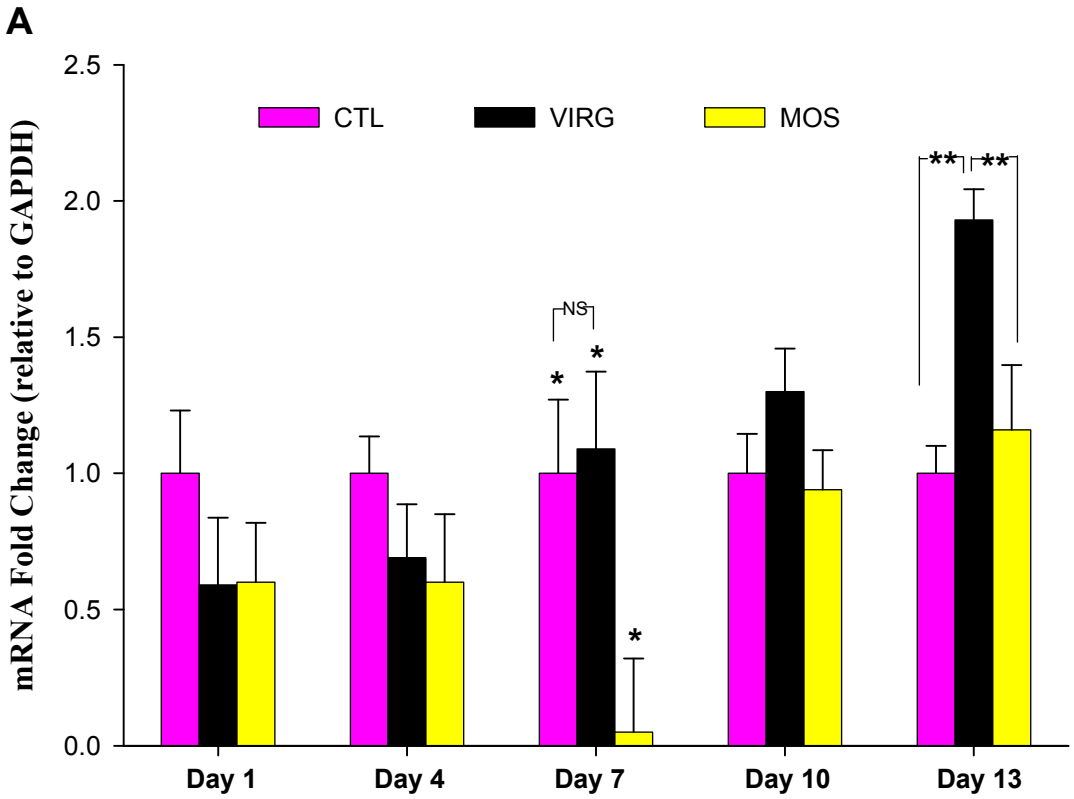


**Figure 3.2.** Expression mRNA levels of interleukin (IL)-12 measured by quantitative real time RT-qPCR at different time points after oral saline inoculation and *S.* Enteritidis infection. In non-infected chicks, *IL 12* was repressed by MOS at d 7, but unexpectedly induced by VIRG at d 13 **(A)**. In *S.* Enteritidis infected chicks, MOS dramatically reduced *IL 12* expression than CTL- and VIRG-fed chicks **(B)**. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). *, $P < 0.05$, **, $P < 0.01$ by Scheffe's $t$ test.



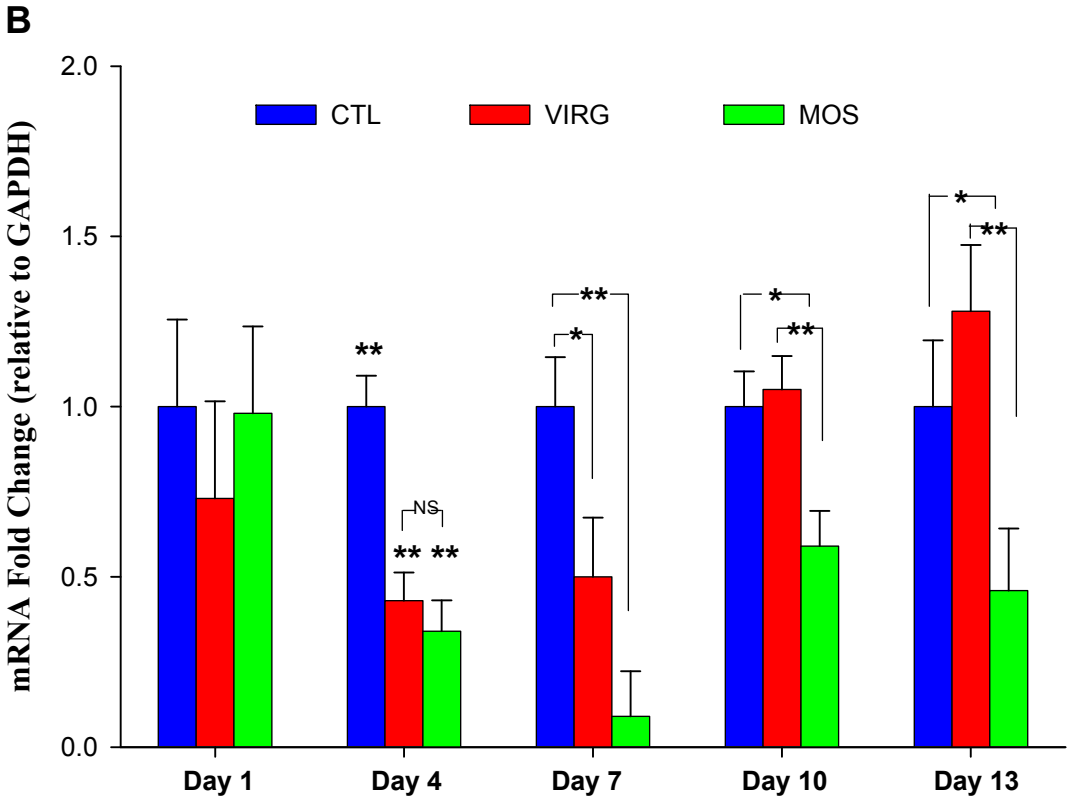
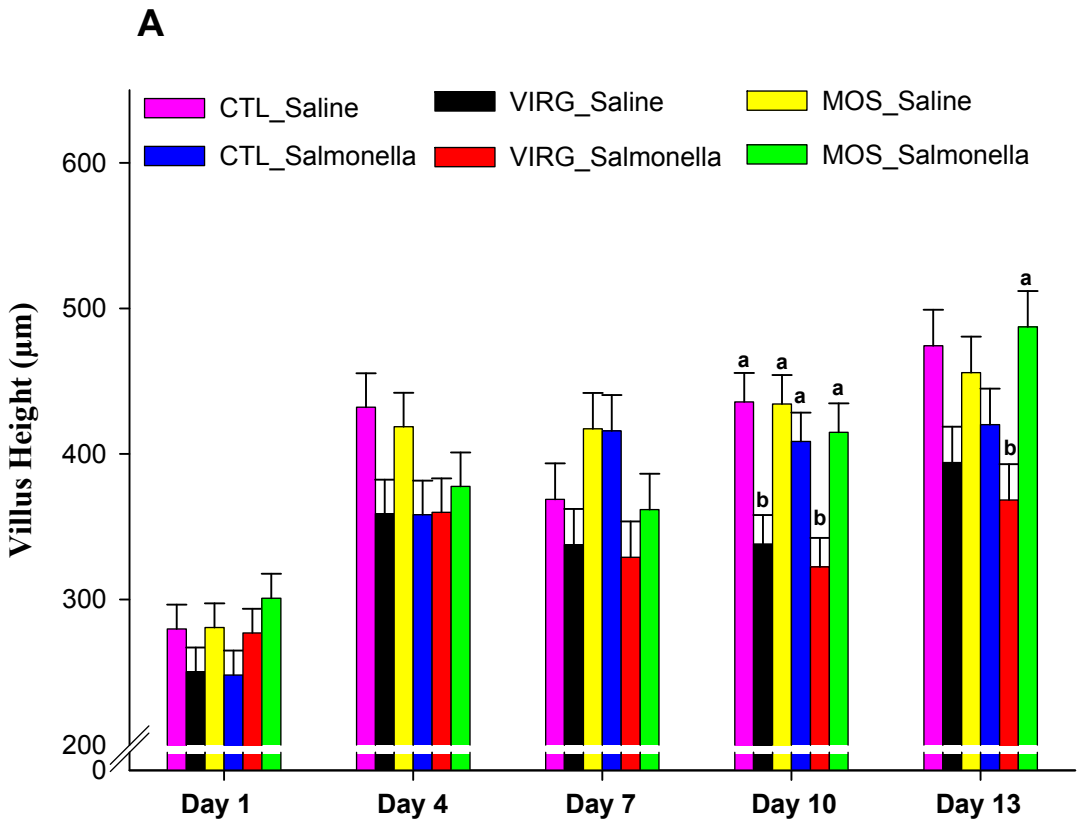
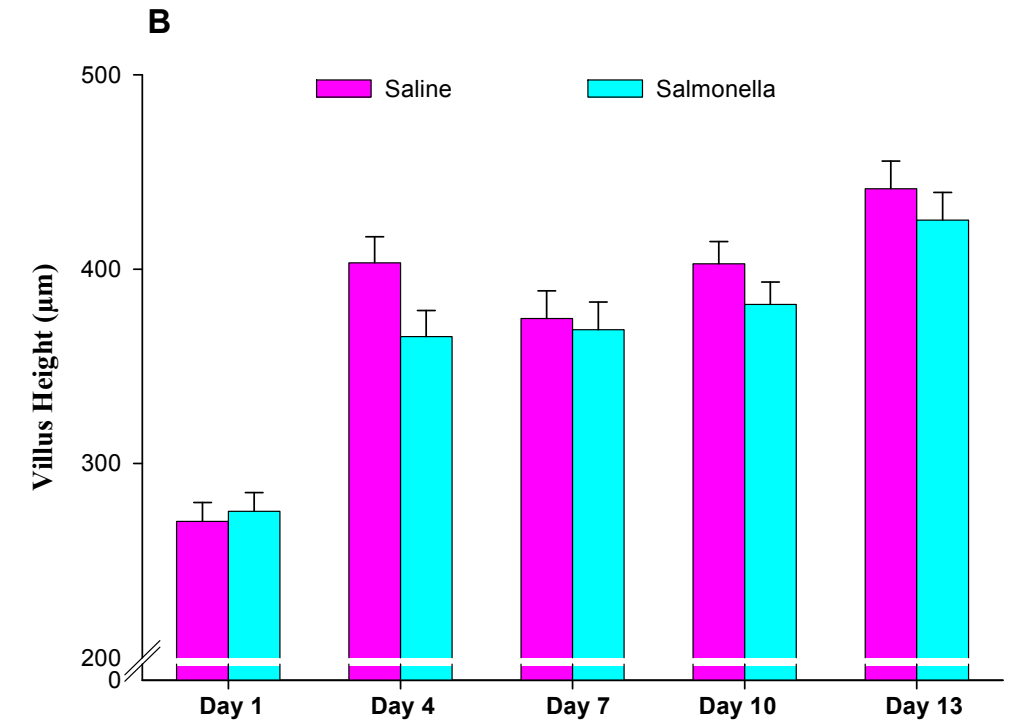

B
mRNA Fold Change (relative to GAPDH)
2.0
1.5
1.0
0.5
0.0
CTL
VIRG
MOS
**
NS
** **
*
**
*
**
*
**
Day 1
Day 4
Day 7
Day 10
Day 13

**Figure 3.3.** Ileal villi height of chicks at different time points after oral saline inoculation and *S.* Enteritidis infection. In comparison to CTL- and MOS-fed chicks, VIRG significantly reduced villi height at d 10 and 13 in non-infected and *S.* Enteritidis infected chicks **(A)** or when both groups pooled together **(C)**. **(B)** *S.* Enteritidis also tended to reduce villi height across dietary treatments. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). (a,b), $P < 0.05$ by Scheffe's $t$ test.




B
Saline
Salmonella
Villus Height (µm)
500
400
300
200
0
Day 1
Day 4
Day 7
Day 10
Day 13

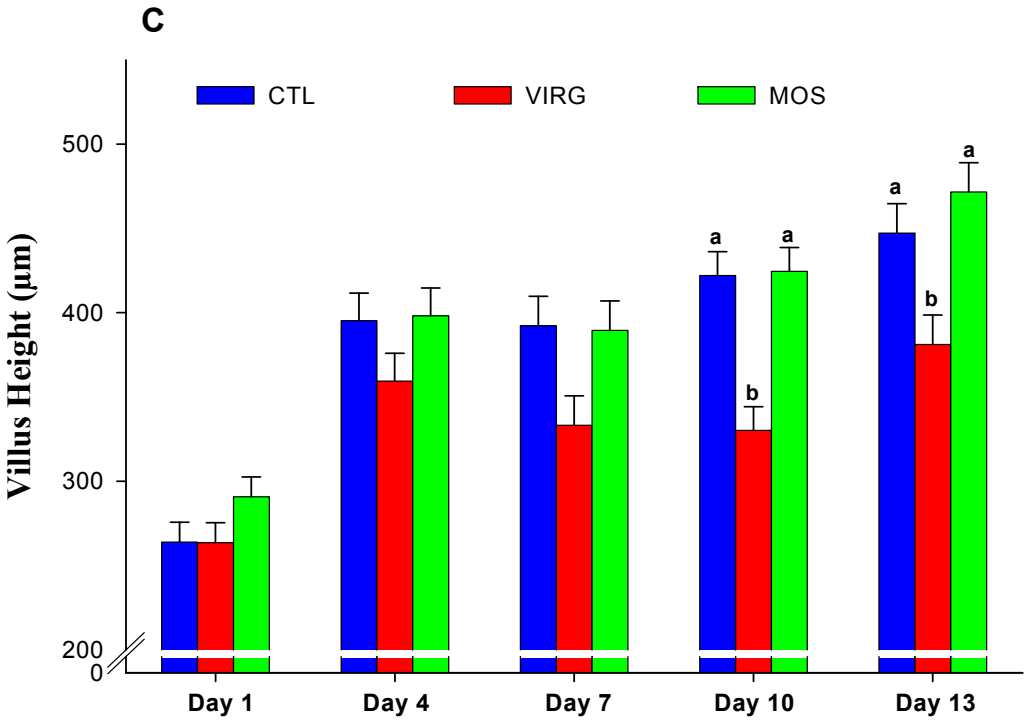
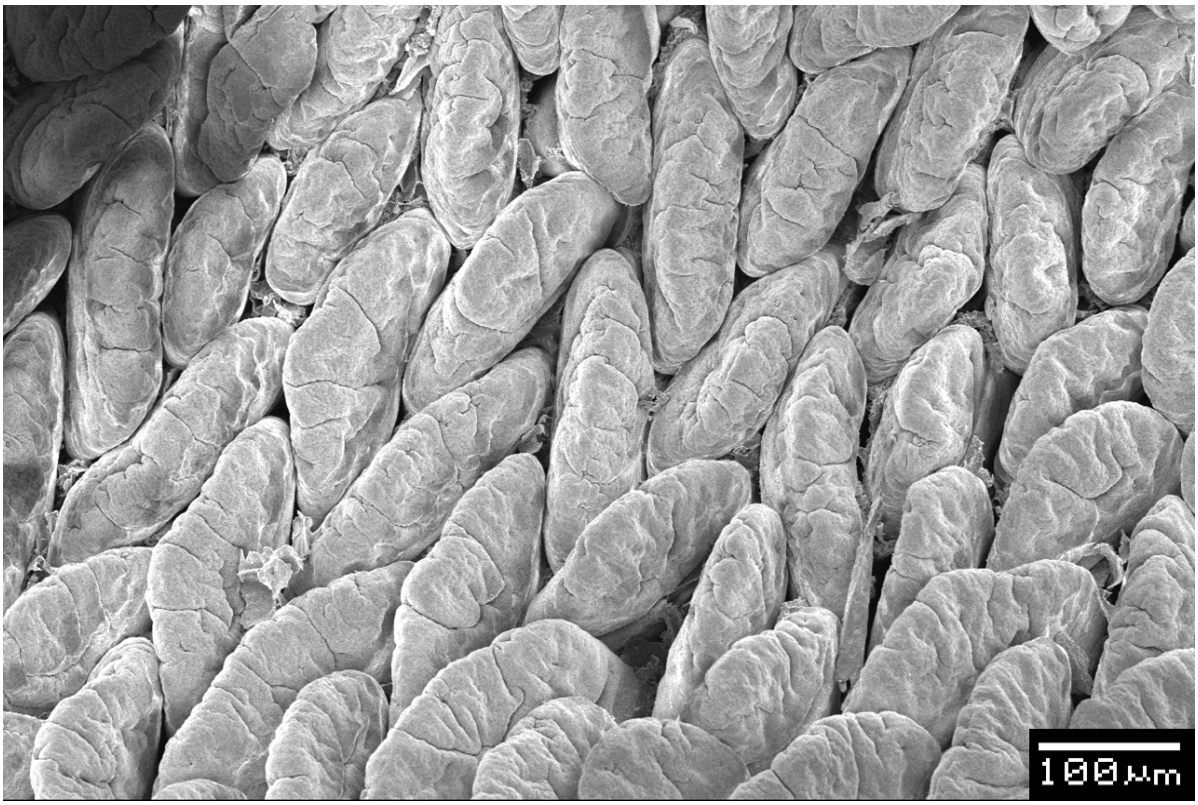

C
CTL
VIRG
MOS
Villus Height (µm)
500
400
300
200
0
a
b
a
a
b
a
Day 1
Day 4
Day 7
Day 10
Day 13

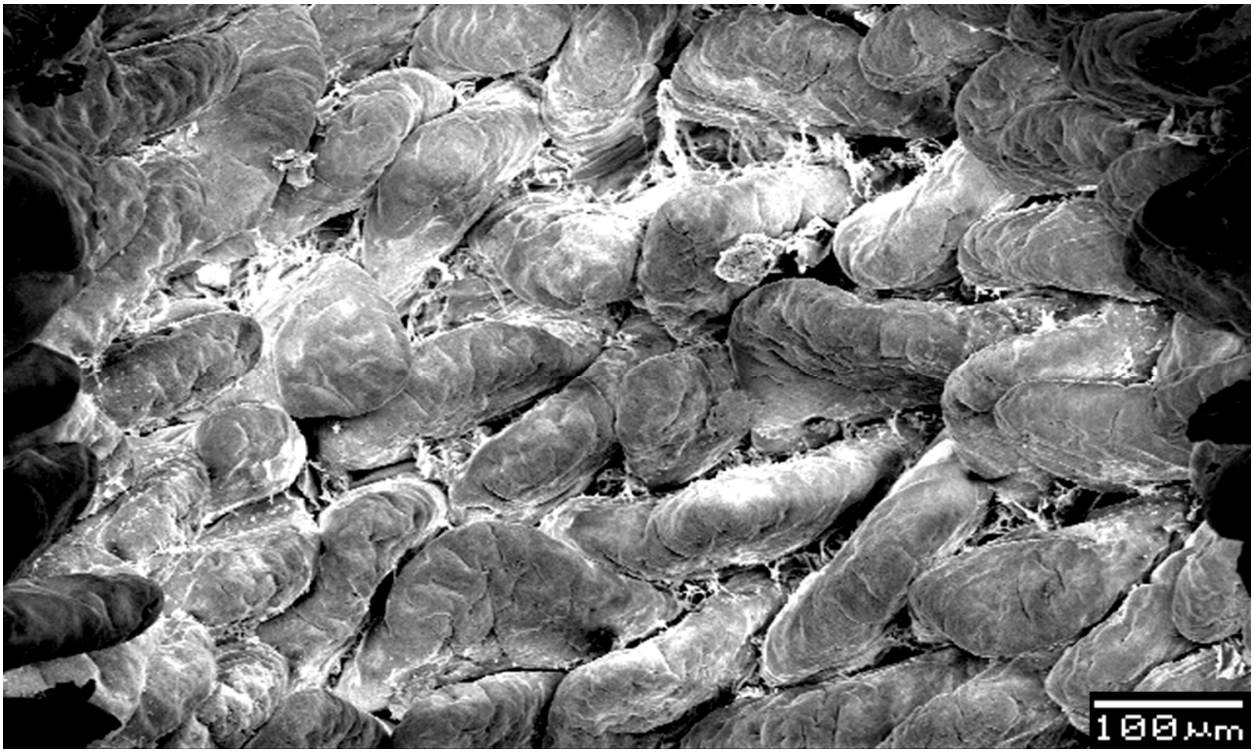
**Figure 3.4.** Scanning electron micrographs of chick ileum at 7 d after oral saline inoculation and *S.* Enteritidis infection. **(A)** In comparison with non-infected chicks fed the CTL diet, villi were longer and wider among MOS-fed chicks but shorter and narrower in VIRG-fed chicks. **(B)** *S.* Enteritidis reduced villi height and villi width, increased villi density and caused sharpening of villi tips in all infected chicks respective to their non-infected controls. Note the healthier and longer villi with a characteristic leaf-shape structure and large surface area among infected chicks fed MOS than CTL or VIRG. Note also the extremely short villi of the already shorter villi due to *S.* Enteritidis in VIRG chicks.

**A**

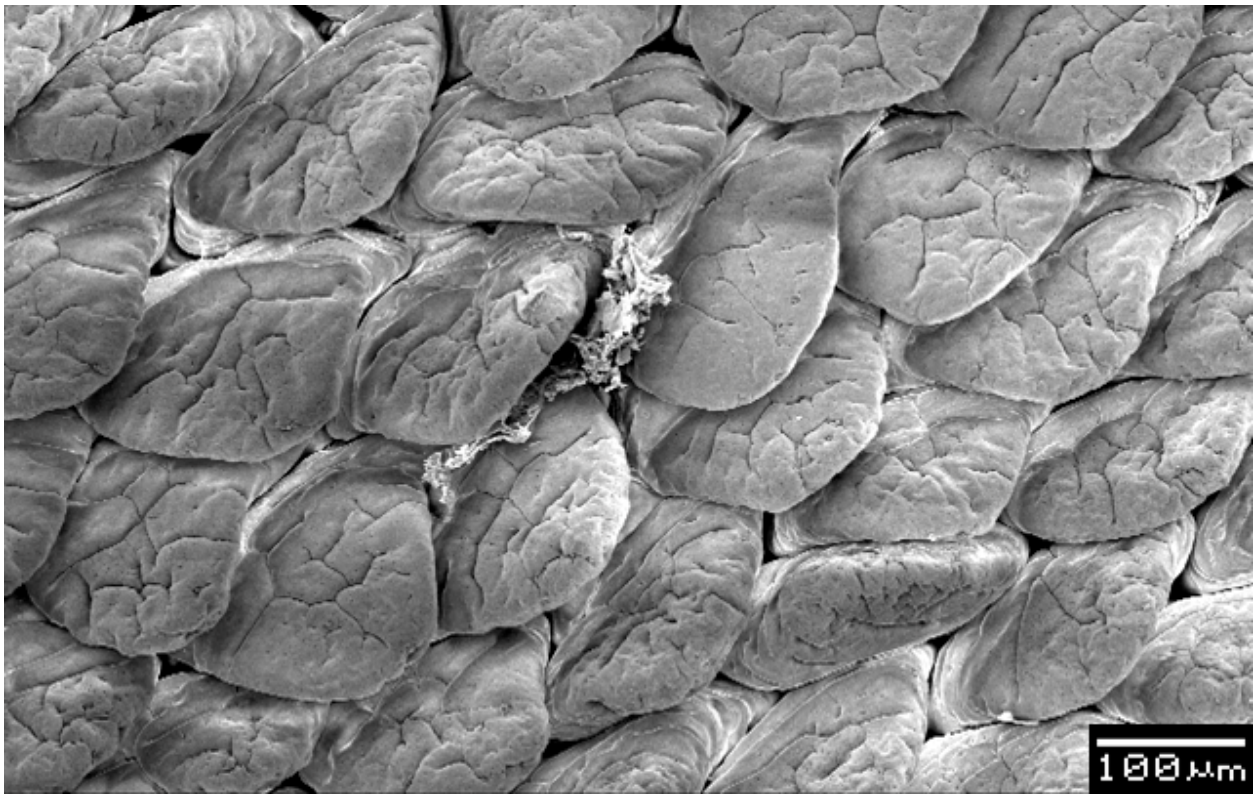
**CTL**



**VIRG**

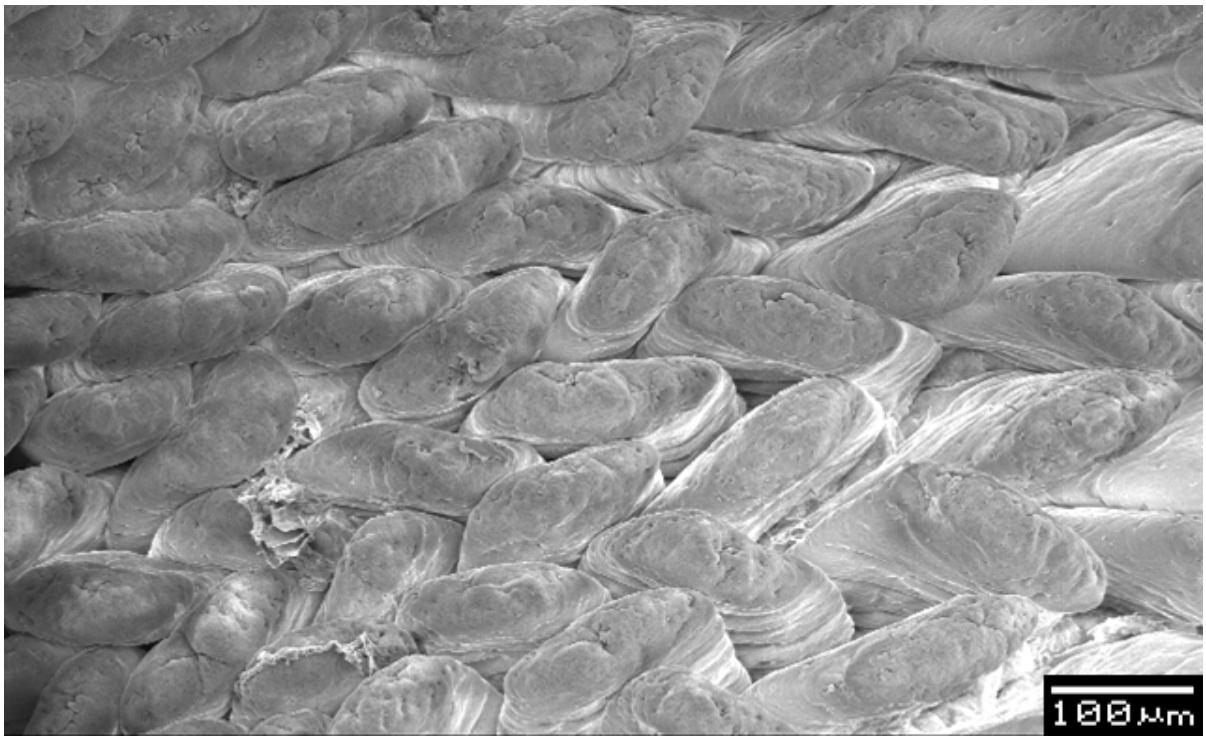



**MOS**



**B**

**CTL**




**VIRG**

**MOS**

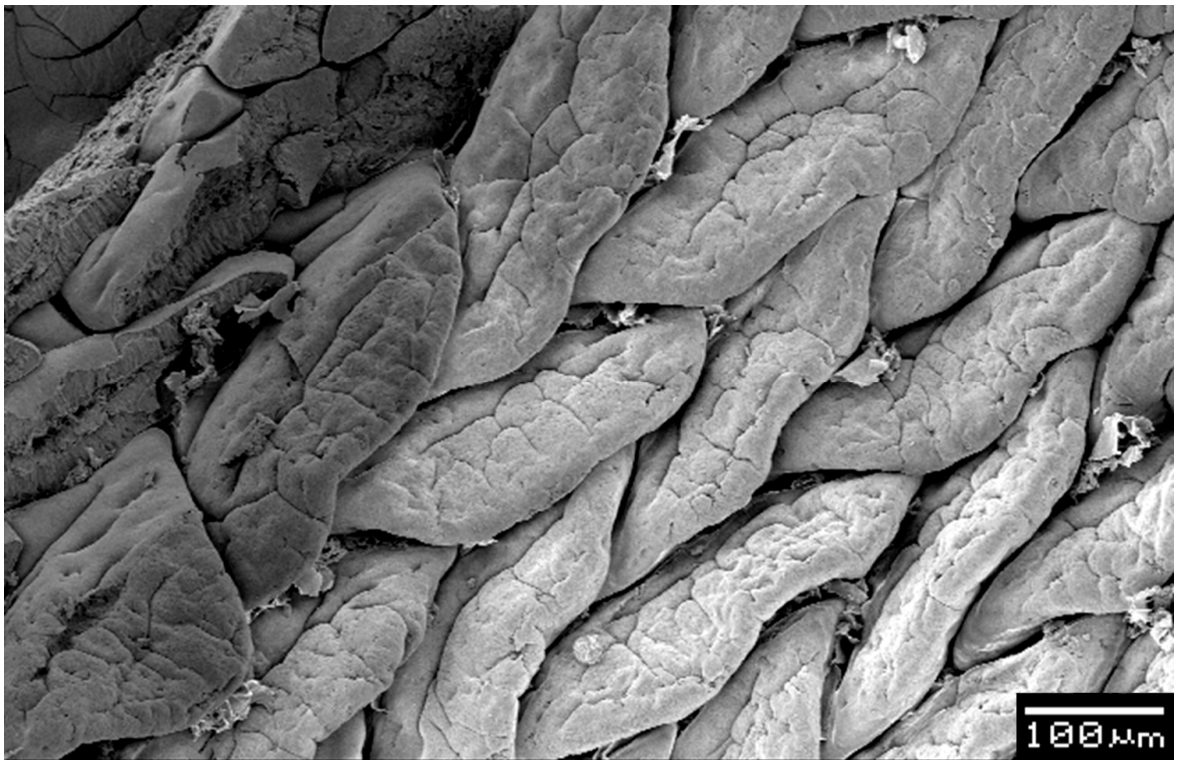
**Figure 3.5.** Scanning electron micrographs of chick ileum at 13 d after oral saline inoculation and *S.* Enteritidis infection. **(A)** Villi were healthy with a characteristic leaf-shape structure among non-infected chicks. But, VIRG caused shortening and enlargement of the less densely populated villi, whereas the extremely long and wider villi in MOS-fed chicks tend to flap over. **(B)** *S.* Enteritidis reduced villi height and villi width, increased villi density and caused sharpening of villi tips in all infected chicks respective with their non-infected controls. However, these detrimental effects on villi integrity more dramatically occurred in CTL-fed chicks. Villi were longer in VIRG hosts. Note the healthier and longer villi with a characteristic leaf-shape structure among infected chicks fed MOS.

**A**

**CTL**

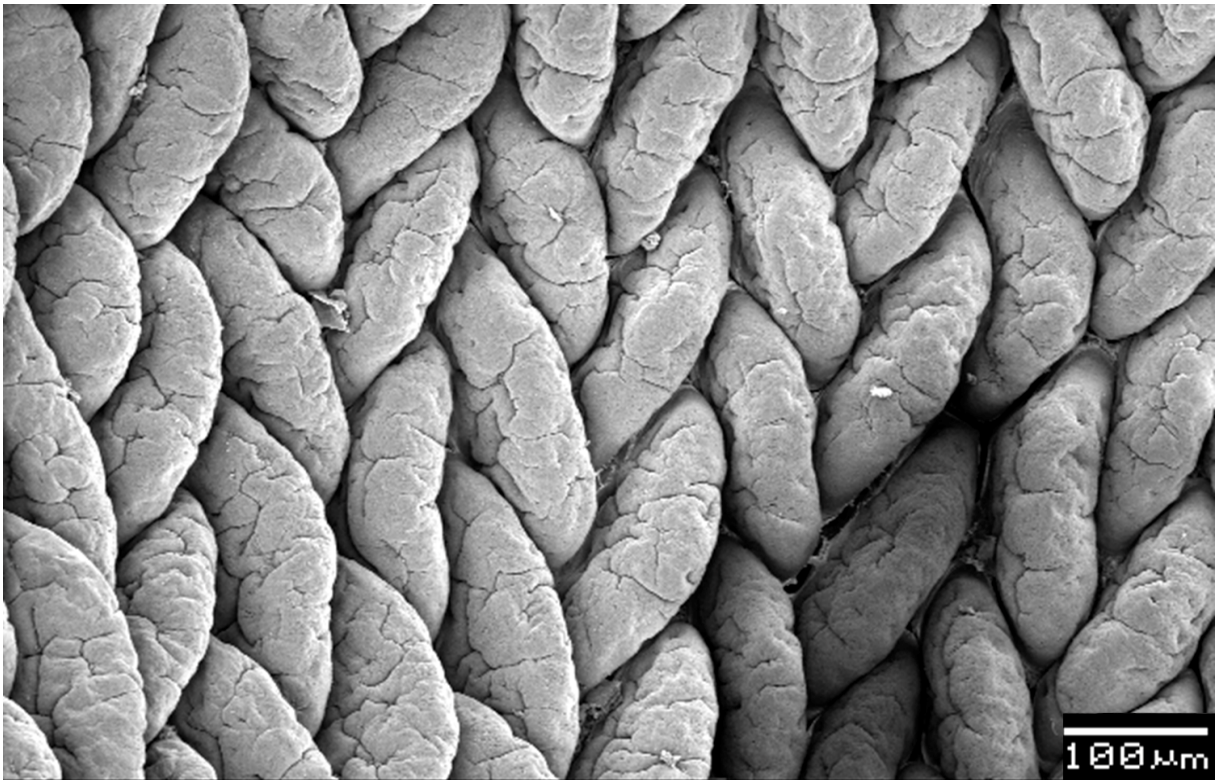
**VIRG**


**MOS**

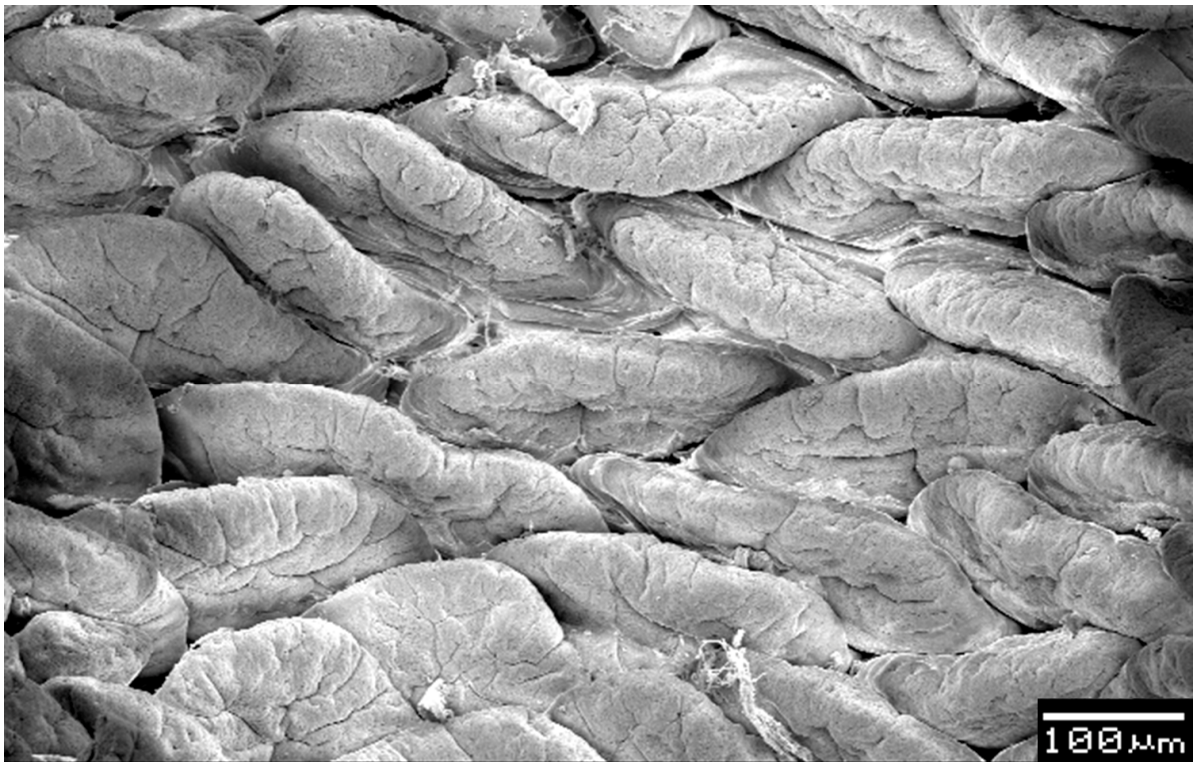


**B**

**CTL**



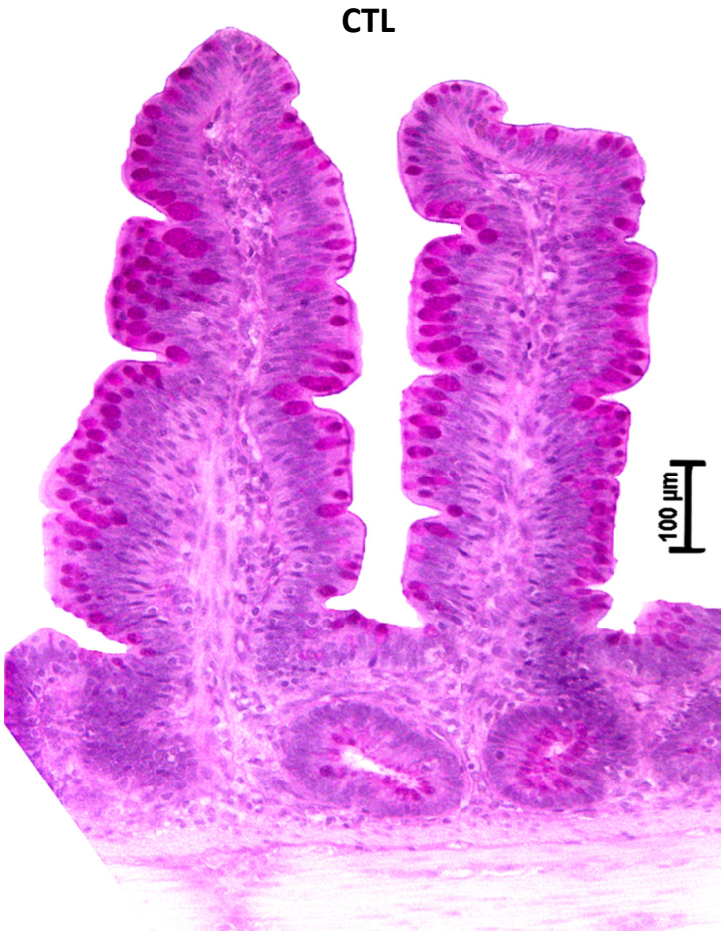
**VIRG**




**MOS**

**Figure 3.6.** PAS-stained ileum sections of chicks at 7 d after oral saline inoculation and *S.* Enteritidis infection. **(A)** Image of the ileum of non-infected chicks fed CTL, VIRG or MOS. **(B)** *S.* Enteritidis caused exfoliation of epithelial cells at villi tips (long arrows) in CTL-fed chicks, which was prevented by dietary VIRG or MOS. Note the presence of cell debris in the intestinal lumen and marked infiltration of inflammatory cells (short arrow), predominately neutrophils and macrophages, in villi lamina propria of infected CTL-fed chicks. Note the longer villi and abundance of neutral goblet cells (red globules) in the villi membrane and crypts of MOS-fed chicks in both the non-infected and infected groups, but considerably shorter villi and reduced goblet cell number among VIRG-fed chicks. In addition, note the bigger size of goblet cells and its mucins-containing and luminal secreting capacity (arrow) due to MOS in infected chicks. Magnification 200X. PAS = periodic acid Schiff stain.

**A**

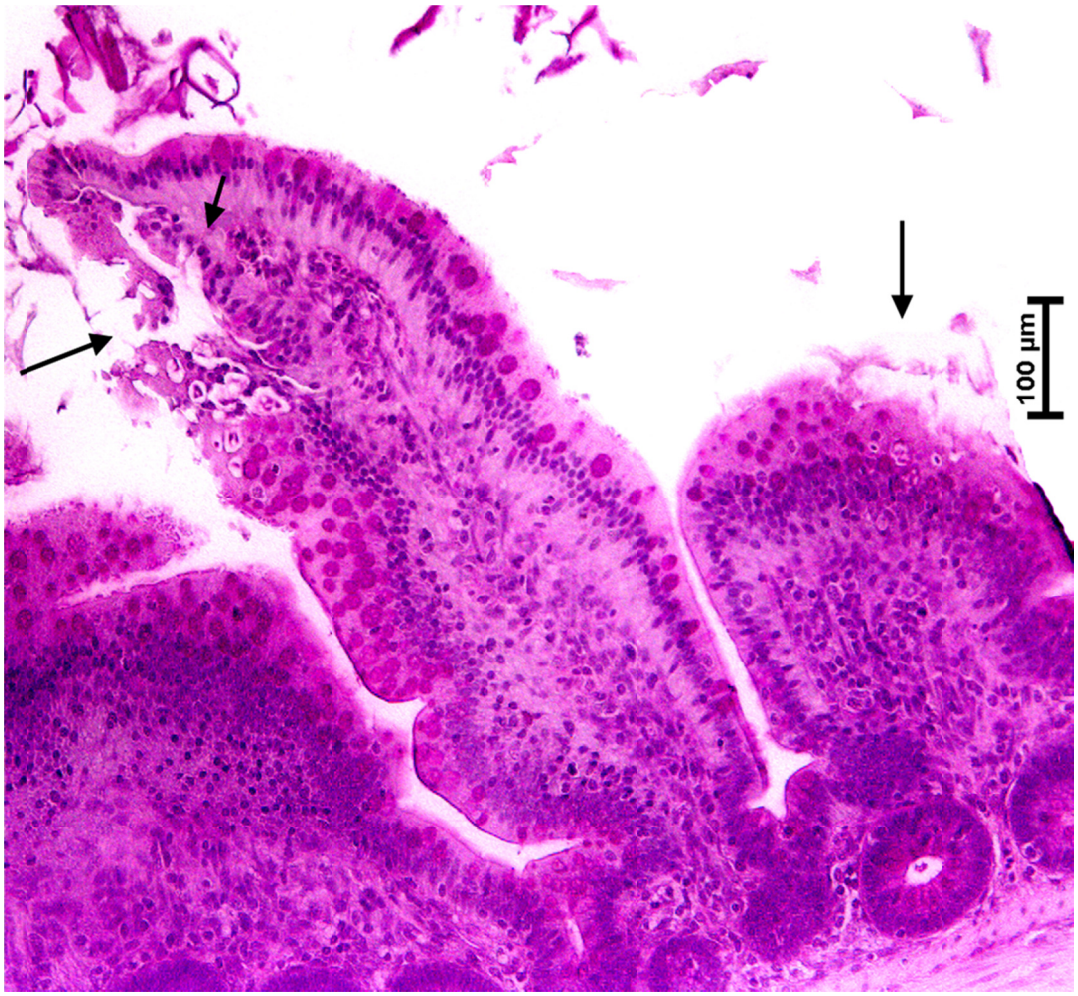


100 µm

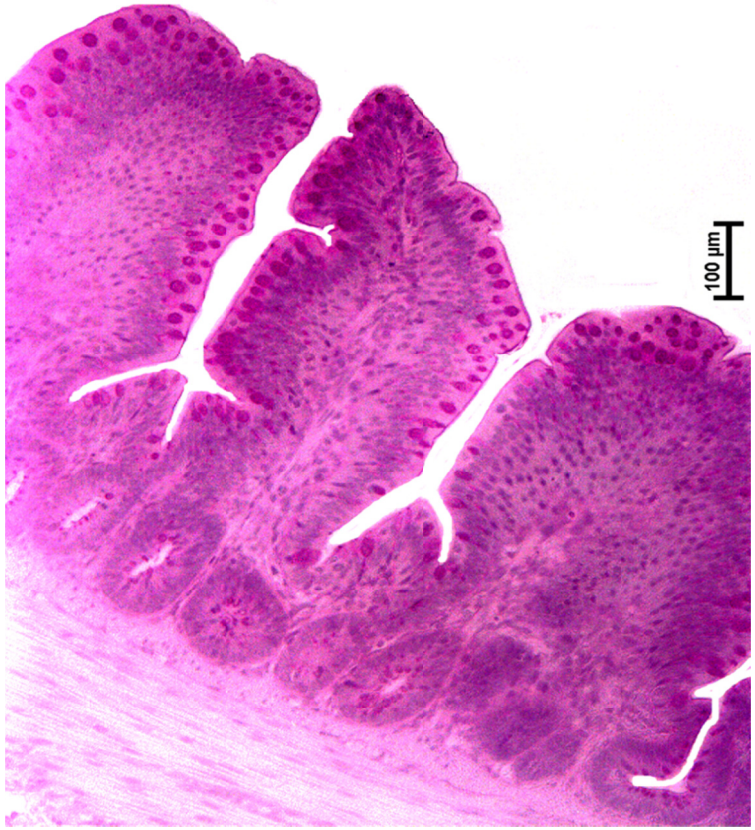

100 µm

**B**

**CTL**



VIRG




MOS

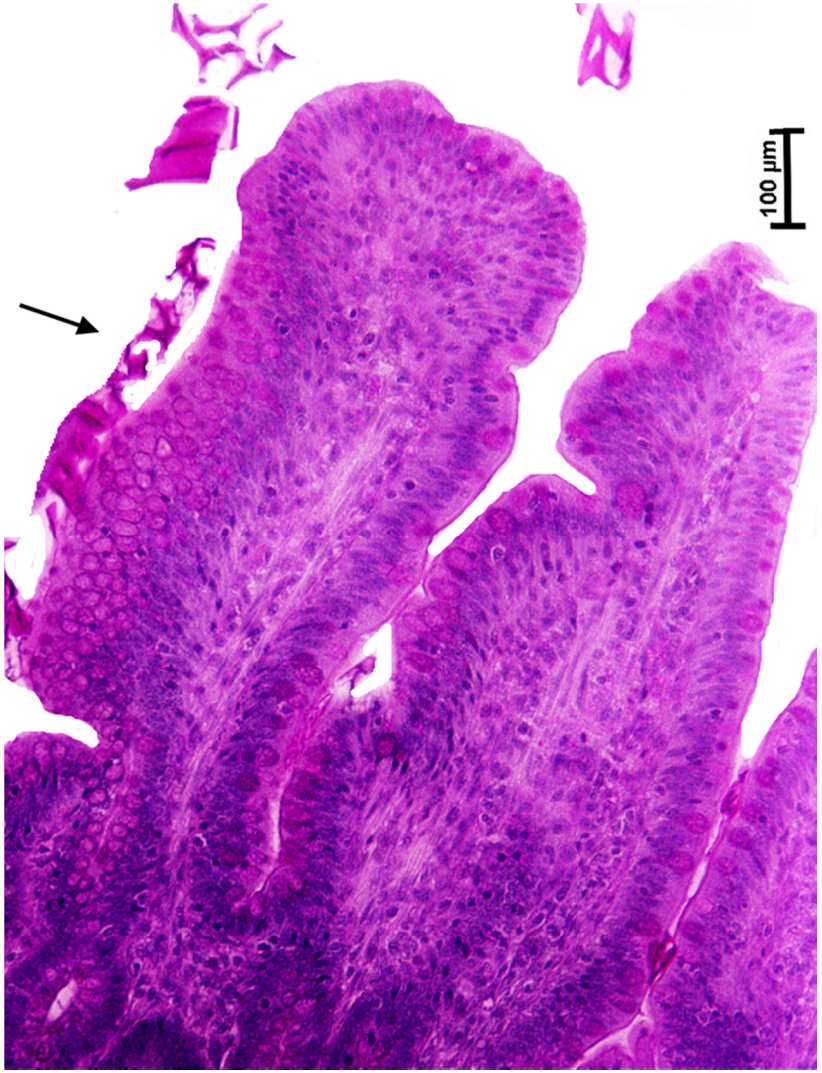
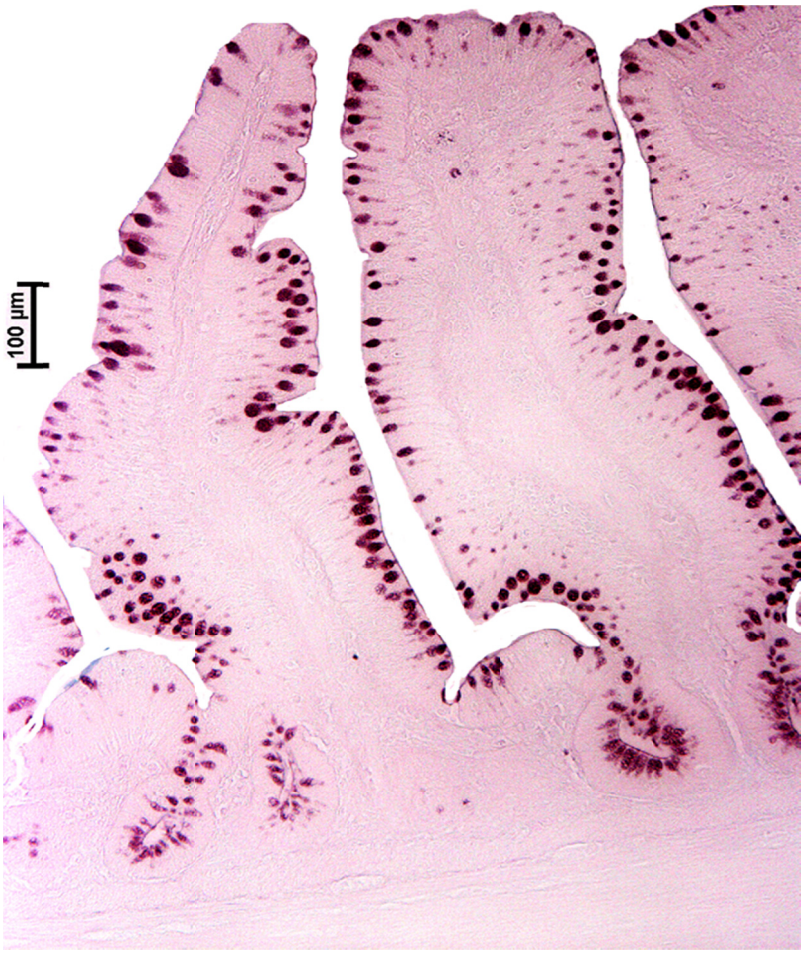
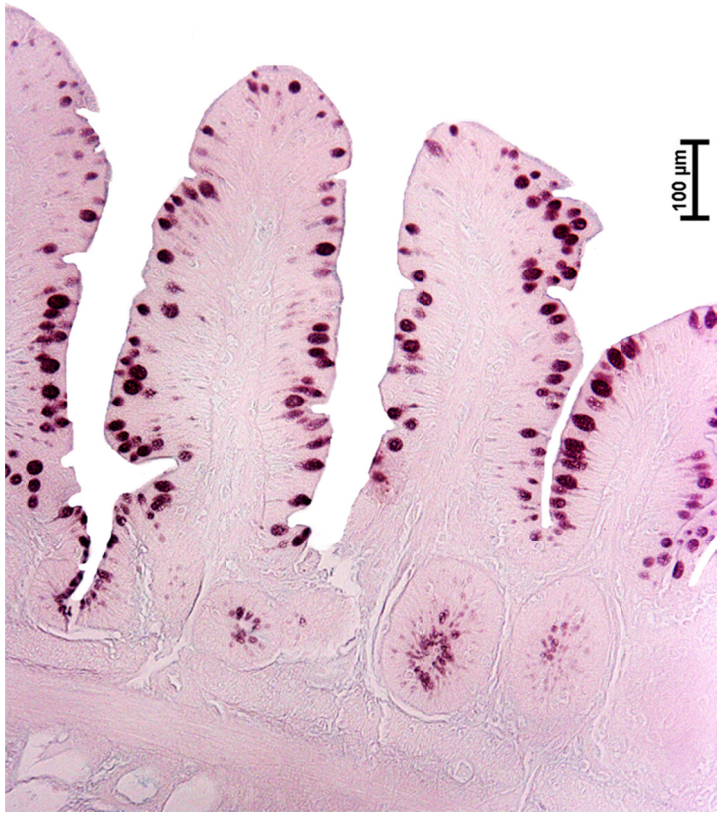
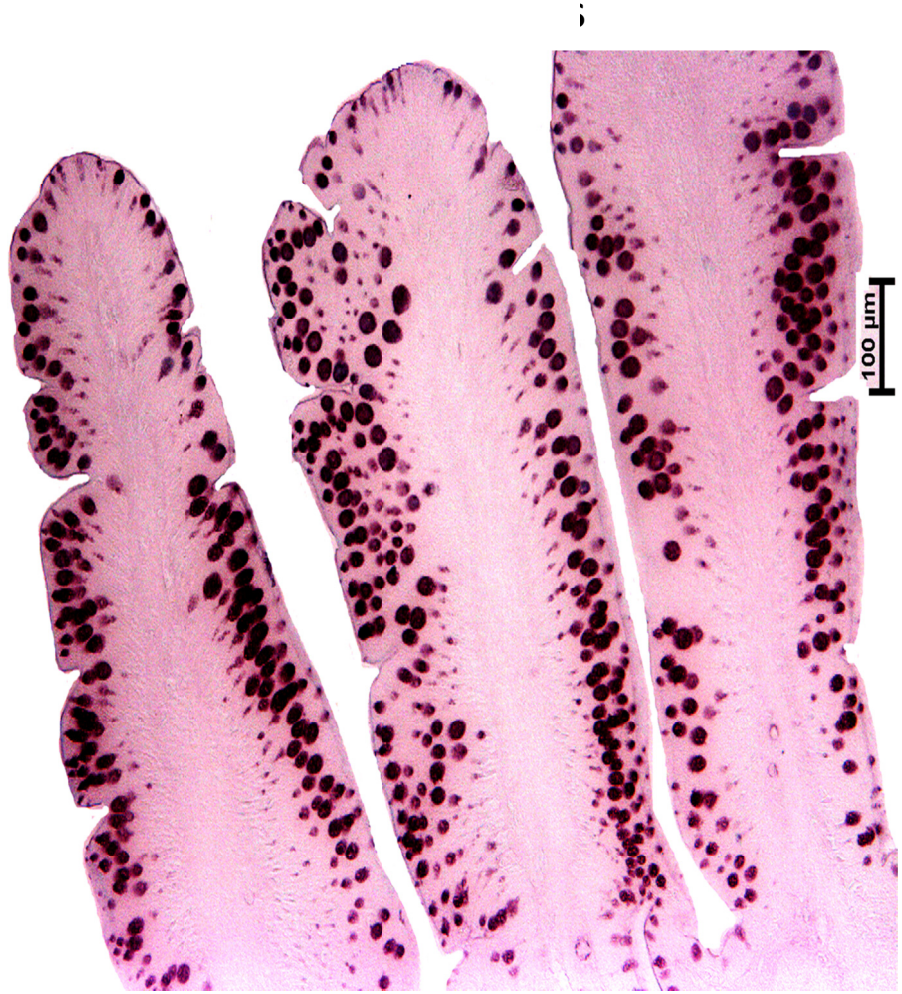


**Figure 3.7.** Image of AB-stained ileum sections of chicks at 7 d after oral saline inoculation **(A)** and *S.* Enteritidis infection **(B)**. MOS markedly increased villi height and acidic goblet cell (black globules) numbers in both the non-infected and infected chicks, whereas VIRG reduced villi height and goblet cell number when compared with CTL chicks. The bigger goblet cells in MOS hosts are indicative of higher mucin containing and secreting capacity. Note the abundance of acidic mucins in the intestinal lumen of infected hosts fed MOS. Also note the exfoliation of epithelial cells caused by *S.* Enteritidis at villi tips (arrows) in CTL-fed chicks, which was prevented by dietary VIRG or MOS. Magnification 200X. AB = highiron diamine-Alcian blue stain.

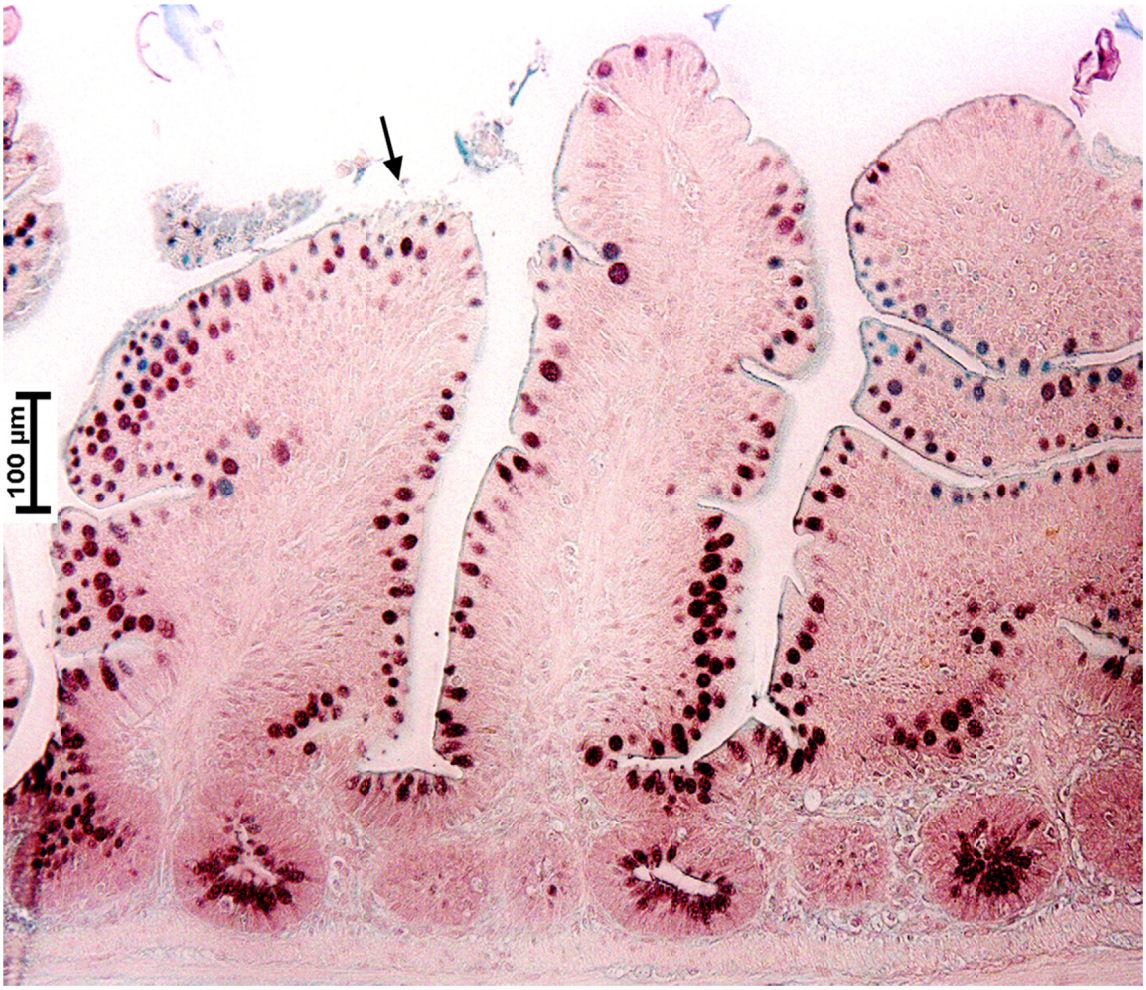
**A**

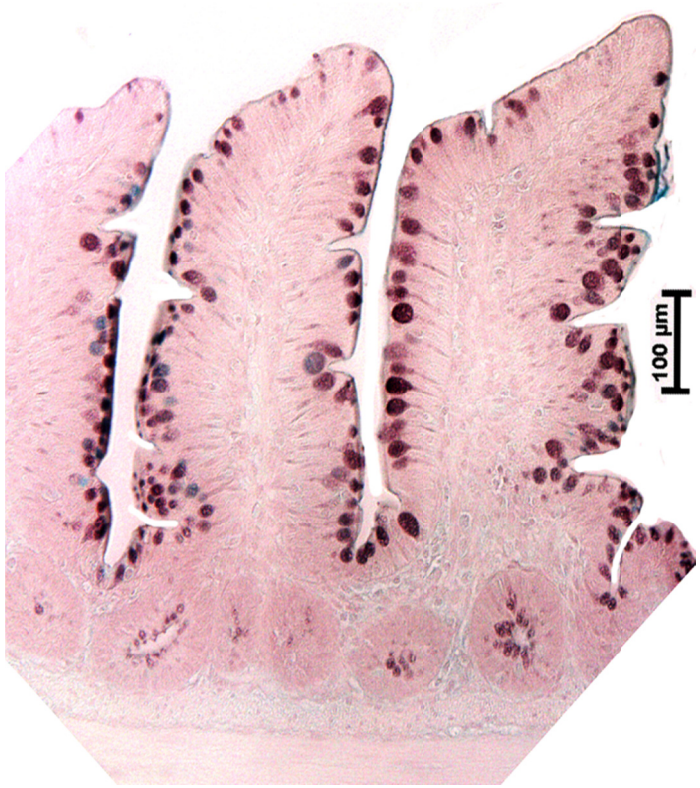



100 µm


100 µm

**B**






MOS

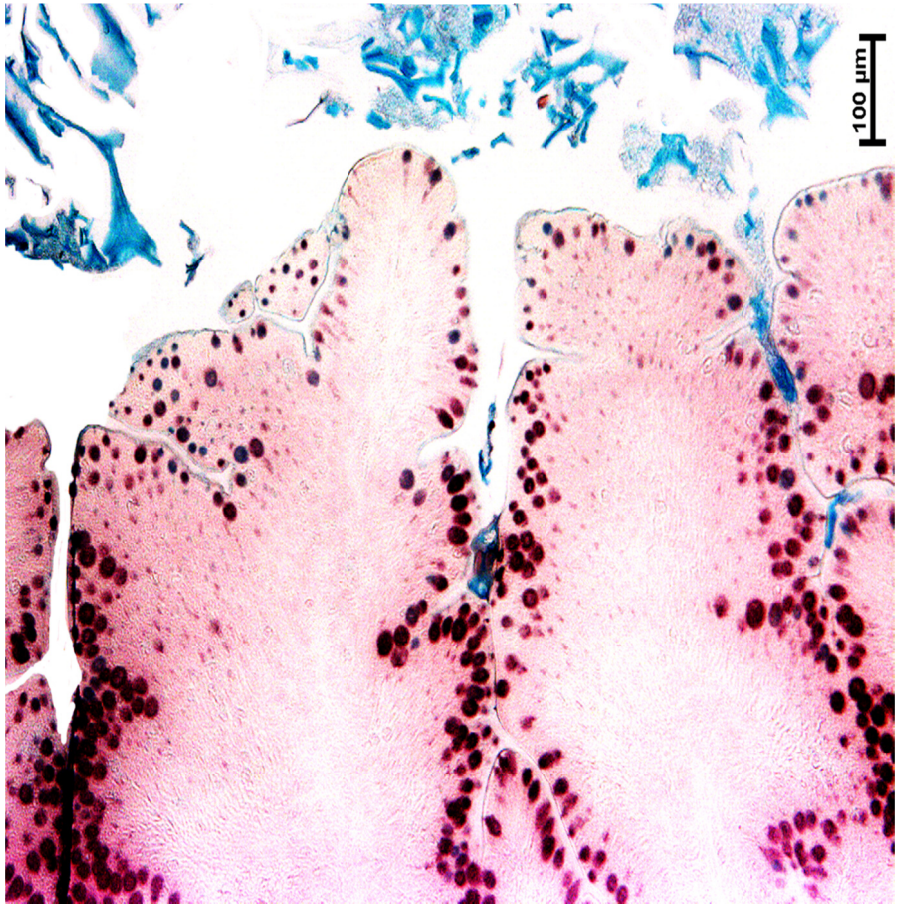
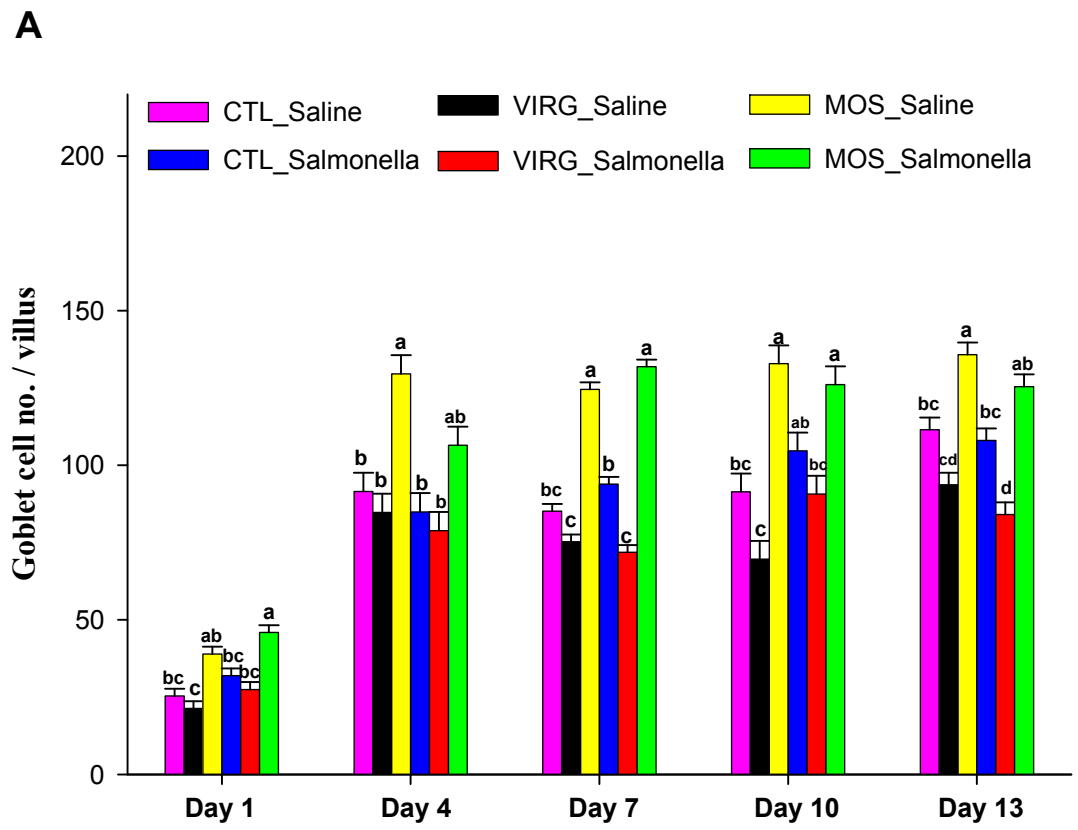
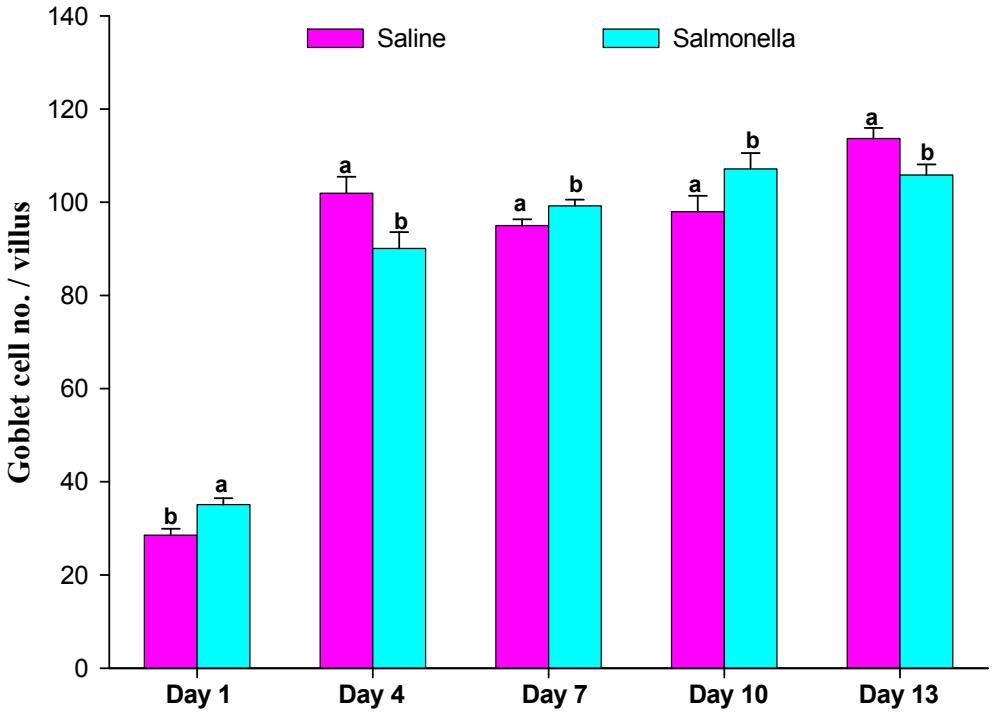


**Figure 3.8.** Neutral goblet cell numbers per villus of the chick ileum at different time points after oral saline inoculation and *S.* Enteritidis infection. In comparison to CTL- and VIRG-fed chicks, MOS increased the number of neutral goblet cells in non-infected and infected chicks **(A)** or when both groups pooled together **(C)**. *S.* Enteritidis had inconsistent effects on neutral goblet cells **(B)**. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). (a-d), $P < 0.05$ by Scheffe's $t$ test.



B


Saline
Salmonella
Goblet cell no. / villus
0
20
40
60
80
100
120
140
Day 1
Day 4
Day 7
Day 10
Day 13
a
b

C

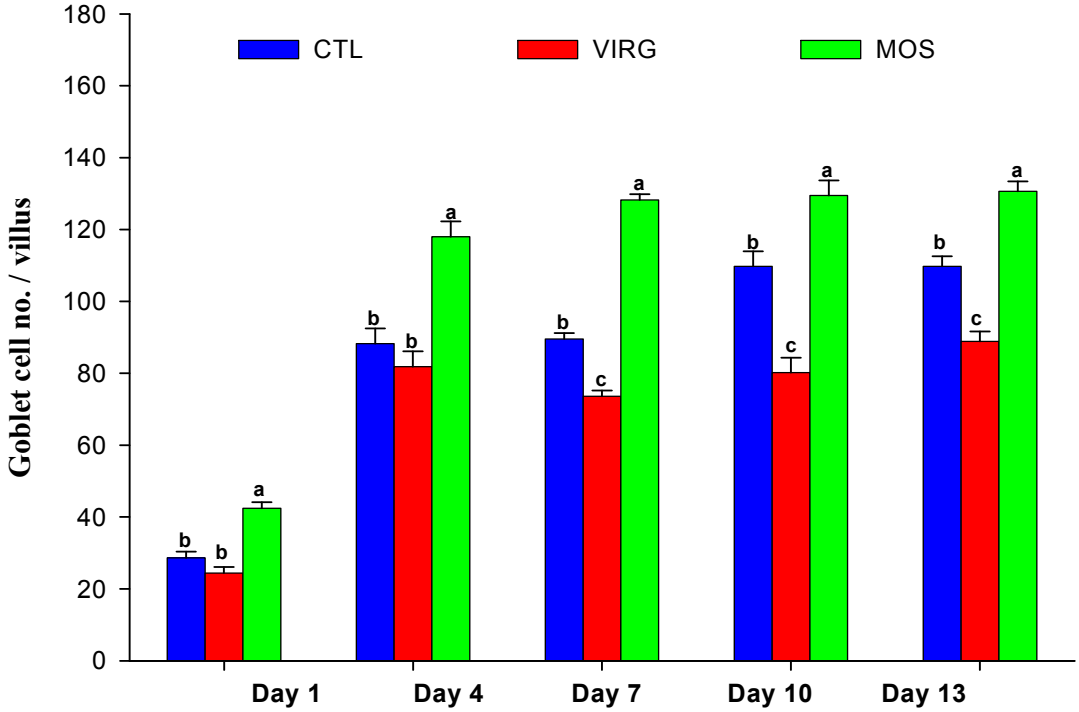
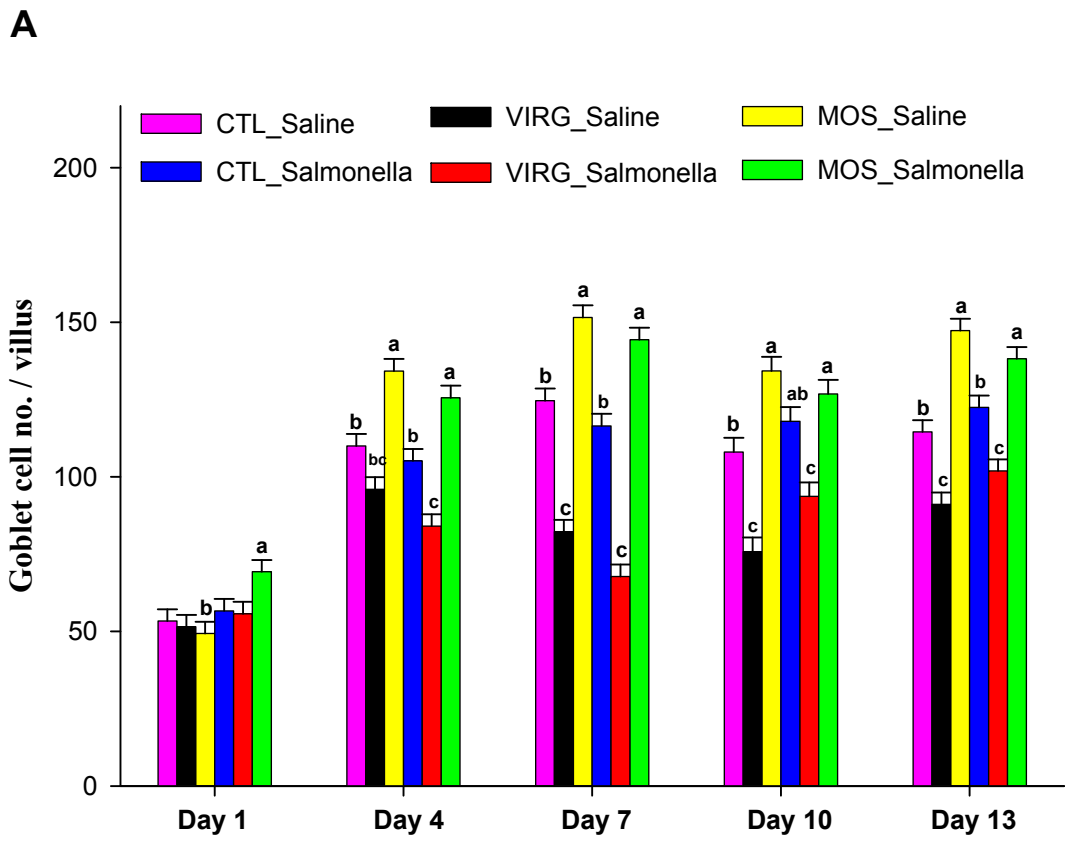
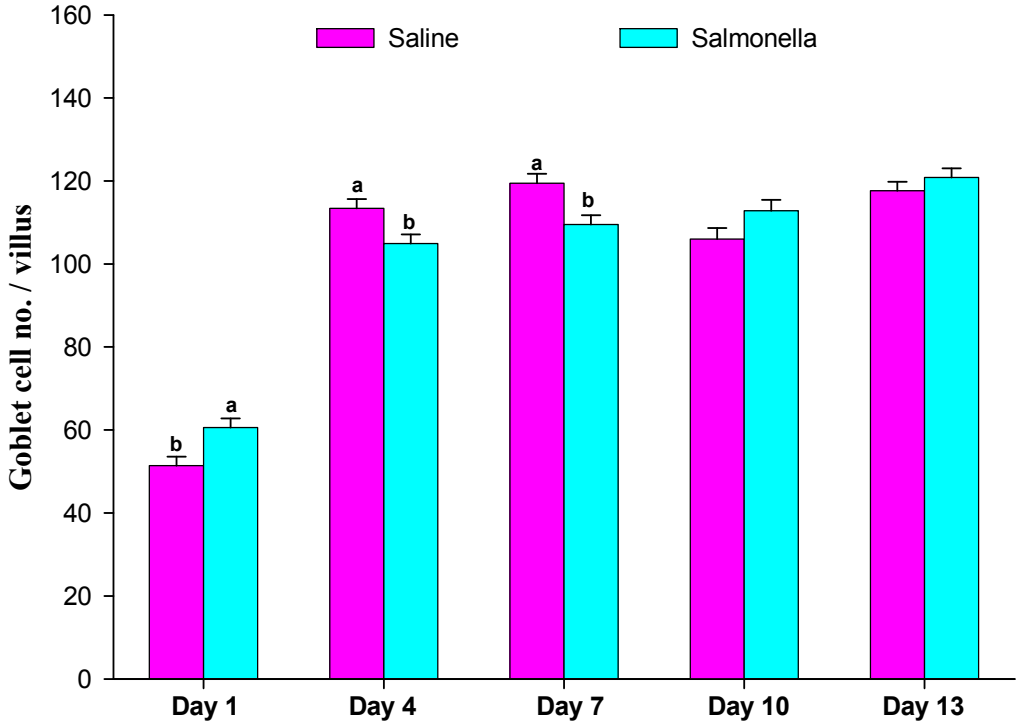

CTL
VIRG
MOS
Goblet cell no. / villus
0
20
40
60
80
100
120
140
160
180
Day 1
Day 4
Day 7
Day 10
Day 13
a
b
c

**Figure 3.9.** Acidic goblet cell numbers per villus of the chick ileum at different time points after oral saline inoculation and *S.* Enteritidis infection. In comparison to CTL- and VIRG-fed chicks, acidic goblet cell numbers were increased by MOS both in non-infected and infected chicks **(A)** or when both groups pooled together **(C)**. The effect of *S.* Enteritidis on acidic goblet cells was variable **(B)**. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). (a-c), $P < 0.05$ by Scheffe's $t$ test.



**B**


Saline
Salmonella
Goblet cell no. / villus
160
140
120
100
80
60
40
20
0
a
b
a
b
a
b
Day 1
Day 4
Day 7
Day 10
Day 13

**C**

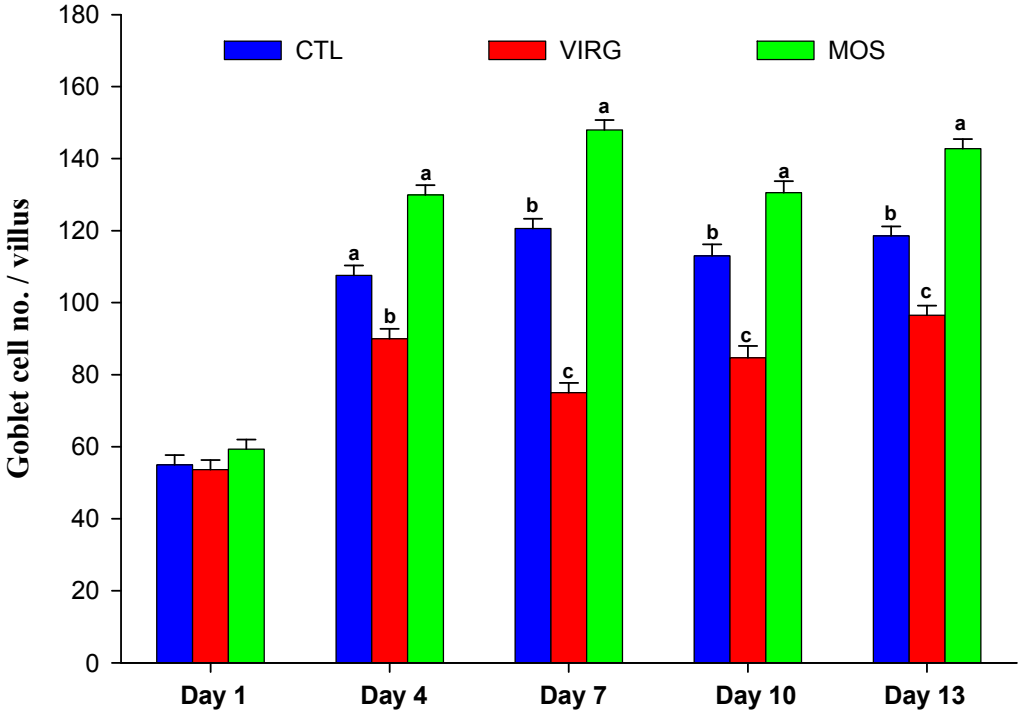
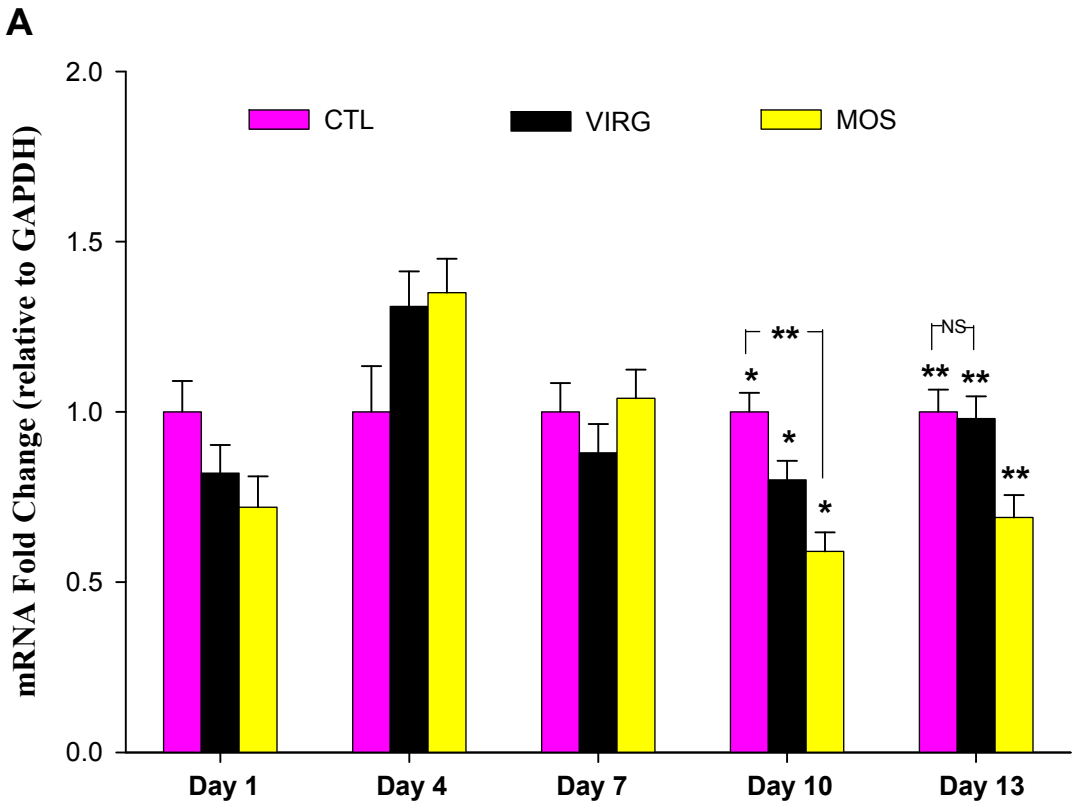

CTL
VIRG
MOS
Goblet cell no. / villus
180
160
140
120
100
80
60
40
20
0
a
b
a
b
c
a
b
c
a
b
c
a
Day 1
Day 4
Day 7
Day 10
Day 13

**Figure 3.10.** Expression mRNA levels of mucin (MUC)-2 measured by quantitative real time RT-qPCR at different time points after oral saline inoculation and *S.* Enteritidis infection. *MUC 2* expression was repressed by MOS among non-infected chicks (**A**), but not differentially regulated by dietary treatments among infected chicks **(B)**. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). *, $P < 0.05$, **, $P < 0.01$ by Scheffe's $t$ test.



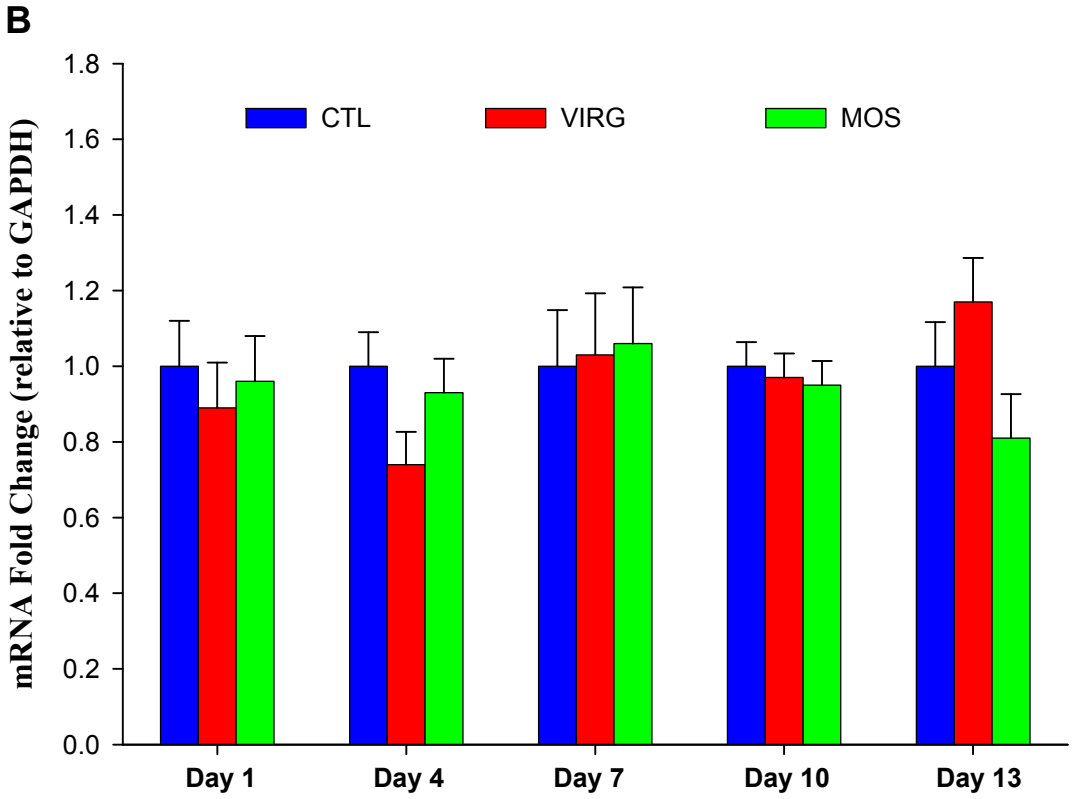
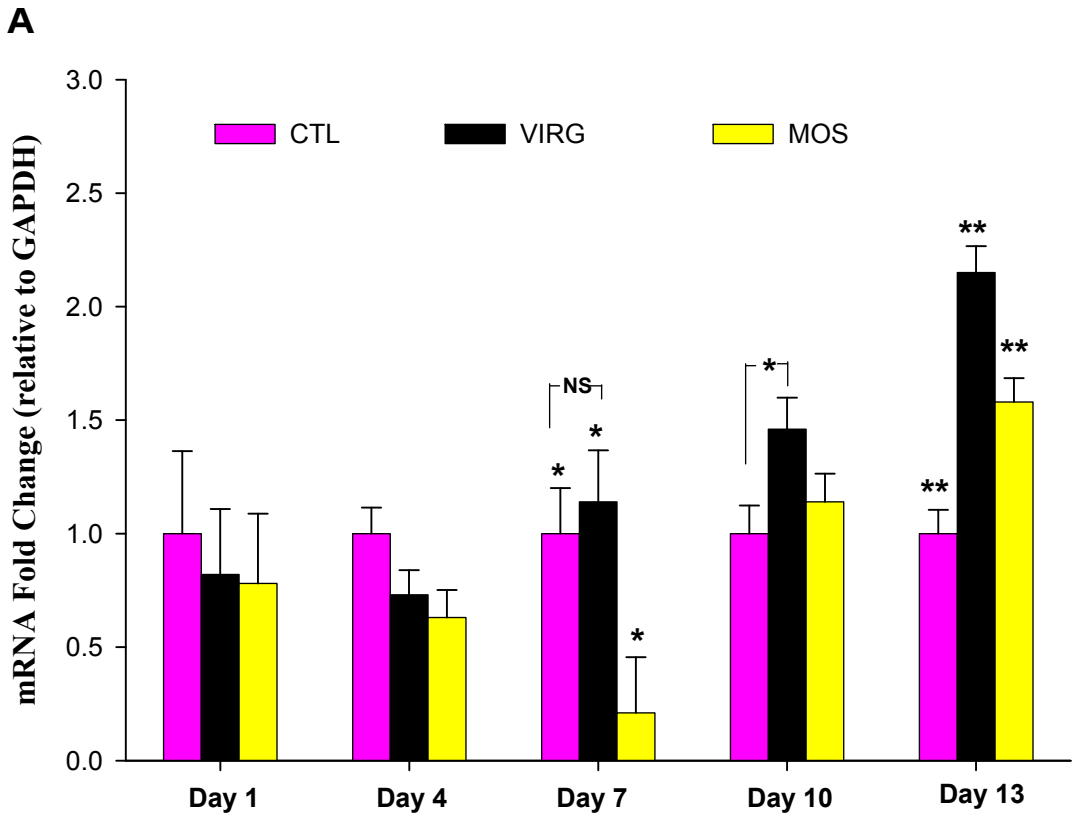
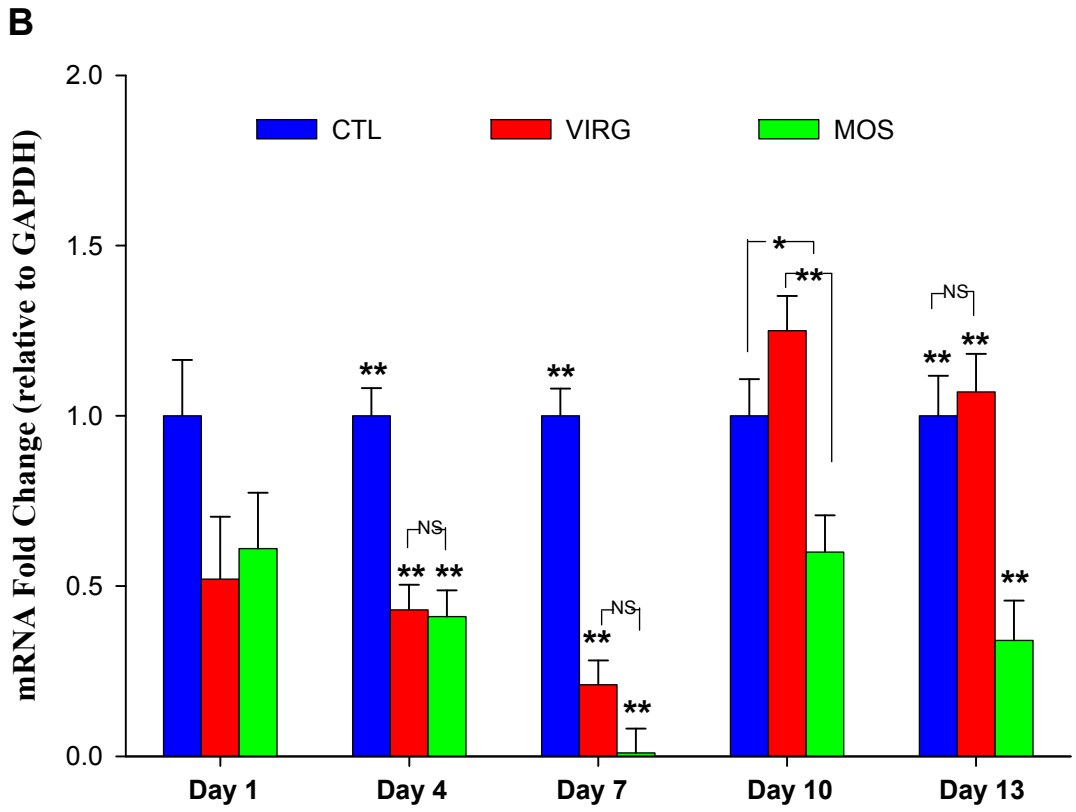

B
mRNA Fold Change (relative to GAPDH)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CTL
VIRG
MOS
Day 1
Day 4
Day 7
Day 10
Day 13

**Figure 3.11.** Expression mRNA levels of mucin (MUC)-1 measured by quantitative real time RT-qPCR at different time points after oral saline inoculation and *S.* Enteritidis infection. In non-infected chicks, *MUC 1* expression was reduced by MOS at d 7, but increased by VIRG at d 13 **(A)**. In infected chicks, MOS considerably reduced *MUC 1* expression than CTL- and VIRG-fed chicks **(B)**. Data are presented as mean ± SEM ($n$ = 6 chicks/diet). *, $P < 0.05$, **, $P < 0.01$ by Scheffe's $t$ test.




B
2.0
1.5
1.0
0.5
0.0
mRNA Fold Change (relative to GAPDH)
CTL
VIRG
MOS
**
**
**
NS
**
**
**
NS
**
*
**
**
NS
**
Day 1
Day 4
Day 7
Day 10
Day 13

## CONNECTING STATEMENT 2

In Chapter III, MOS considerably improved intestinal innate defense mechanisms against *S.* Enteritidis than VIRG, principally mediated by increased development of neutral- and acidic-mucins secreting goblet cells, which ultimately secreted higher neutral and acidic mucins into the intestinal lumen, respectively. MOS's higher mucins-mediated defense mechanisms more significantly abrogated *S.* Enteritidis damage of intestinal epithelial cells and terminated *S.* Enteritidis -induced enteric inflammation earlier than VIRG. As a confirmation of improved intestinal health due to MOS, we observed longer and healthier intestinal villi in hosts fed MOS than VIRG.

In Chapter IV, considering that *Salmonella* invasion of intestinal epithelial cells often causes systemic infection and inflammation, we were curious to know whether MOS, in contrast to VIRG, may beneficially modulate innate immunity to mitigate systemic inflammation. Moreover, we were interested to explore the effects of MOS and VIRG on liver and intestinal metabolic activities. A *Salmonella* LPS-induced systemic inflammation chicken model and microarray analysis were used to elucidate the mechanisms of MOS and VIRG actions on innate immunity and glucose metabolism during the period of late inflammation.

# CHAPTER IV. CELL WALLS OF *SACCHAROMYCES CEREVISIAE* DIFFERENTIALLY MODULATED INNATE IMMUNITY AND GLUCOSE METABOLISM DURING LATE SYSTEMIC INFLAMMATION[1]

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[1]Accepted for publication in PLoS ONE

## 4.1 ABSTRACT

***Background*:** *Salmonella* causes acute systemic inflammation by using its virulence factors to invade the intestinal epithelium. But, prolonged inflammation may provoke severe body catabolism and immunological diseases. *Salmonella* has become more life-threatening due to emergence of multiple-antibiotic resistant strains. Mannose-rich oligosaccharides (MOS) from cells walls of Saccharomyces cerevisiae have shown to bind mannose-specific lectin of Gram-negative bacteria including Salmonella, and prevent their adherence to intestinal epithelial cells. However, whether MOS may potentially mitigate systemic inflammation is not investigated yet. Moreover, molecular events underlying innate immune responses and metabolic activities during late inflammation, in presence or absence of MOS, are unknown.

***Methods and Principal Findings:*** Using a *Salmonella* LPS-induced systemic inflammation chicken model and microarray analysis, we investigated the effects of MOS and virginiamycin (VIRG, a sub-therapeutic antibiotic) on innate immunity and glucose metabolism during late inflammation. Here, we demonstrate that MOS and VIRG modulated innate immunity and metabolic genes differently. Innate immune responses were principally mediated by intestinal IL-3, but not TNF-α, IL-1 or IL-6, whereas glucose mobilization occurred through intestinal gluconeogenesis only. MOS inherently induced IL-3 expression in control hosts. Consequent to LPS challenge, IL-3 induction in VIRG hosts but not differentially expressed in MOS hosts revealed that MOS counteracted LPS's detrimental inflammatory effects. Metabolic pathways are built to elucidate the mechanisms by which VIRG host's higher energy requirements were met: including gene up-regulations for intestinal gluconeogenesis (*PEPCK*) and liver glycolysis (*ENO2*), and intriguingly liver fatty acid synthesis through *ATP citrate synthase* (*CS*) down-regulation and *ATP citrate lyase* (*ACLY*) and *malic enzyme* (*ME*) up-regulations. However, MOS host's lower energy demands were sufficiently met through TCA citrate-derived energy, as indicated by *CS* up-regulation.

***Conclusions:*** MOS terminated inflammation earlier than VIRG and reduced glucose mobilization, thus representing a novel biological strategy to alleviate *Salmonella*-induced systemic inflammation in human and animal hosts.

## 4.2 INTRODUCTION

*Salmonella* is a leading human food-borne pathogen, worldwide (CDC, 2011). The pathogen invades the intestinal epithelium by using its specialized Type III secretory systems (T3SS) to cause acute systemic or extra-intestinal inflammation (Griffin and McSorley, 2011). Indeed, the intestine is the portal of entry through which *Salmonella* triggers systemic infections. However, although life-threatening, treatment of *Salmonella*-induced systemic inflammation has received very little interests in scientific investigations. The disease is frequently caused by consumption of undercooked contaminated poultry meat and meat products (Zhao et al., 2001), which accidentally occur upon exposure to intestine-residing *Salmonella* during chicken processing.

Over decades, low doses of sub-therapeutic antibiotics such as virginiamycin (**VIRG**) have been administered daily in diets of food-producing animals, including poultry, to control intestinal pathogens. Unlike therapeutic antibiotics, sub-therapeutic antibiotics are macromolecules that exert localized bactericidal effects in the intestines only. However, according to the World Health Organization (**WHO**), such practice has debatably been associated with emergence of multiple antibiotic-resistant strains of *Salmonella* (WHO, 2011). Today, not only has *Salmonella* become more difficult to control in poultry production, but antibiotic treatment of *Salmonella*-induced gastrointestinal and systemic infections has become less successful among hospitalized patients, causing higher death rates (CDC, 2011; WHO, 2011). Therefore, the development of natural immuno-modulators that can prevent or treat *Salmonella* infections in both poultry and humans is highly desirable. Evidence exist that mannose-rich oligosaccharides (**MOS**), purified from cells walls of *Saccharomyces cerevisiae*, competitively binds mannose-specific lectin, namely FimH, of Gram-negative bacteria expressing the Type 1 fimbriae, including *Salmonella*, thereby reducing their adherence to mannose-containing glycoprotein receptors on intestinal epithelial cells in humans and chickens (Ofek and Beachey, 1978; Baurhoo et al., 2007).

Innate immunity represents the first line of immune defense against invading pathogens in both mammals and avian species. Extracellular Toll-like receptor 4 (TLR-4) of innate immune cells, including macrophages and dendritic cells, recognizes the LPS-endotoxin in outer membranes of Gram-negative bacteria (Hoshino et al., 1999). The engagement of LPS to TLR-4 triggers a cascade of transduction signaling resulting in inflammatory responses characterized by secretion of pro-inflammatory cytokines, including IL-1 and IL-6 that orchestrate pathogen clearance (Akira et al., 2006). But, innate immune responses must be regulated exceptionally tightly because high IL-1 and IL-6 levels cause fever, anorexia and bodyweight (**BW**) losses (Xie et al., 2000; Harden et al., 2008), catabolism of skeletal muscles (Flores et al., 1989; Holecek et al., 1995; Doyle et al., 2011) and adipose tissues (Feingold et al., 1992), and immunological diseases (Cook et al., 2004) in chickens, rats and humans. Therefore, it is clear that an ideal immune response would be one that can clear pathogens or antigens and be terminated soon after infection. However, despite significant advances in our understanding about inflammatory responses, molecular events of innate immunity and metabolic activities during the period of late inflammation are still not clear. Moreover, whether modulation of intestinal mucosal immunity due to dietary MOS may suppress *Salmonella*-induced systemic inflammation and reduce nutrient mobilization is unknown. The regulatory immune response between intestinal mucosal and systemic immunity is well recognized (Clarke et al., 2011; Galdeano et al., 2011). Therefore, considering the human health havoc due to sub-therapeutic antibiotic utilization among food-producing animals, this study evaluated the effects of MOS and sub-therapeutic antibiotics on innate immunity and nutrient metabolism during late *Salmonella* LPS-induced systemic inflammation.

The experiment reported herein, conducted in a chicken model, a frequently utilized biological model in nutrigenomic scientific investigations (Stern, 2005), and using chicken-specific microarrays, reveals that MOS and the VIRG antibiotic differently regulated expressions of genes involved in innate immunity and metabolic pathways during late systemic inflammation. Innate immune responses were principally mediated by intestinal IL-3, but not IL-1 or IL-6. In contrast to VIRG, MOS inherently induced

innate immune responses in non-challenged control hosts. Interestingly, however, MOS terminated innate immune responses earlier than VIRG and reduced glucose mobilization.

## 4.3. MATERIALS AND METHODS

### *Chickens, Experimental Diets and LPS Challenge*

Hatched chicks (Cobb 500 broilers) were raised in two groups (n=64/group). In each bird group (8 cages/diet), half was fed a diet containing MOS (2 kg/ton BioMos®; Alltech Inc., Nicholasville, KY) or virginiamycin (16.5 mg/kg), as described (Baurhoo et al., 2009). To induce an acute inflammatory response, group 1 hosts (n=64) were injected i.p. with 3ml of *Salmonella* Typhimurium LPS (100 mg LPS/L, Sigma-Aldrich, ON, Canada) whereas group 2 control hosts were saline-injected at 14 d of age. All animal procedures were approved by the McGill Animal Care Committee (protocol number 5399). All birds had free access to feed and water.

### *Bodyweight, Feed Intake and Body Temperature Measurements*

All non-challenged control (saline-injected) and LPS-challenged hosts were individually weighed at 0 (initial BW), 12, 24 and 48 h post-injection to determine BW gain relative to initial BW. Average feed consumption of chickens was calculated at similar time points. Body temperatures were recorded after 0, 2, 4, 6, 8, 12, 24 and 48 h of saline or LPS injection using a thermocouple rectal probe thermometer (Physitemp Instruments Inc., Clifton, NJ).

### *Liver Weights and Tissue Samples Collection*

Chickens (n = 8/diet/group) were randomly euthanized at 12, 24 and 48 h post-injection and liver weights of respective chickens were expressed relative to their final BW. At 24 h after saline and LPS injections, liver, intestine (jejunum) and skeletal muscle (breast meat) samples (n=6 /diet/group) were immediately snap frozen in liquid nitrogen, and stored at -80 ºC for later RNA extraction.

***Microarray Analysis***

After 24 h, total RNA was extracted from liver, intestine (jejunum) and skeletal muscle (breast) tissues using Trizol reagent and Purelink RNA Mini Kit (Invitrogen). Isolated total RNA was quantified on the basis of its absorption at 260 nm using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and visualized on an agarose gel to check quality. RNA was reverse transcribed into Cy3 or Cy5 aminoallyl labelled cDNA and hybridized onto chicken-specific focused oligonucleotides microarrays. The microarray platform used (accession number GPL13457) and data files (accession number GSE28959) are registered at the MIAME compliant National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) archive (http://ncbi.nlm.nih.gov/projects/geo). Briefly, 70mers chicken-specific oligonucleotides, obtained from Operon Biotechnologies Inc. (Germantown, MD), were spot printed on UltraGAPS Amino-Silane Coated Slides (Corning Inc., Acton, MA) as described (Druyan et al., 2008). Each oligonucleotide sequence (probe) was replicated 12 times per array.

***cDNA Labeling and Microarray Hybridization***

A total of 12 microarrays was used per tissue and chicken group (n=6/diet) in a 2 x 2 factorial design and complete interwoven loop arrangement (Appendix 1; Garosi et al., 2005). First, RNA was retrotranscribed into aminoallyl labelled cDNA using the ChipShot Indirect Labelling and Clean-Up System Kit (Promega, Madison, WI) and Cy3 or Cy5 fluorescent dye (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's recommendations. Reverse transcription was carried out at 42°C for 2 h, followed by RNase H digestion for 15 min at 37°C. Briefly, a reactive amine derivative of 5-(3-aminoallyl)-2`-deoxyuridine 5`-triphosphate was incorporated during reverse transcription. Subsequent to reverse transcriptase reaction, succinimidyl esters of Cy3 or Cy5 were covalently bonded to aminoallyl-labelled cDNAs. Cy-3 and Cy-5 labelled cDNA were then purified, combined and hybridized to the array for 24 h in darkness by making use of the Pronto Plus! Microarray Hybridisation Kit (Corning Inc., NY).

***Microarray Data Analysis***

Hybridized arrays were scanned twice at 65% (Cy3) and 50% (Cy5) laser power using a ScanArray GX PLUS Microarray Scanner (PerkinElmer Life and Analytical Sciences, Shelton, CT) to obtain Cy3:Cy5 intensity ratios of labelled cDNA hybridized to complementary oligonucleotide sequences on the array. Spot intensity data were extracted using the ScanAlyze Software (Standford University, Standford, CA) and analyzed using the JMP Genomics software (SAS Institute Inc., Cary, NC). Data were $\log_2$ transformed prior to normalization by using locally weighted regression and smoothing, first within array (ratio analysis) and then across arrays (Lowess normalization). Normalized data were monitored by distribution analysis of the transformed data. Finally, the normalized $\log_2$ transformed data were analyzed using a two-way ANOVA, as described (Wolfinger et al., 2001). Expression values were modeled as: $Y_{ijklm} = \mu + A_i + C_j + D_k + I_l + DI_{kl} + e_{ijklm}$, where $\mu$ represents the overall mean value, $A_i$: random effect for arrays (i = 1, 2…..12), $C_j$: main effect of Cy-dye (j = Cy-3 or Cy-5), $D_k$: main effect of diet (k = MOS or VIRG), $I_l$: main effect of injection (l = saline or LPS), and $DI_{kl}$: interaction effect between diet and injection, and $e_{ijklm}$: random error. Mean intensities were tested using the false discovery rate (FDR) multiple comparison *t* test and differentially expressed genes were declared at $P < 0.05$. Finally, for each pairwise comparison, significantly different genes were filtered based on their mean intensity values to determine up- or down-regulated genes due to diet, injection and diet*injection.

***Real-time quantitative PCR Analysis***

Real-time quantitative PCR (RT-qPCR) was used for validation of differential gene expressions observed in microarrays. Total RNA was retrotranscribed into cDNA by using 1 µg total RNA and iScript cDNA Synthesis Kit (Bio-Rad, ON, Canada), following the manufacturer's instructions. RT-qPCR was performed using the Bio-Rad CFX384 RT-qPCR Detection System, SsoFast Evagreen Supermix (Bio-Rad) and primer-set sequences (Table 4.1). RT-qPCR reactions were performed at 95 $^{o}$C for 5 min, followed by 39 cycles of 95 $^{o}$C for 15 s and 60 $^{o}$C for 30 s. A melting curve program was included at the end of each RT-qPCR to verify presence of a unique product. Relative intestinal (*PEPCK*) and liver (*ACLY*, *ME*, *CS* and *FAS*) gene expression levels were normalized to

*GAPDH* or *beta-2 microglobulin*, respectively. Samples were analyzed in technical duplicates, and differential gene expressions were determined using the comparative standard curve method.

***Liver Metabolites Measurements***

Liver citrate and pyruvate levels were measured by specific enzymatic reactions using the Citrate and Pyruvate Assay Kits (BioVision, CA, USA), following the manufacturer's instructions with few modifications. Briefly, 0.5 g of liver tissues were homogenized completely by sonication in 700 µL of respective buffer solutions and then centrifuged at 15,000 g for 10mins to remove cell debris. After the supernatant was deproteinized using the Deproteinizing Sample Preparation Kit (BioVision), a 100 µL sample volume was used for analysis. Reaction mix was prepared without buffer dilution.

***Statistical Analysis***

Except for microarray data, all data were analyzed as a two-way ANOVA and a 2 x 2 factorial arrangement to determine the main effects of diet and injection, and their interaction effects by using the MIXED procedure of SAS (SAS Institute, 2003). For bodyweight, liver weight and body temperature data, a Nested Model Design was also employed with cages nested within diet*injection, as follows: $Y_{ijkl} = \mu + Diet_i + Injection_j + Diet_i*Injection_j + Cage_{ijk} + e_{ijkl}$, where µ represents the overall mean value, $Diet_{i:}$ fixed effect of diet (i = MOS or VIRG), $Injection_j$: fixed effect of injection (j = saline or LPS), $Diet_i$ * $Injection_j$: interaction effect between diet and injection, $Cage_{ijk}$: random effect of cage nested within diet*injection (k = 1,2,...8), and $e_{ijkl}$: random error. Differences among treatment means were tested using Scheffe's *t* test and statistical significance declared at $P < 0.05$.

## 4.4 RESULTS

LPS induced pathological symptoms, reduced feed intake and BW, and increased liver size in MOS- and VIRG-fed chickens. However, to make a clear distinction between the effects of MOS and VIRG among hosts within the physiological (non-challenged controls) and inflammatory (LPS-challenged) conditions, we relied on microarray results that detailed the coordinately regulated biological mechanisms underlying innate immunity and nutrient metabolism. Tissue-specific RNA extracted from the intestines, liver and skeletal muscles at 24 h post-LPS challenge were analyzed using chicken-specific microarrays. All data files from this experiment have been deposited into the MIAME compliant Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/projects/geo (accession no. GSE28959).

### *LPS Induced Clinical Symptoms in Antibiotic- and MOS-Fed Chickens*

To provoke a systemic inflammatory response, chickens were injected i.p. with a sublethal dose of LPS (3ml of 100 mg LPS/L). The reaction to LPS is a well-characterized innate immune response (Lopez-Bojorquez et al., 2004). Whether hosts were fed MOS or VIRG, LPS caused symptoms of drowsiness, lethargy, ruffled feathers, moderate diarrhea, starvation and withdrawal from water at 6 h post-LPS injection, thus demonstrating success of our challenge model. These clinical signs of innate immune response were most evident around 8 h after LPS injection. Clinical and behavioral changes due to LPS injection have previously been reported in different animal species, including chickens (Xie et al., 2000). No signs of inflammatory responses were observed among non-challenged control hosts. To assess pathological changes further, body temperatures were measured at 0, 2, 4, 6, 8, 12, 24 and 48 h post-LPS injection. Body temperature was similar among all hosts prior (0 h) to LPS challenge (Figure 4.1*A*). But, LPS markedly increased body temperatures of MOS- and VIRG-fed hosts after 4 h of LPS challenge, and this effect persisted through 24 h (Figure 4.1*A* and *B*). After 48 h, however, all hosts regained their homeostatic state after termination of inflammatory responses (Figure 4.1*A* and *B*).

***LPS Reduced Feed Intake and Bodyweight Gain, and Increased Liver Weight in Antibiotic- and MOSc-Fed Chickens***

LPS markedly reduced feed intake at 12 h (Figure 4.2*A*), but not at 24 and 48 h, post-challenge despite VIRG and MOS supplementations. In addition, LPS's effects in reducing feed consumption and inducing profuse diarrhea at 12 h led to severe loss in BW gain (growth) (Figure 4.3*A* and *C*). However, depressed BW gain persisted through 24 and 48 h post-LPS challenge. On the other hand, increased liver weights were observed at 12 and 24 h among LPS-challenged hosts (Figure 4.4*A* and *C*). But, the more profound increase in liver weights that occurred after 12 h rather than 24 h (+0.66 % vs +0.40 % of BW) indicated that higher liver metabolic activities might have occurred at 12 h post-LPS challenge responsive to host's higher energy demands. Moreover, given the similarities in body temperatures, feed intake, and liver weights between LPS-challenge and non-challenge control hosts, it is clear that inflammatory responses were abated at 48 h post-LPS challenge. All of these findings indicated that inflammatory responses were more intense earlier than 24 h post-LPS challenge. Therefore, based on the similarity in feed intake, depressed growth and increased liver weights, but persistence in elevated body temperatures, we concluded that 24 h post-LPS treatment corresponded to late inflammation.

***Main Effects: LPS Increased Innate Immune Responses***

Our results revealed that LPS significantly increased innate immune responses in intestinal tissues (Table 4.2) characterized by IL-3 up-regulation, and down-regulation of the gene for zinc finger CCCH-type containing 15 (ZC3H15), which negatively regulates macrophage activation (Liang et al., 2008). Additionally, the gene coding for the signal transducer and activator of transcription 2 (STAT2), a signaling pathway that augments macrophage's phagocytic activities against pathogenic bacteria by inducing inducible nitric oxide synthase (iNOS) and lysosomal enzymes (Decker et al., 2002), was induced in the liver. But, down-regulation of genes were observed for TLR 2 precursor (TLR2-2) that also recognizes and binds LPS (Kirschning et al., 1998), gallinacin-1 alpha (Gal-1), a wide spectrum antimicrobial peptide functionally equivalent to human β-defensins (Hasenstein and Lamont, 2007), and putative CXCR1, an IL-8 receptor that binds the IL-

8 chemoattractant expressed by macrophages, monocytes and neutrophils. Indeed, gene expression for 2′-5′-oligoadenylate synthetase A (OAS*A), which is involved in viral RNA cleavage inhibiting IFN-γ-mediated viral infections (Behera et al., 2002), was intestinally down-regulated in the absence of viral infection. However, differential immune gene expressions as observed in the intestines and liver were not detected in muscle tissues.

***Main Effects: LPS Increased Glucose Mobilization and Modified Fatty Acid Metabolism***

During acute inflammation, starvation alters host's carbohydrate, protein and fat metabolisms that are orchestrated by synergistically-operated pro-inflammatory cytokines, to meet the body's energy requirements. Subsequent to rapid glucose absorption and oxidative utilization, blood glucose level is maintained by liver glycogenolysis, catabolism of skeletal muscles that generates and mobilizes amino acids for liver gluconeogenesis (Flores et al., 1989; Holecek et al., 1995), and catabolism of adipose tissues that triggers liver lipolysis (Feingold et al., 1992).

However, here, we observed that glucose mobilization occurred differently during late inflammation than during immunologically non-challenged conditions. LPS significantly increased intestinal gluconeogenesis by increasing gene expression for phosphoenolpyruvate carboxykinase 1 (PEPCK), a key gluconeogenic enzyme that synthesizes phosphoenolpyruvate from oxaloacetate (Table 4.2). Evidently, intestinal glycolysis and cholesterol synthesis were repressed as indicated by gene down-regulations for phosphopyruvate hydratase (ENO2), which converts 2-phosphoglycerate into phosphoenolpyruvate, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which catalyzes the rate-limiting step in the mevalonate pathway converting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into mevalonate (Goldstein and Brown, 1990), respectively. The high rates of glucose synthesis and glucose trafficking across intestinal epithelial cells enhanced intestinal contractions as indicated by *myosin heavy polypeptide 7 cardiac muscle* (*MYH7*) and *myosin light polypeptide 9 regulatory* (*MYL9*) up-regulations (van Rooji et al., 2009). Contrary to expectations, gluconeogenesis did not occur in the liver. Intriguingly, LPS up-regulated genes coding for malic enzyme (ME), a

key enzyme involved in fatty acid synthesis catalyzing the oxidative decarboxylation of malate to pyruvate, NADPH and carbon dioxide (Hillgartner and Charron, 1998), and enoyl-CoA hydratase (EHHADH), a key enzyme involved in β-oxidation of fatty acids (Waterson and Hill, 1972). Therefore, metabolic energy to support late inflammation was derived mostly from increased fatty acid *de-novo* biosynthesis followed by its catabolism. Unexpectedly, LPS up-regulated gene for 5′-AMP-activated protein kinase gamma 2 (PRKAG2), a low energy sensor that represses acetyl-CoA carboxylase and HMG-CoA reductase to inhibit fatty acid and cholesterol biosynthesis, respectively (Hardie, 2003). In muscle tissues, down-regulation of the gene for 6-phosphofructokinase (PFKM), a regulatory enzyme that converts fructose-6-phosphate into fructose-1,6-biphosphate, suppressed the glycolytic pathway, whereas *atrial natriuretic factor precursor* (*NPPA*) up-regulation caused vasodilatation to increase blood flow (Nsenga et al., 2009). Taken together, all these findings reveal a clear disassociation between glucose mobilization and the biosynthesis and β-oxidation of fatty acids. These discrepancies could be attributed to MOS's immune-stimulatory effects among non-challenged control hosts, as discussed later.

***MOS Increased Innate Immune Responses in Non-Challenged Control Chickens***

Because MOS increases *Salmonella* and *E. coli* clearance of the intestines (Ofek and Beachey, 1978), we thought that MOS may suppress innate immune responses under inflammatory conditions rather than VIRG thereby reducing catabolism of body reserves. However, MOS significantly increased innate immune responses among non-challenged control hosts than VIRG. For instance, several innate immune genes were induced by MOS in the intestines, including *IL-3*, *TLR-3*, *TLR2-2* and *Gal-1* (Table 4.3). Moreover MOS up-regulated liver genes for putative CXCR1, IL 13 receptor alpha 2 (IL13RA2), which is a specific IL-13 receptor, and CD3 glycoprotein (CD3), which increases T cell activation and signaling of humoral immunity (Letourneur and Klausner, 1992). But, *STAT2* was down-regulated.

### *MOS Increased Glucose Mobilization and Metabolism in Non-Challenged Control Chickens*

Augmentation in immune responses by MOS among non-challenged control hosts significantly increased glucose mobilization and metabolism. MOS down-regulated the gene for heat shock protein 1 (HSPE1), which folds and activates newly synthesized linear proteins into functional 3-D proteins (Ostermann et al., 1989); thus, deactivated proteins were increasingly utilized in intestinal gluconeogenesis as mediated by *PEPCK* up-regulation (Table 4.3). Increased intestinal contractions, in part due to increased glucose absorption across epithelial cells, were mediated by *MYH7* up-regulation but not *MYH11* and *MYL9*. Correspondingly, *NPPA* was up-regulated to ascertain high glucose flux into the liver via the hepatic portal vein. Therefore, to increase the glucose-uptake capacity of liver cells, MOS induced the gene for deiodinase type 2 (DIO2), reported to reduce insulin resistance by increasing intracellular triiodothyronine (T3) levels (Chidakel et al., 2005). Furthermore, down-regulation of *potassium voltage-gated channel shaker-related subfamily 3* (*KCNA3*) significantly increased insulin-stimulated glucose uptake through the GLUT4 glucose transporter, as reported by (Xu et al., 2004). As evidenced by *ENO2* up-regulation, high liver glucose increased liver glycolytic activities for energy generation. *ME* and *EHHADH* down-regulations repressed liver fatty acid biosynthesis and β-oxidation, respectively. Therefore, elevated intestinal gluconeogenesis and liver glycolysis were sufficient to meet the host's energy demands.

### *LPS Mediated Innate Immune Responses Differently within MOS and Antibiotic Chicken Groups*

So far, we reported inherent immune-stimulatory effects due to independent LPS and MOS treatments. Therefore, simultaneous administration of these treatments was expected to intensify the inflammatory responses. Interestingly, however, our results revealed that MOS counteracted the detrimental effects of LPS on innate immunity. Although we observed intestinal down-regulation of the gene for IL-10, an anti-inflammatory cytokine that causes negative-feedback on secretions of pro-inflammatory cytokines (Taylor et al., 2006), neither *IL-3*, as observed due to LPS (Table 4.2) treatment alone, nor any other pro-inflammatory cytokines were induced (Table 4.4). To further

support MOS's effect in alleviating inflammatory responses, we observed down-regulations of *TLR2-2*, *TLR-3* and *OAS***A*, and *IL13RA2* and *CD3* in intestinal and liver tissues, respectively. In MOS-fed hosts, innate immune responses after LPS challenge were principally mediated by *ZC3H15* down-regulation that enhanced macrophage activation. In VIRG hosts, however, intestinal *IL-3* and *TLR-3* up-regulations (Table 4.5) revealed higher LPS-induced inflammatory responses.

***LPS Mobilized Energy Differently within MOS and Antibiotic Chicken Groups***

To further evidence that MOS reduced innate immune responses, here we report that LPS failed to induce gluconeogenesis or any other major nutrient mobilization processes among MOS-fed hosts (Table 4.4). Previously, however, we observed increased intestinal gluconeogenesis due to LPS in the absence of MOS (Table 4.2). These results, together with reduced liver glucose uptake mediated by *DIO2* down-regulation and *KCNA3* up-regulation, led us to believe that liver glucose levels were sufficiently high to meet the host's energy demands. However, reduced glycolytic activities, due to *ENO2* down-regulation, demonstrated that liver glucose levels were abnormally low. Intriguingly, despite reduced glycolysis, we observed up-regulation of the gene for ATP citrate synthase (CS), which catalyzes citrate synthesis from acetyl Co-A and oxaloactetate. Given that citrate is the key regulatory substrate of the TCA cycle, our results indicated that host's energy demands were likely met mainly via the TCA cycle. We also observed that glucose utilization for energy caused down-regulation of *UDP glucose pyrophosphorylase 2* (*UGP2*), which reduces liver glycogen synthesis (Ferrer et al., 2003), whereas *PRKAG2* up-regulation repressed fatty acid and cholesterol biosynthesis. Finally, MOS down-regulated the gene for α-actin 2 in intestinal smooth muscles (ACTA2) that reduced intestinal contractions (Guo et al., 2007), whereas *MYH11* and *MYL9* were up-regulated. Down-regulation of *HMGCR* repressed intestinal cholesterol synthesis.

Contradictorily, despite VIRG supplementation, LPS challenge profoundly increased gluconeogenesis, as indicated by *PEPCK* up-regulation in the intestines (Table 4.5). Evidently, *MYH7* was up-regulated whereas *ENO2* and *HMGCR* were down-regulated to suppress intestinal glycolysis and cholesterol synthesis, respectively. But,

high glucose influx into the liver increased glycolytic activities through *ENO2* up-regulation. Therefore, glucose metabolites were most increasingly utilized in TCA cycle for energy generation. Surprisingly, *CS* was down-regulated and citrate was instead utilized in fatty acid biosynthesis, as demonstrated by increased expressions of genes for ATP citrate lyase (ACLY), which catalyzes citrate cleavage into acetyl Co-A and oxaloacetate (Cheema-Dhadli et al., 1973; Pearce et al., 1998), ME and fatty acid synthetase (FAS; as shown in Figure 4.5*A*). *PFKM* down-regulation and *NPPA* up-regulation occurred in muscle tissues of both MOS- and VIRG-fed hosts.

***MOS Increased Innate Immune Responses in LPS-Challenged Chickens***

In comparisons to LPS-challenged hosts fed VIRG, the additive immune stimulatory effects of LPS and MOS significantly increased innate immune responses as demonstrated by intestinal *IL-3* up-regulation, and down-regulation of *IL-10* and *ZC3H15* (Table 4.6). But, *TLR-3*, the corresponding receptor to IL-3, was repressed in the intestines. *STAT2* was up-regulated in the liver.

***MOS Mobilized Energy Differently than Antibiotic in LPS-Challenged Chickens***

Despite increased immune responses in LPS-challenged hosts fed MOS, these chickens faced no detrimental nutrient mobilization processes when compared to LPS-injected hosts fed VIRG. Intestinal gluconeogenesis did not occur although newly-synthesized proteins were deactivated by *HSPE1* down-regulation (Table 4.6). Again, our results about *CS* up-regulation demonstrate that energy was essentially derived from increasingly synthesized citrate in the liver. Under the effects of LPS, MOS repressed glycogen synthesis by down-regulating *UGP2*; glycolysis by down-regulating *ENO2*; fatty acids biosynthesis by down-regulating *ACLY*, *ME* and *FAS* (Figure 4.9); and cholesterol biosynthesis by up-regulating *PRKAG2*. Liver *KCNA3* was up-regulated in the absence of high glucose influx. Indeed, these findings are very similar to those observed when comparing MOS-fed hosts in the LPS-challenged and non-challenged control groups.

***Real-time quantitative PCR and Liver Metabolite Measurements***

To confirm our microarray data, we performed quantitative RT-qPCR analysis on three differentially expressed genes, and measured concentrations of specific liver metabolites. Figure 4.5 and Tables 4.3, 4.5 and 4.6 show that *PEPCK*, *ACLY* and *ME* expression patterns correlated strongly with microarray results. Moreover liver citrate and pyruvate levels were in agreement with gene expression results (Figure 4.6). *FAS*, not present on the array utilized in this study, expression (Figure 4.5) was determined by RT-qPCR. All RT-qPCR efficiency (E) values were in between 93 to 100 %.

## 4.5 DISCUSSION

Molecular events underlying late inflammation and subsequently nutrient mobilization, in response to pathogens or antigens, are still not clear. Interestingly, at 24 h post-LPS challenge, microarray results revealed that innate immune responses were principally mediated by IL-3, a pro-inflammatory cytokine that has received little scientific investigations, together with other innate immune mediators (Table 4.2). Few studies reported IL-3 as playing key roles in linking innate and adaptive immunity. IL-3 is critical for the differentiation of monocytes into dendritic cells, and contributes in proliferation and survival of dendritic cells (Ebner et al., 2002); dendritic cells are involved in Th cell response. While *IL-1* and *IL-6* were consistently up-regulated during intense inflammatory responses in poultry (Kogut et al., 2005) and mice (Park et al., 2010), here we report that these pro-inflammatory cytokines were not differentially expressed during late inflammation. These results evidenced that inflammation is a time-dependent biological immune reaction, regulated by different immune mediators. Most interestingly, our results revealed that dietary MOS modulated innate immune responses and nutrient metabolisms differently than VIRG.

Our finding that MOS increased immune responses of non-challenged control hosts, but here principally mediated by intestinal IL-3, is consistent with published data (Gao et al., 2008; Gomez-Verduzco et al., 2009; Szymanska-Czerwinska et al., 2009), thereby revealing its inherent immune-stimulatory properties. Although the mechanism by which MOS inherently stimulates immunity is unclear, it may be associated with the antigenic properties of yeast cell walls. In contrast, VIRG did not confer such immune

stimulatory effects because antibiotics lack antigenic properties. During immune stimulation, an energy-demanding biological process, and the consequential reduction in feed intake, the host's metabolic activities were coordinately regulated to increase energy availability for metabolism. Liver gluconeogenesis, involving muscle catabolism, usually occurs during intense inflammation and starvation (Flores et al., 1989). However, here, we observed that gluconeogenesis occurred only in the intestines. Additionally, during the process of glucose synthesis, the preferential utilization of amino acids from ingested feed spared skeletal muscle catabolism. Glucose, mobilized to the liver, was then rapidly metabolized via increased glycolytic activities to meet host's elevated energy demands during the inflammatory response (summarized in Figure 4.7). Nevertheless, as previously reported (Baurhoo et al., 2007; 2009), MOS's immune-stimulatory effects did not profoundly mobilize glucose and had no detrimental effects on feed intake or growth.

Both LPS and MOS triggered elevated innate immune responses and glucose mobilization. However, our results that none of the pro-inflammatory cytokines were up-regulated due to continual MOS intake followed by LPS challenge (Table 4.4) revealed that MOS counteracted LPS's detrimental effects on immunity. We also observed that energy demands of hosts fed MOS were sufficiently met by increased TCA cycle-derived energy. Contrastingly, VIRG failed to counteract or reduce LPS's inflammatory effects, as indicated by increased *IL-3* expression (Table 4.5). The higher energy demands of VIRG hosts necessitated glucose mobilization through intestinal gluconeogenesis and increased liver glycolytic activities. Based on these findings, we conclude that dietary MOS helped terminate LPS-induced inflammation earlier than VIRG. This beneficial effect of MOS may be explained by its inherent immune-stimulatory properties that caused mild immune stimulation, thereby 'arming' the body's defense mechanisms to rapidly and efficiently clear the endotoxin.

However, increased TCA activities surprisingly occurred among hosts fed MOS despite their reduced glycolytic activities. Although fatty acid and cholesterol synthesis genes are coordinately down-regulated during LPS-triggered systemic inflammation (Yoo and Desiderio, 2003), we observed increased liver *de novo* fatty acid synthesis among hosts fed VIRG despite increased intestinal gluconeogenesis and liver glycolysis. Generally, fatty acid synthesis, which converts excess energy into energy reserves, occurs

only when dietary carbohydrate intake exceeds immediate energy requirements. But, we observed a reduction in feed intake due to LPS challenge. Because inflammation is a dynamic biological immune reaction, molecular events at 24 h post-LPS challenge are a consequence of earlier immunological events. To help explain these apparently conflicting observations, we will briefly consider nutrient mobilization during intense inflammation. Whereas glycogenolysis and gluconeogenesis are frequently reported during intense inflammation, significant mobilization and catabolism of glucose may have significantly increased liver glucose and its glucose metabolites, including acetyl Co-A, pyruvate and citrate, levels in both MOS- and VIRG-fed hosts. In the absence of innate immune responses at 24 h post-LPS challenge, increased activity of CS, a key enzyme involved in TCA cycle, revealed that energy demands of MOS-fed hosts were mainly derived from liver glucose/glucose metabolites that accumulated earlier. Evidently, intestinal gluconeogenesis and liver glycolysis were not necessary and repressed (summarized in Figure 4.8).

In VIRG-fed hosts, however, elevated innate immune responses at 24 h post-LPS challenge required higher energy. Insufficient energy derived from accumulated liver glucose/glucose metabolites necessitated further glucose mobilization and catabolism through intestinal gluconeogenesis and liver glycolysis, respectively. However, as evidenced by *ACLY* up-regulation, exceptionally high liver citrate levels, which accumulated during intense inflammation, triggered *CS* down-regulation. Citrate is well recognized as a potent allosteric negative-feedback inhibitor of CS activity and plays a crucial role in liver metabolic activities. Evidently, to rapidly catabolize and deplete the accumulated liver cytosolic citrate after its efflux from the mitochondria where it is synthesized, (i) *ACLY* up-regulation generated high acetyl Co-A levels, (ii) *ME* up-regulation increased liver NADPH concentrations, (iii) whereas *FAS* up-regulation synthesized palmitate, the major fatty acid that ultimately yields long fatty acid chains, from acetyl Co-A, NADPH and malonyl Co-A, which is synthesized from acetyl Co-A by acetyl Co-A carboxylase (summarized in Figure 4.9). ACLY, ME and FAS are key lipogenic enzymes that convert liver cytoplasmic citrate into fatty acids. The preferential acetyl Co-A and NADPH utilization in *de novo* fatty acid biosynthesis mediated by *ACLY*, *ME* and *FAS* up-regulations is consistent with published reports (Morral et al.,

2007). In previously fasted and refed rats and chickens, increased liver lipogenesis was also mediated by *ACLY*, *ME* and *FAS* up-regulations (Kochan et al., 1997; Wang et al., 2009). While increased glucose mobilization and decreased fatty acid synthesis have frequently been reported during intense inflammation (Yoo and Desiderio, 2003; Ceciliani et al., 2002), here we report that the liver rapidly metabolized citrate into fatty acids to restore its citrate homoeostatic level during late inflammation in addition to glucose mobilization for body energy requirements.

In agreement with O'Hea and Leveille (O'Hea and Leveille, 1968), we observed that livers in chickens derived most of the NADPH required for fatty acid synthesis from the ME reaction, whereas livers in rats obtained about 65 % of NADPH from the pentose phosphate pathway (Flatt and Ball, 1966). Collectively, these findings indicate that significantly more glucose was mobilized from the intestine and more glucose metabolites accumulated in the liver of VIRG hosts during the period of intense inflammation than MOS-fed hosts, and that VIRG failed to terminate innate immune responses earlier. But, when challenged with LPS, we observed an elevation in innate immune responses, principally mediated by intestinal IL-3, among hosts fed MOS than VIRG. Although these results were not surprising considering the additive immune-stimulatory effects of MOS and LPS, no major nutrient mobilization processes occurred among LPS-challenged hosts fed MOS (summarized in Figure 4.10). TCA-derived energy from high liver glucose and glucose metabolites which accumulated earlier than 24 h of LPS challenge was sufficient to meet energy demands of the hosts fed MOS.

Livers and kidneys are well-recognized gluconeogenic organs in humans and mice (Stumvoll et al., 1998; Eid et al., 2006). Whereas the intestine is equivocally reported as a gluconeogenic organ in mice (Croset et al., 2001; Martin et al., 2007), we are among the first to demonstrate that the chicken small intestine, but not skeletal muscles, is also a gluconeogenic organ that was regulated by *PEPCK*. We have discussed the increased intestinal gluconeogenesis at 24 h post-LPS challenge. Given that MOS and VIRG are not absorbed across the intestinal epithelium, these macromolecules produce localized effects in the intestines. Therefore, all our findings evidenced cross talks between intestinal mucosal immunity and systemic immunity. This is the first study demonstrating that

MOS can beneficially modulate innate immunity and nutrient metabolism during late systemic inflammation.

In summary, late inflammation was principally modulated by IL-3. In contrast to antibiotics like VIRG, MOS elicited several beneficial responses: (i) terminated *Salmonella* LPS-induced systemic inflammation earlier, presumably due to its inherent intestinal innate immune-stimulatory properties; and (ii) reduced the magnitude of glucose mobilization. Therefore, this study potentiates the use of natural immuno-modulators, such as a MOS, to attenuate *Salmonella*-induced systemic inflammation both among human and animal hosts, and without posing the risk of antibiotic-resistance development.

## 4.6 ACKNOWLEDGEMENTS

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**Table 4.1.** Primers-set sequences used to analyze gene expression by quantitative PCR.

| Gene | Forward primer[1] | Reverse primer[1] | Amplicon length (bp) | PubMed Accession No. |
|---|---|---|---|---|
| PEPCK | CTGCTGGTGTGCCTCTTGTA | TTCCCTTGGCTGTCTTTCC | 259 | NM_205471 |
| ACLY | GGCGTGAATGAACTGGCTAAC | TAGTCTTGGCATAGTCATAGGTCTGTTG | 79 | NM_001030540 |
| ME | TGCCAGCATTACGGTTTAGC | CCATTCCATAACAGCCAAGGTC | 175 | NM_204303 |
| FAS | TGAAGGACCTTATCGCATTGC | GCATGGGAAGCATTTTGTTGT | 96 | NM_205155 |
| GAPDH | TGCCATCACAGCCACACAGAAG | ACTTTCCCCACAGCCTTAGCAG | 123 | NM_204305 |
| Beta-2 microglobulin | AAGGAGCCGCAGGTCTA | CTTGCTCTTTGCCGTCATAC | 151 | Z48921 |

[1] Sequences are indicated from 5′ end to 3′ end of oligonucleotides.

**Table 4.2.** Genes identified as differentially expressed due to main LPS effects[1]

| Gene | Gene symbol | Gene ID | Fold change | *P*-value | Gene regulation by LPS[2] |
|---|---|---|---|---|---|
| Intestine | | | | | |
| Immune response | | | | | |
| Interleukin 3 | IL-3 | 474356 | 1.03 | 0.00328 | + |
| Toll-like receptor 2 precursor | TLR2-2 | 769014 | 0.98 | 0.00596 | - |
| Zinc finger CCCH-type containing 15 | ZC3H15 | 423992 | 0.98 | 0.03575 | - |
| 2'-5' oligoadenylate synthetase A | OAS*A | 395908 | 0.96 | 0.02579 | - |
| Gallinacin-1 alpha | Gal-1 | 395841 | 0.99 | 0.09106 | - |
| Metabolism | | | | | |
| Phosphoenolpyruvate carboxykinase 1 | PEPCK | 396458 | 1.05 | 0.00497 | + |
| Phosphopyruvate hydratase | ENO2 | 395689 | 0.94 | 0.00018 | - |
| 3-hydroxy-3-methylglutaryl-CoA Reductase | HMGCR | 395145 | 0.95 | 0.00004 | - |
| Others | | | | | |
| Myosin, heavy polypeptide 7, cardiac Muscle | MYH7 | 395350 | 1.03 | 0.01938 | + |
| Actin alpha 2, smooth muscle, aorta | ACTA2 | 423787 | 0.98 | 0.04217 | - |
| Myosin light polypeptide 9 regulatory | MYL9 | 396215 | 1.04 | 0.02469 | + |
| Liver | | | | | |
| Immune response | | | | | |
| Signal transducer and activator of transcription 2 | STAT2 | 6773 | 1.04 | 0.00018 | + |
| Putative CXCR1 isoform I and II (IL-8 receptor) | CXCR1 | 430652 | 0.97 | 0.02406 | - |
| Metabolism | | | | | |
| Enoyl-CoA hydratase | EHHADH | 424877 | 1.05 | 0.00017 | + |
| Protein phosphatase 1 | PPP1R8 | 419564 | 1.03 | 0.00818 | + |
| Malic enzyme 1 | ME | 374189 | 1.03 | 0.03684 | + |
| 5'-AMP-activated protein kinase gamma-2 | PRKAG2 | 420435 | 1.03 | 0.00190 | + |
| Others | | | | | |
| Deiodinase Type 2 | DIO2 | 373903 | 0.95 | 0.00043 | - |
| Iroquois homeobox protein 1 | IRX1 | 374185 | 1.05 | 0.00136 | + |
| Potassium voltage-gated channel shaker-related subfamily No 3 | KCNA3 | 404303 | 1.06 | 0.00008 | + |
| Breast | | | | | |
| Metabolism | | | | | |
| 6-phosphofructokinase (PFK-1) | PFKM | 374064 | 0.95 | 0.00001 | - |
| Others | | | | | |
| Atrial natriuretic factor precursor | NPPA | 395765 | 1.03 | 0.00005 | + |

[1]Pooled LPS-treated birds: prebiotic + antibiotic groups. [2]+: up-regulated genes by LPS; -: down-regulated genes by LPS.

**Table 4.3.** Genes identified as differentially expressed due to MOS in non-challenged control birds[1]

| Gene | Gene symbol | Gene ID | Fold change | *P*-value | Gene regulation by MOS[2] |
|---|---|---|---|---|---|
| Intestine | | | | | |
| Immune response | | | | | |
| Interleukin 3 | IL-3 | 474356 | 1.05 | 2.98E-05 | + |
| Toll-like receptor 3 | TLR3 | 422720 | 1.04 | 0.00687 | + |
| Toll-like receptor 2 precursor | TLR2-2 | 769014 | 1.03 | 0.00984 | + |
| 2'-5' oligoadenylate synthetase A | OAS*A | 395908 | 1.15 | 0.00000 | + |
| Gallinacin-1 alpha | Gal-1 | 395841 | 1.03 | 0.00145 | + |
| Metabolism | | | | | |
| Phosphoenolpyruvate carboxykinase 1 | PEPCK | 396458 | 1.11 | 0.00007 | + |
| Others | | | | | |
| Myosin, heavy polypeptide 7, cardiac Muscle | MYH7 | 395350 | 1.04 | 0.01797 | + |
| Secreted protein acidic cysteine-rich | SPARC | 386571 | 0.94 | 0.00023 | - |
| Myosin, heavy chain 11, smooth muscle | MYH11 | 396211 | 0.87 | 0.00000 | - |
| Heat shock 10kDa protein 1 | HSPE1 | 395948 | 0.90 | 0.00057 | - |
| Myosin light polypeptide 9 regulatory | MYL9 | 396215 | 0.93 | 0.00117 | - |
| Atrial natriuretic factor precursor | NPPA | 395765 | 1.05 | 0.00005 | + |
| Iron regulatory protein 1 | IRP1 | 373916 | 0.92 | 0.00000 | - |
| Liver | | | | | |
| Immune response | | | | | |
| Interleukin 13 Receptor Alpha 2 | IL13RA2 | 422219 | 1.05 | 0.00002 | + |
| Signal transducer and activator of transcription 2 | STAT2 | 6773 | 0.97 | 0.02042 | - |
| Putative CXCR1 isoform I and II (IL-8 receptor) | CXCR1 | 430652 | 1.07 | 0.00018 | + |
| CD3 glycoprotein | CD3D | 396518 | 1.04 | 0.00010 | + |
| Metabolism | | | | | |
| Enoyl-CoA hydratase | EHHADH | 424877 | 0.95 | 0.01192 | - |
| Protein phosphatase 1 | PPP1R8 | 419564 | 0.97 | 0.04035 | - |
| Phosphopyruvate hydratase | ENO2 | 395689 | 1.05 | 0.00362 | + |
| Malic enzyme 1 | ME | 374189 | 0.96 | 0.05583 | - |
| Others | | | | | |
| Deiodinase Type 2 | DIO2 | 373903 | 1.05 | 0.02708 | + |
| Potassium voltage-gated channel shaker-related subfamily No 3 | KCNA3 | 404303 | 0.95 | 0.00649 | - |
| Breast | | | | | |
| Others | | | | | |
| NK2 transcription factor related locus 5 | NKX2-5 | 396073 | 1.18 | 0.00000 | + |
| Desmin | DES | 395906 | 0.89 | 0.00011 | - |

[1]Control birds: MOS (prebiotic)-fed birds v/s VIRG (antibiotic)-fed birds; [2]+: up-regulated genes by MOS; -: down-regulated genes by MOS.

**Table 4.4.** Genes identified as differentially expressed due to LPS within MOS-fed birds[1]

| Gene | Gene symbol | Gene ID | Fold change | *P*-value | Gene regulation by LPS[2] |
|---|---|---|---|---|---|
| Intestine | | | | | |
| Immune response | | | | | |
| Interleukin 10 | IL-10 | 428264 | 0.97 | 0.00343 | - |
| Toll-like receptor 3 | TLR3 | 422720 | 0.95 | 0.00024 | - |
| Toll-like receptor 2 precursor | TLR2 -2 | 769014 | 0.96 | 0.00036 | - |
| Zinc finger CCCH-type containing 15 | ZC3H15 | 423992 | 0.95 | 0.00012 | - |
| 2'-5'-oligoadenylate synthetase A | OAS*A | 395908 | 0.82 | 0.00000 | - |
| Gallinacin-1 alpha | Gal-1 | 395841 | 0.96 | 0.00013 | - |
| Metabolism | | | | | |
| Phosphopyruvate hydratase | ENO2 | 395689 | 0.93 | 0.00434 | - |
| 3-hydroxy-3-methylglutaryl-CoA reductase | HMGCR | 395145 | 0.95 | 0.00338 | - |
| Others | | | | | |
| Secreted protein acidic cysteine-rich | SPARC | 386571 | 1.04 | 0.01425 | + |
| Myosin, heavy chain 11, smooth muscle | MYH11 | 396211 | 1.10 | 0.00015 | + |
| Actin alpha 2, smooth muscle, aorta | ACTA2 | 423787 | 0.94 | 0.00006 | - |
| Myosin light polypeptide 9 regulatory | MYL9 | 396215 | 1.10 | 0.00006 | + |
| Iron regulatory protein 1 | IRP1 | 373916 | 1.04 | 0.01284 | + |
| Liver | | | | | |
| Immune response | | | | | |
| Interleukin 13 Receptor Alpha 2 | IL13RA2 | 422219 | 0.97 | 0.00431 | - |
| Signal transducer and activator of transcription 2 | STAT2 | 6773 | 1.07 | 0.00000 | + |
| Putative CXCR1 isoform I and II (IL-8 receptor) | CXCR1 | 430652 | 0.93 | 0.00007 | - |
| CD3 glycoprotein | CD3D | 396518 | 0.98 | 0.04308 | - |
| Metabolism | | | | | |
| UDP Glucose Pyrophosphorylase 2 | UGP2 | 373900 | 1.00 | 0.00492 | - |
| Enoyl-CoA hydratase | EHHADH | 424877 | 1.10 | 0.00000 | + |
| Protein phosphatase 1 | PPP1R8 | 419564 | 1.06 | 0.00017 | + |
| Phosphopyruvate hydratase | ENO2 | 395689 | 0.91 | 0.00000 | - |
| ATP citrate synthase | CS | 1431 | 1.07 | 0.00092 | + |
| 5'-AMP-activated protein kinase gamma-2 | PRKAG2 | 420435 | 1.07 | 0.00001 | + |
| Others | | | | | |
| Deiodinase Type 2 | DIO2 | 373903 | 0.94 | 0.00134 | - |
| Iroquois homeobox protein 1 | IRX1 | 374185 | 1.09 | 0.00001 | + |
| Endothelial PAS domain protein 1 | EPAS1 | 395596 | 1.10 | 0.00033 | + |
| Potassium voltage-gated channel shaker-related subfamily No 3 | KCNA3 | 404303 | 1.13 | 0.00000 | + |
| Breast | | | | | |
| Metabolism | | | | | |
| 6-phosphofructokinase (PFK-1) | PFKM | 374064 | 0.95 | 0.00058 | - |
| Others | | | | | |
| Atrial natriuretic factor precursor | NPPA | 395765 | 1.03 | 0.00417 | + |

[1]Birds fed prebiotic (MOS): LPS-injected v/s saline-injected; [2]+: up-regulated genes by LPS; -: down-regulated genes by LPS.

**Table 4.5.** Genes identified as differentially expressed due to LPS within antibiotic-fed birds[1]

| Gene | Gene symbol | Gene ID | Fold change | *P*-value | Gene regulation by LPS[2] |
|---|---|---|---|---|---|
| Intestine | | | | | |
| Immune response | | | | | |
| Interleukin 3 | IL-3 | 474356 | 1.04 | 0.00404 | + |
| Toll-like receptor 3 | TLR3 | 422720 | 1.03 | 0.01264 | + |
| 2'-5'-oligoadenylate synthetase A | OAS*A | 395908 | 1.13 | 0.00000 | + |
| Metabolism | | | | | |
| Phosphoenolpyruvate carboxykinase 1 | PEPCK | 396458 | 1.12 | 0.00002 | + |
| Phosphopyruvate hydratase | ENO2 | 395689 | 0.94 | 0.01255 | - |
| 3-hydroxy-3-methylglutaryl-CoA reductase | HMGCR | 395145 | 0.95 | 0.00369 | - |
| Others | | | | | |
| Myosin, heavy polypeptide 7, cardiac muscle | MYH7 | 395350 | 1.03 | 0.04746 | + |
| Myosin, heavy chain 11, smooth muscle | MYH11 | 396211 | 0.92 | 0.00094 | - |
| Atrial natriuretic factor precursor | NPPA | 395765 | 1.03 | 0.00679 | + |
| Iron regulatory protein 1 | IRP1 | 373916 | 0.96 | 0.02641 | - |
| Liver | | | | | |
| Metabolism | | | | | |
| ATP citrate lyase | ACLY | 395373 | 1.04 | 0.04584 | + |
| Phosphopyruvate hydratase | ENO2 | 395689 | 1.05 | 0.00869 | + |
| Malic enzyme 1 | ME | 374189 | 1.06 | 0.01385 | + |
| ATP citrate synthase | CS | 1431 | 0.96 | 0.03024 | - |
| Breast | | | | | |
| Metabolism | | | | | |
| 6-phosphofructokinase (PFK-1) | PFKM | 374064 | 0.95 | 0.00246 | - |
| Others | | | | | |
| Atrial natriuretic factor precursor | NPPA | 395765 | 1.03 | 0.00416 | + |

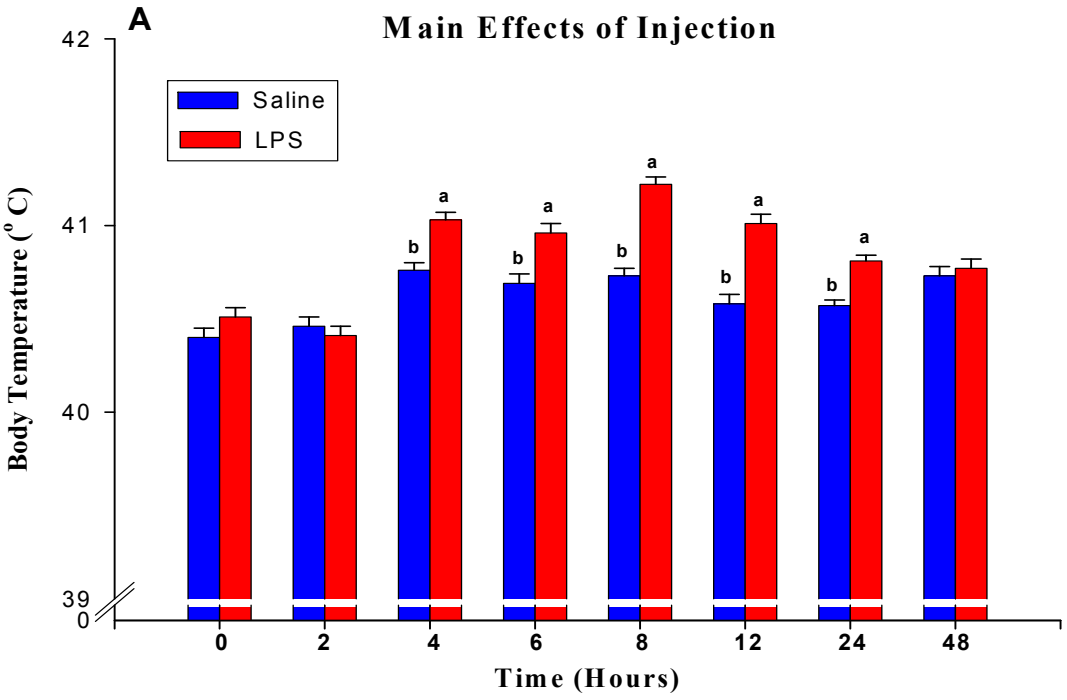
[1]Birds fed antibiotic (VIRG): LPS-injected v/s saline-injected; [2]+: up-regulated genes by LPS; -: down-regulated genes by LPS.

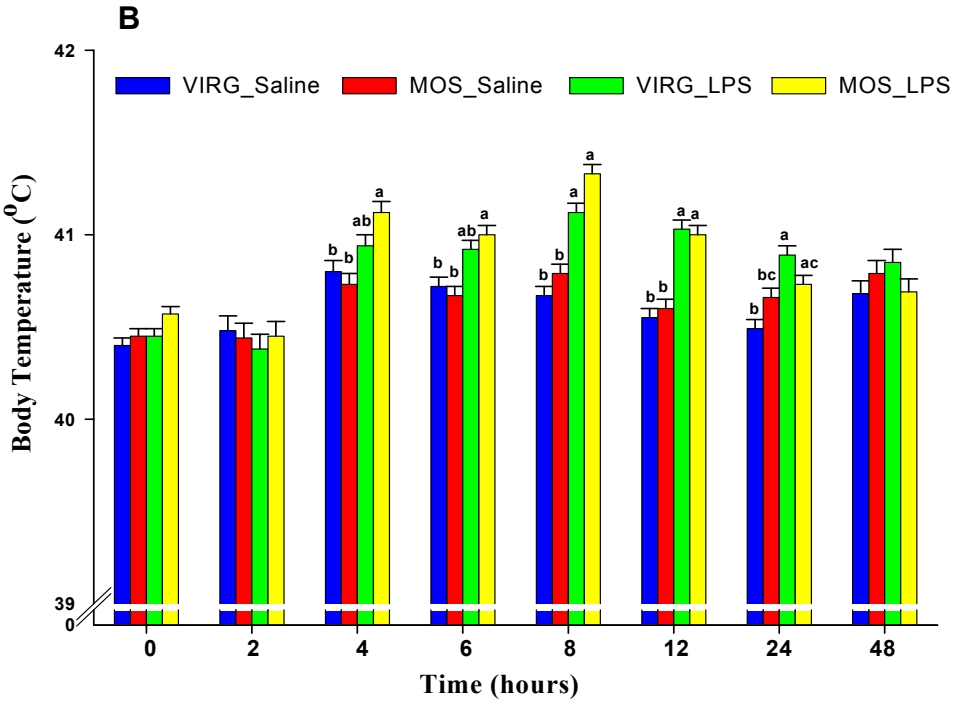
**Table 4.6.** Genes identified as differentially expressed due to LPS between MOS- and antibiotic-fed birds[1]

| Gene | Gene symbol | Gene ID | Fold change | *P*-value | Gene regulation by MOS[2] |
|---|---|---|---|---|---|
| Intestine | | | | | |
| Immune response | | | | | |
| Interleukin 3 | IL-3 | 474356 | 1.03 | 0.00871 | + |
| Interleukin 10 | IL-10 | 428264 | 0.96 | 0.00028 | - |
| Toll-like receptor 3 | TLR3 | 422720 | 0.96 | 0.00053 | - |
| Zinc finger CCCH-type containing 15 | ZC3H15 | 423992 | 0.96 | 0.00100 | - |
| 2'-5'-oligoadenylate synthetase A | OAS*A | 395908 | 0.84 | 0.00000 | - |
| Gallinacin-1 alpha | Gal-1 | 395841 | 0.98 | 0.03227 | - |
| Others | | | | | |
| Actin alpha 2 smooth muscle aorta | ACTA2 | 423787 | 0.94 | 0.00017 | - |
| Heat shock 10kDa protein 1 | HSPE1 | 395948 | 0.92 | 0.00756 | - |
| Liver | | | | | |
| Immune response | | | | | |
| Signal transducer and activator of transcription 2 | STAT2 | 6773 | 1.03 | 0.02089 | + |
| Metabolism | | | | | |
| UDP Glucose Pyrophosphorylase 2 | UGP2 | 373900 | 0.92 | 0.00009 | - |
| ATP citrate lyase | ACLY | 395373 | 0.93 | 0.00017 | - |
| Phosphopyruvate hydratase | ENO2 | 395689 | 0.91 | 0.00000 | - |
| Malic enzyme 1 | ME1 | 374189 | 0.92 | 0.00012 | - |
| ATP citrate synthase | CS | 1431 | 1.11 | 0.00000 | + |
| 5'-AMP-activated protein kinase gamma-2 | PRKAG 2 | 420435 | 1.05 | 0.00290 | + |
| Others | | | | | |
| Iroquois homeobox protein 1 | IRX1 | 374185 | 1.06 | 0.00268 | + |
| Endothelial PAS domain protein 1 | EPAS1 | 395596 | 1.09 | 0.00158 | + |
| Potassium voltage-gated channel shaker-related subfamily No 3 | KCNA3 | 404303 | 1.07 | 0.00045 | + |
| Breast | | | | | |
| Others | | | | | |
| NK2 transcription factor related locus 5 | NKX2-5 | 396073 | 1.14 | 0.00000 | + |

[1]LPS treated birds: MOS-fed birds v/s antibiotic-fed birds; [2]+: up-regulated genes by MOS; -: down-regulated genes by MOS.

**Figure 4.1.** Innate immune-stimulatory effects of LPS caused elevation in body temperatures. Antibiotic (VIRG)- and prebiotic (MOS)-fed birds were injected i.p. with LPS and body temperatures recorded at different stages of inflammation. At 0, 2 and 48 h after injection, body temperatures were not different between LPS- and saline-injected birds (*A*), or between VIRG- and MOS-fed birds within the LPS or saline group, respectively (*B*), or between birds fed VIRG and MOS (*C*). However, in comparison with control (saline) birds, LPS significantly increased ($P < 0.05$) body temperatures at 4, 6, 8, 12 and 24 h post-injection (*A*). But, such increase in body temperatures were not observed between VIRG and MOS birds within the LPS or saline group, respectively (*B*), or between VIRG- and MOS-fed birds irrespective of injection type (*C*). Results are expressed as mean ± SEM. Supercripts: (a,b) denote statistical differences among treatment means at a particular time point, $P < 0.05$, Scheffe's multi-comparison *t*-test.

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VIRG_Saline
MOS_Saline
VIRG_LPS
MOS_LPS
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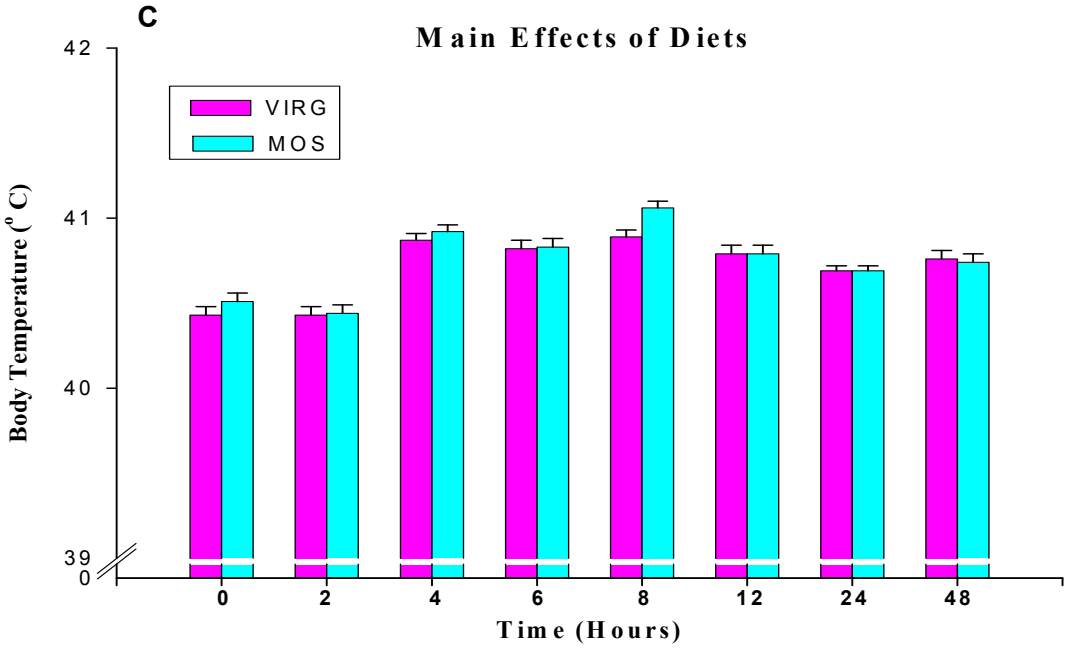
41
40
39
0
0
2
4
6
8
12
24
48
Time (hours)


C
Main Effects of Diets
VIRG
MOS
Body Temperature (° C)
42
41
40
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24
48
Time (Hours)

**Figure 4.2.** The effects of LPS injected i.p. on feed intake in antibiotic- (VIRG) and prebiotic- (MOS) fed birds at different stages of inflammation. LPS significantly reduced feed intake ($P < 0.05$) at 12 h post-injection (*A*). However, feed intake was not different between birds fed the VIRG and MOS diet (*B*), or between VIRG and MOS birds within the LPS or saline group, respectively (*C*). At 24 and 48 h post-injection, feed intake was not different between LPS and control birds (*A*), birds fed the VIRG and MOS diet (*B*), or between VIRG and MOS birds within the LPS or saline group, respectively (*C*). Results are expressed as mean ± SEM. Supercripts: (a,b) denote statistical differences among treatment means at a particular time, $P < 0.05$, Scheffe's multi-comparison *t*-test.

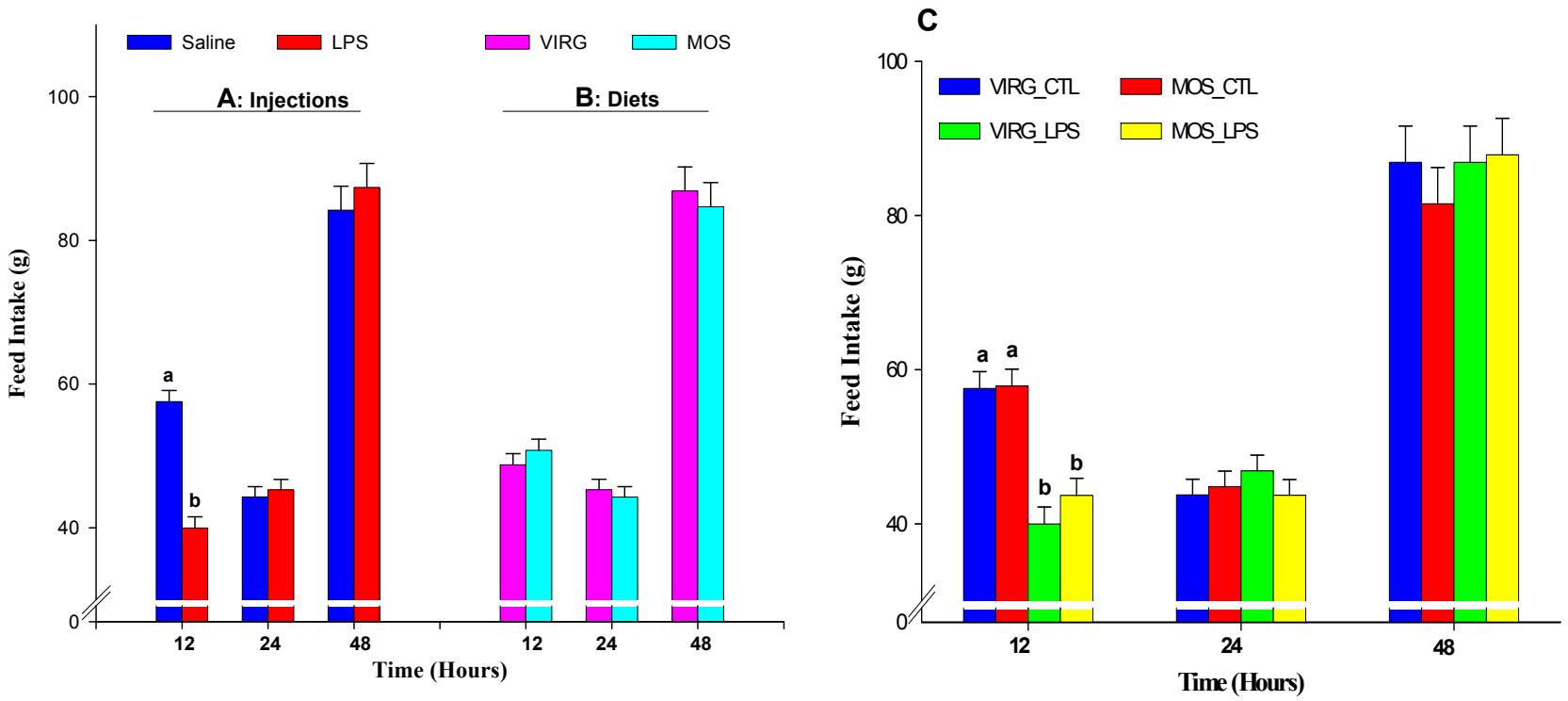


**Figure 4.3.** The effects of LPS injected i.p. on bodyweight (BW) gain in antibiotic- (VIRG) and prebiotic- (MOS) fed birds at different stages of inflammation. LPS significantly reduced BW ($P < 0.05$) after 12, 24 and 48 h of injection (*A*). In contrast to MOS-fed birds, those fed VIRG grew faster ($P < 0.05$) at 48 h only (*B*). But, BW gain was not different between VIRG and MOS birds within the LPS or saline group, respectively (*C*). Results are expressed as mean ± SEM. Supercripts: (a,b) denote statistical differences among treatment means at a particular time point, $P < 0.05$, Scheffe's multi-comparison *t*-test.

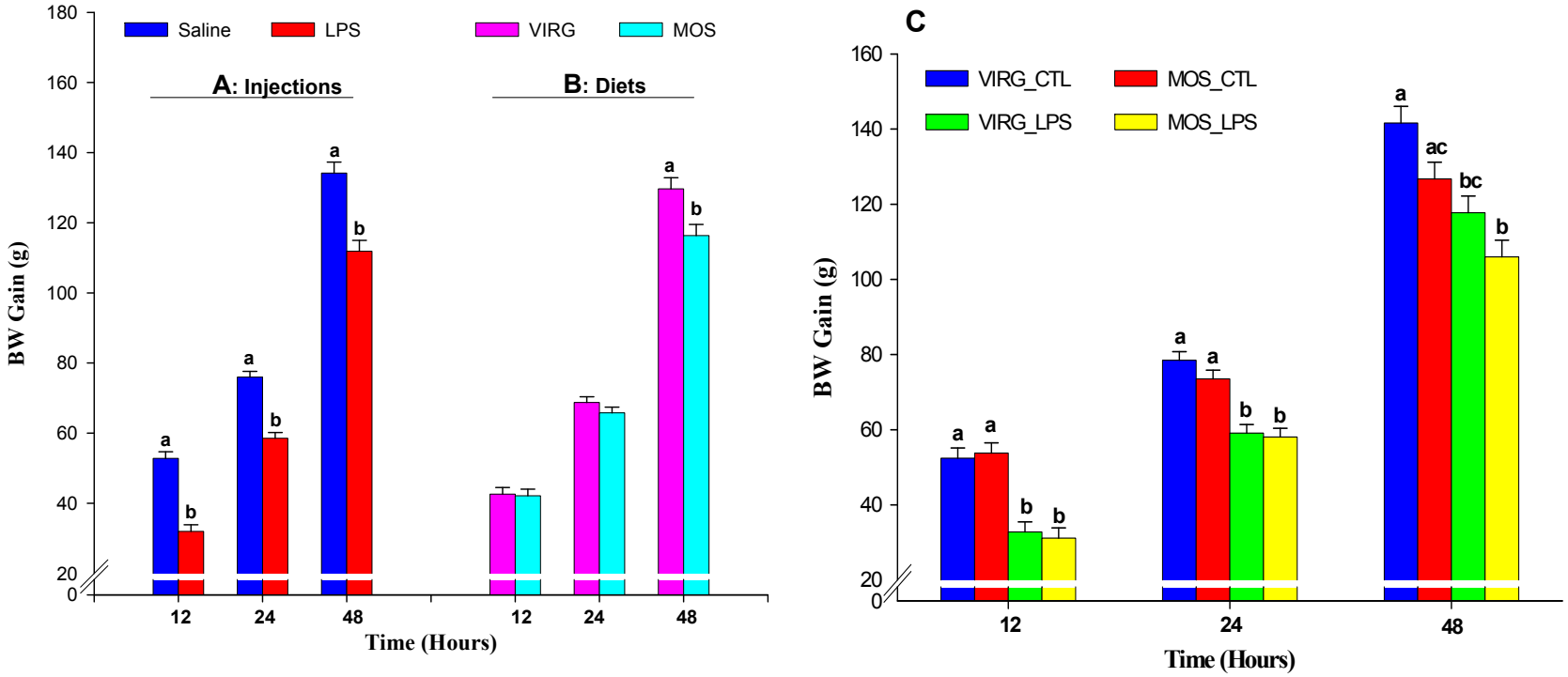


**Figure 4.4.** The effects of LPS injected i.p. on liver weights of antibiotic- (VIRG) and prebiotic- (MOS) fed birds at different stages of inflammation. LPS significantly increased ($P < 0.05$) liver weights at 12 and 24 h post-injection, but not after 48 h (*A*). However, at all times, liver weights were not different between birds fed the VIRG and MOS diet (*B*), or between VIRG and MOS birds within the LPS or saline group, respectively (*C*). Results are expressed as mean ± SEM. Supercripts: (a,b) denote statistical differences among treatment means at a particular time, $P < 0.05$, Scheffe's multi-comparison *t*-test.

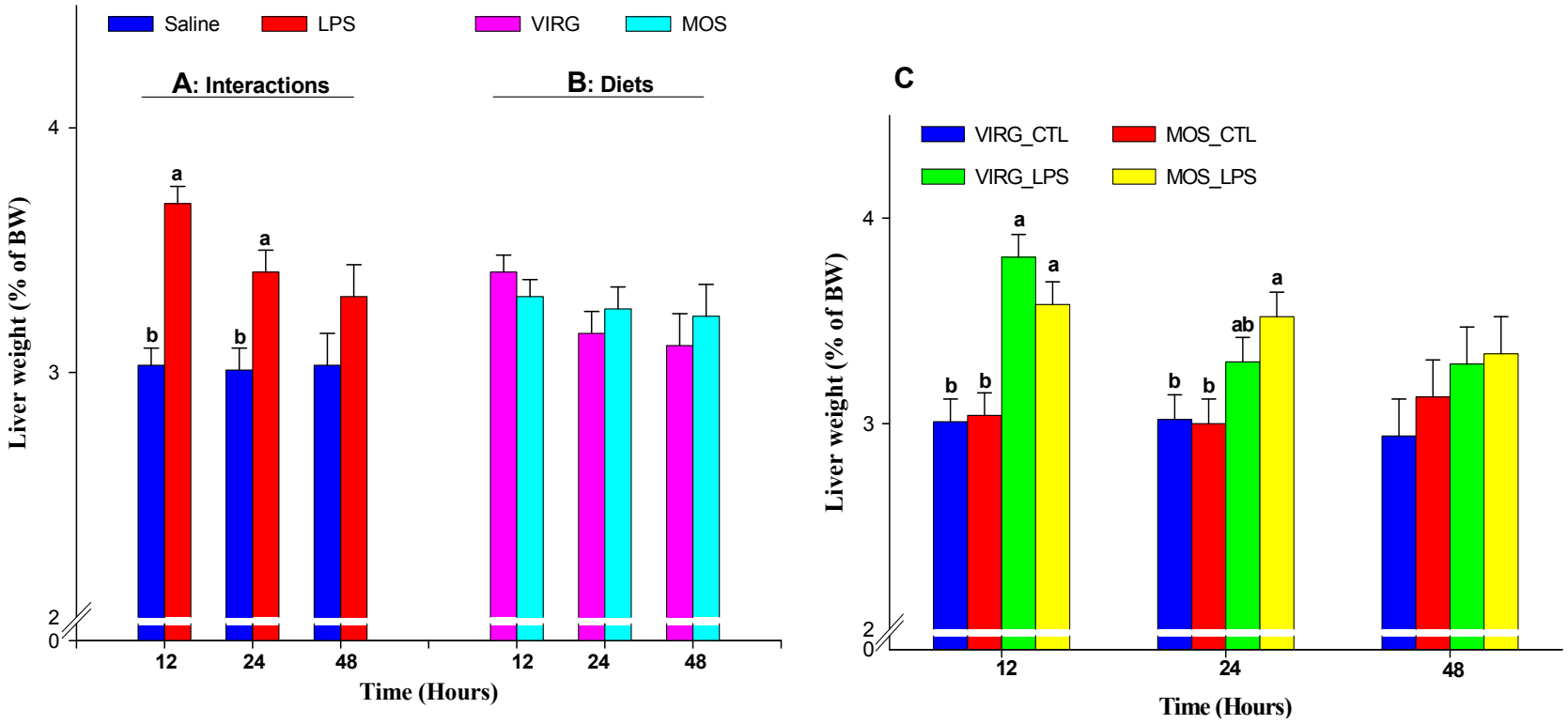


**Figure 4.5.** RT-qPCR validation of microarray data. LPS up-regulated liver *ACLY*, *ME* and *FAS* among VIRG-fed hosts (*A*). But, LPS down-regulated these genes in MOS- than VIRG-fed host (*B*). Intestinal *PEPCK* was up-regulated among control hosts fed MOS (*C*), and by LPS among VIRG-fed hosts (*D*). Data are presented as mean ± SEM ($n = 6$). *, $P < 0.01$ by Scheffe's *t* test.

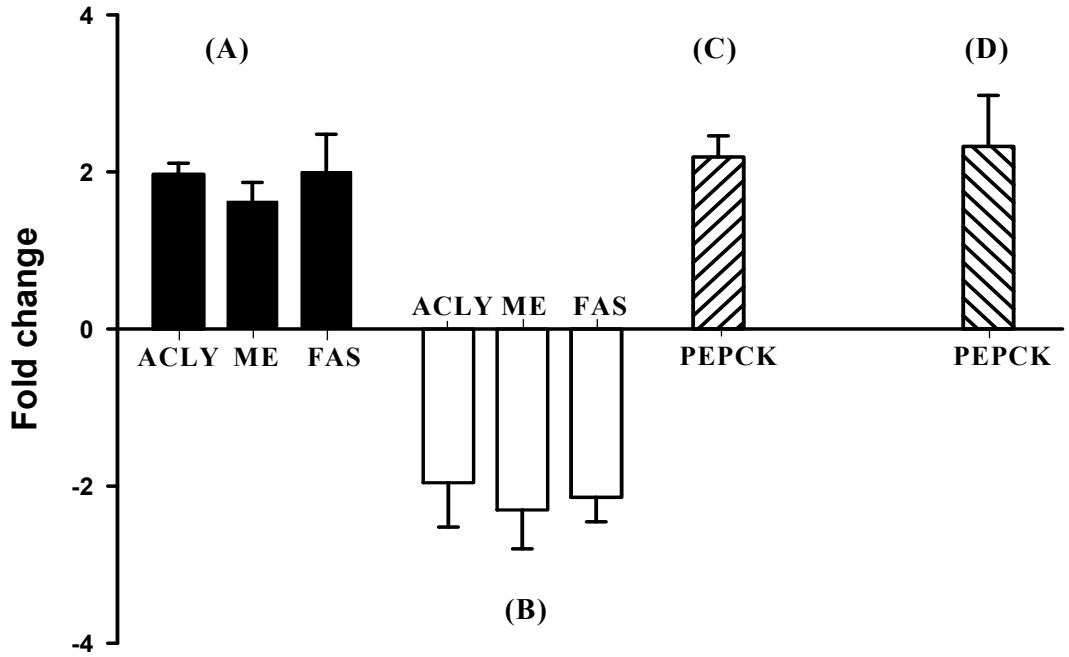


**Figure 4.6.** Concentrations of liver metabolites. Among VIRG-fed hosts, LPS reduced liver citrate, but increased pyruvate levels (*A*). However, higher citrate and lower pyruvate levels were observed in liver of LPS-challenged hosts fed MOS than VIRG (*B*). Data are presented as mean ± SEM ($n = 6$). *, $P < 0.05$, **, $P < 0.01$ by Scheffe's *t* test.

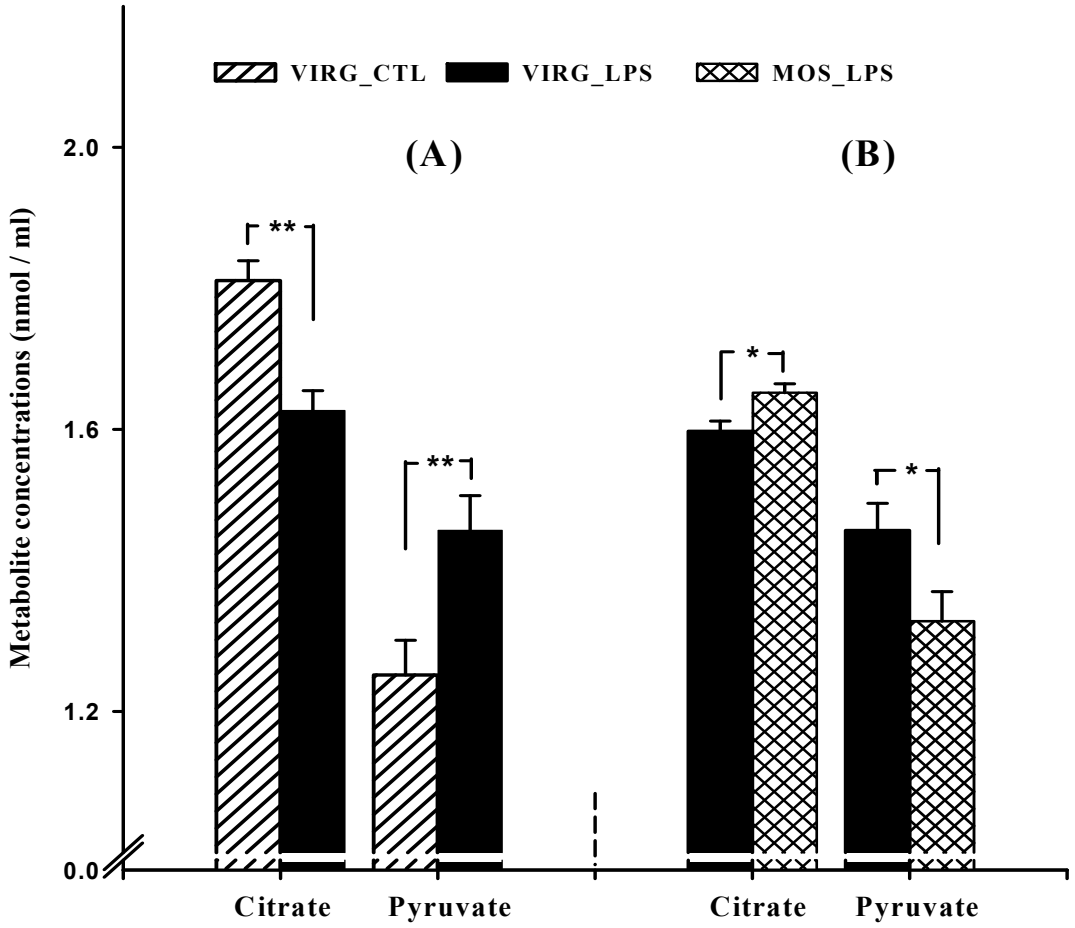


**Figure 4.7.** Schematic illustration of the effects of MOS versus VIRG on glucose metabolism in control hosts. (i) MOS increased intestinal gluconeogenesis by up-regulating *PEPCK*; (ii) the high glucose influx into the liver was rapidly metabolized by glycolysis as mediated by *ENO2* up-regulation; (iii) TCA-derived energy from glycolytic substrates, down-regulated *ME* and *EHHADH* which reduced fatty acid synthesis and β-oxidation, respectively.

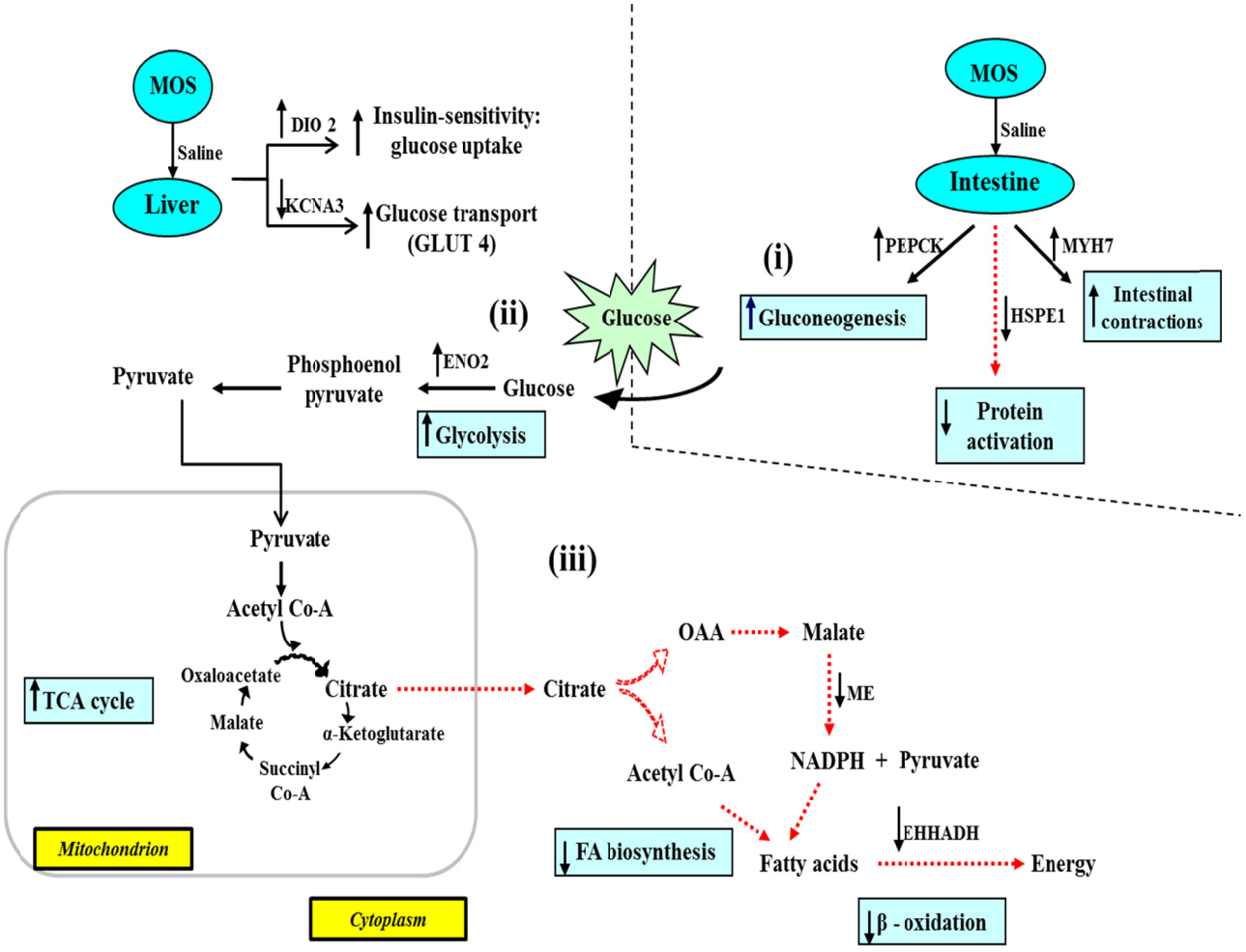


**Figure 4.8.** Schematic illustration of MOS effects on glucose metabolism between control and LPS-challenged hosts. (i) LPS triggered no major intestinal metabolic activities; (ii) in absence of glucose mobilization, liver glucose uptake and transport were repressed by *DIO2* down-regulation and *KCNA3* up-regulation, respectively; (iii) glycolysis and glycogen synthesis were coordinately reduced by *ENO2* and *UGP2* down-regulation, respectively; (iv) *CS* up-regulation increased TCA-derived energy from high liver pyruvate; (v) *PRKAG2* up-regulation inhibited fatty acid and cholesterol biosynthesis.

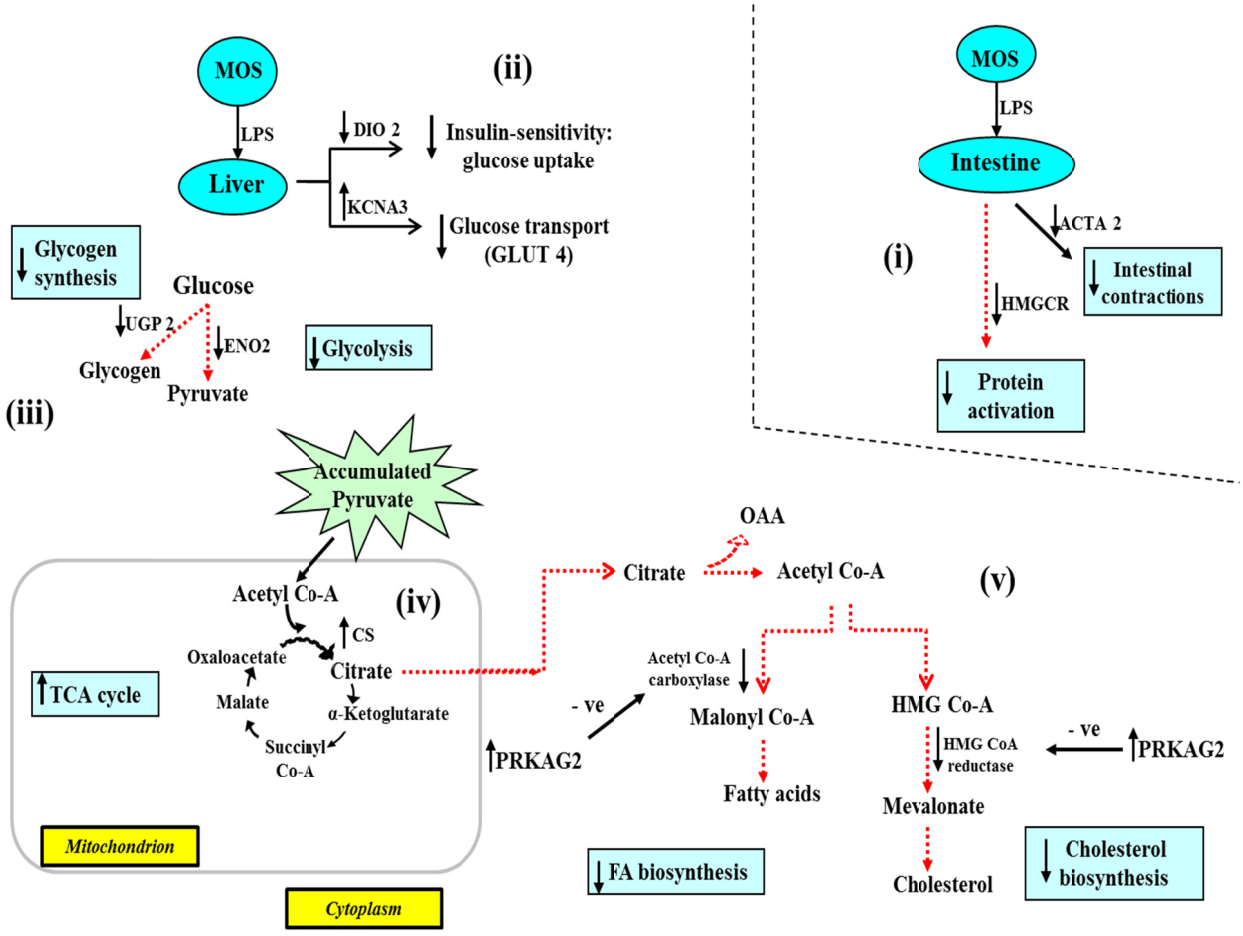


**Figure 4.9.** Schematic illustration of VIRG effects on glucose metabolism between control and LPS-challenged hosts. (i) LPS increased intestinal gluconeogenesis by up-regulating *PEPCK*; (ii) mobilized glucose increased liver glycolytic activities through *ENO2* up-regulation; (iii) *CS* down-regulation reduced utilization of glycolytic substrates by the TCA cycle for energy; (iv) *ACLY*, *ME* and *FAS* up-regulations increased liver fatty acid biosynthesis from high liver citrate.

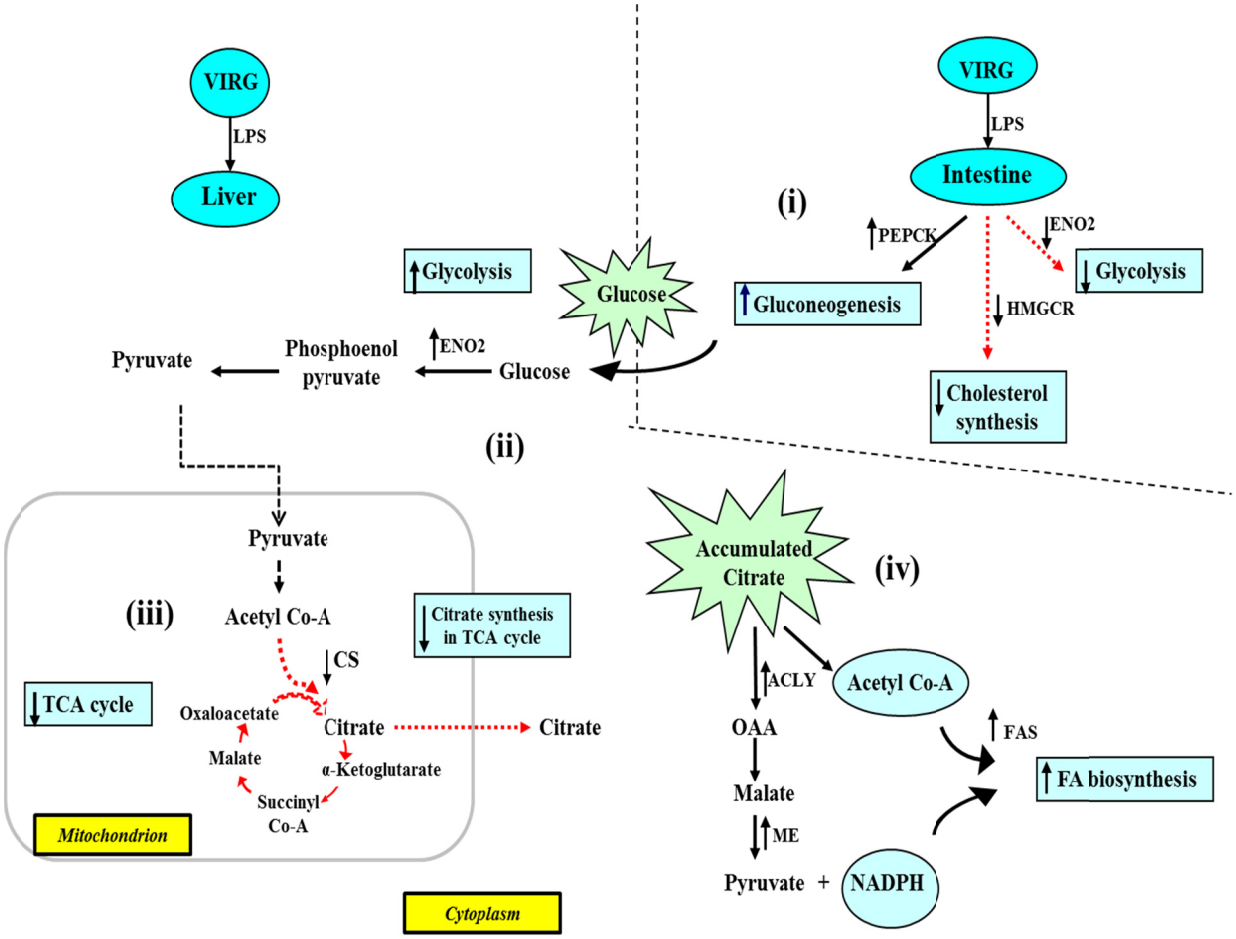
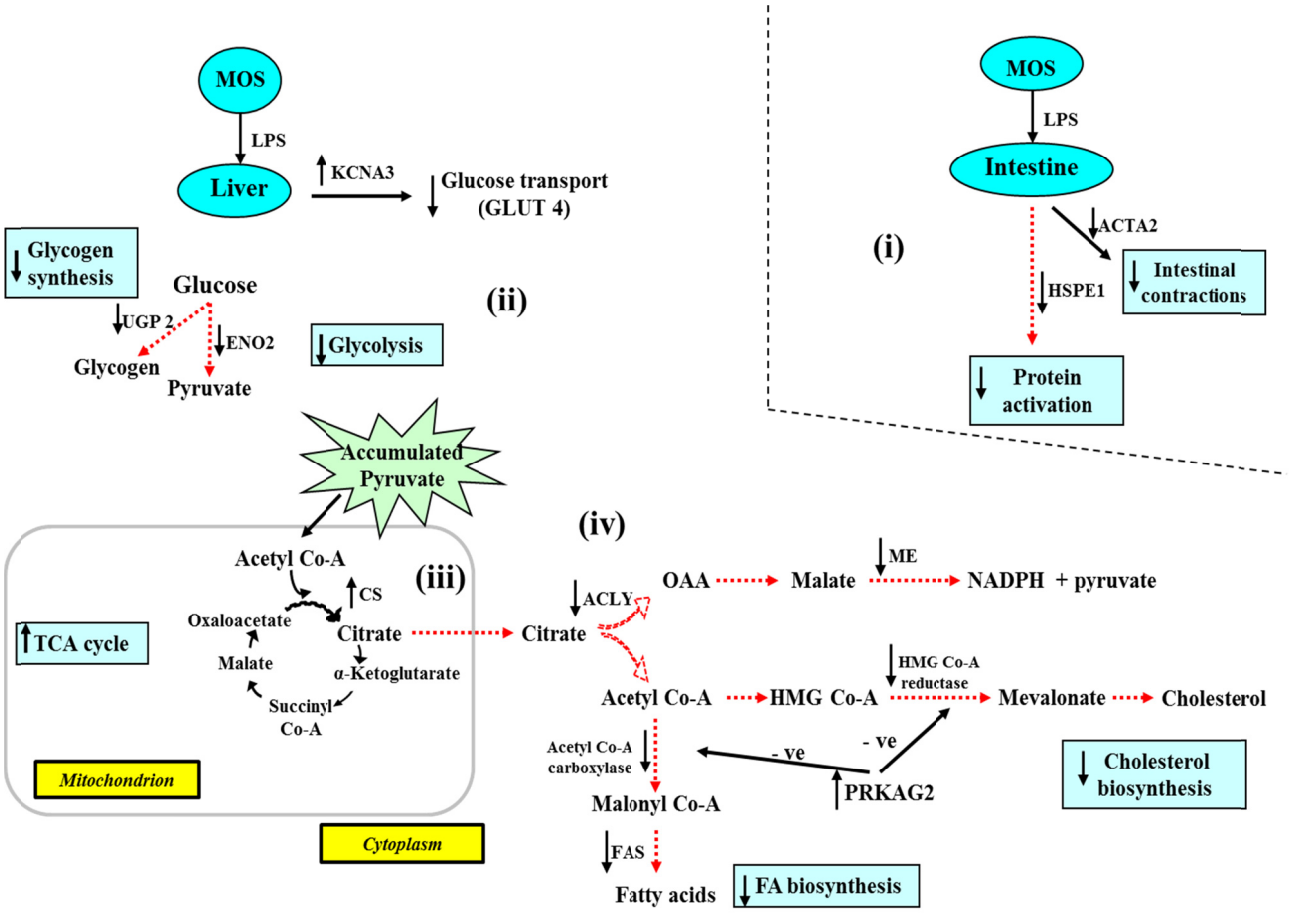


**Figure 4.10.** Schematic illustration of LPS effects on glucose metabolism between MOS- and VIRG-fed hosts. (i) LPS caused no major intestinal metabolic activities in MOS-fed hosts; (ii) in absence of liver glucose mobilization, *KCNA3* was up-regulated, whereas *ENO2* and *UGP2* down-regulation reduced glycolysis and glycogen synthesis, respectively; (iii) *CS* up-regulation increased TCA cycle-derived energy from high liver pyruvate; (iv) *ACLY*, *ME* and *FAS* down-regulations inhibited liver fatty acid biosynthesis; whereas PRKAG2 up-regulation inhibited fatty acid and cholesterol biosynthesis.



# CHAPTER V. GENERAL DISCUSSION AND CONCLUSION

Human *S.* Enteritidis-induced salmonellosis, characterized by profuse diarrhea and life-threatening acute gastro-intestinal inflammation (CDC, 2011), is principally caused by the consumption of undercooked contaminated retail poultry meat (Galanis et al., 2011) and eggs (Patrick et al., 2004). In addition, *S.* Enteritidis causes enteric diseases and varying mortality rates in young chicks (Desmidth et al., 1997; Goldberg and Rubin, 1988). In order to ensure safety of poultry meat and eggs, there always has been urgency to control *S.* Enteritidis on poultry farms. However, despite laborious efforts exerted by poultry veterinarians and medical specialists, salmonellosis still remains an ever-increasing human health problem. In fact, the emergence of multiple-antibiotic resistant *S.* Enteritidis has not only made *S.* Enteritidis become more difficult to control in poultry production, but death rates have increased among hospitalized patients due to inefficacious antibiotic treatment of *S.* Enteritidis-induced salmonellosis (CDC, 2011).

To cut the link between antibiotic-resistance transfer from poultry to humans has become the priority objective that scientists are required to tackle. According to the WHO, the first milestone is to discontinue administering sub-therapeutic antibiotics to healthy chickens for growth promotion and disease prevention purposes (WHO, 2011). Therefore, investigation about MOS usage attempting to improve innate defense mechanisms against intestinal *S.* Enteritidis has been the primary objective of this study. Our study was founded on the principle that, while the reverse is also true, antibiotic-resistant *S.* Enteritidis undergoes reversion mutation in absence of antibiotic pressure to diminish or abrogate antibiotic resistance (O'Regan et al., 2010). Therefore, our study falls in line with the objective set by the WHO.

Research, so far, conducted with MOS in poultry has focussed mainly on growth performance, and very little consideration has been given to intestinal health. We demonstrated that dietary MOS (0.2 %) significantly enhanced innate defense mechanisms against intestinal pathogens than VIRG or BACT in healthy broilers raised under good sanitary conditions, by increasing the development of mucin-secreting goblet cells (Table 2.5) and favored microflora enrichment with probiotic bacteria, especially bifidobacteria (Figure 2.2). It is known that mucin's specific mannosyl receptors competitively bind mannose-specific lectin at the tip of Type 1 fimbriae of gram-negative

pathogens such as *Salmonella*, *E. coli* and *Campylobacter* (Sajjan and Forstner, 1990; Vimal et al., 2000; Edelman et al., 2003) thus reducing their adherence to mannose-containing glycoprotein receptors on intestinal epithelial cells, whereas probiotic bacteria exhibit innate defense against intestinal pathogens through competitive exclusion (Rolfe, 2000; van der Wielen et al., 2002), and secretion of antimicrobial substances such as bacteriocins and organic acids (Gibson and Wang, 1994; Jin et al., 1996). Improved intestinal health conditions due to MOS than antibiotics were evidenced by typically longer and healthier villi (Table 2.4).

Interestingly, MOS had equivalent effects to VIRG or BACT in the control of intestinal *E. coli* (Figure 2.3). But, it appears that MOS and antibiotics actions to control intestinal pathogens are governed by different mechanisms. This was later deeply investigated in our next study (Chapter III). Taken together, all of these findings provided confirmation to our previous reports (Baurhoo et al., 2007a,b) about MOS's profound intestinal health benefits. In the absence of sub-therapeutic antibiotics, poultry specialists and industrialists questioned whether higher MOS dosage might confer additional protection against intestinal pathogens. To clarify such uncertainty, we included a higher MOS dosage (0.5 %) as another dietary treatment in our study. For the first time, we showed no additional intestinal health benefits when feeding broilers MOS at 0.5 % than 0.2 %. These findings were of particular interest to the broiler industry with regards to broiler health and the economics of broiler production. Based on findings of this study, MOS (0.2 %) was used in our following studies (Chapters III and IV).

As a novel approach, we next investigated whether MOS may improve innate defense mechanisms to sufficiently prevent or treat *S.* Enteritidis-induced intestinal inflammation, and elucidated the mechanisms of MOS and VIRG action on innate defense. To induce acute intestinal inflammation, young chicks were voluntarily infected with *S.* Enteritidis. Young chicks were specifically utilized considering their susceptibility to *Salmonella* invasion of the intestinal epithelium, because these are biologically devoid of a fully functional protective intestinal microflora (Gong et al., 2008) and adaptive immunity (Beal et al., 2004; Lowry et al., 2005). Interestingly, whereas CTL-fed chicks suffered from drowsiness, diarrhea, starvation, exfoliation of epithelial cells, damaged villi integrity and infiltration of *S.* Enteritidis-invaded villi by immune cells such as

neutrophils and macrophages, these classic signs of *Salmonella*-induced enteric inflammation (Jung et al., 1995; Clark et al., 1998; Gewirtz et al., 1999) were abrogated by MOS and VIRG (Figure 4.6). Most importantly, however, MOS down-regulated gene expressions for the IL 12 pro-inflammatory cytokine (Figure 4.2), which is typically secreted by intracellular macrophages of epithelial cells during inflammation (Walter et al., 2001; Trinchieri, 2003), thus revealing that MOS terminated *S.* Enteritidis-induced enteric inflammation earlier than VIRG. The longer and healthier intestinal villi in both non-infected and *S.* Enteritidis infected chicks fed MOS (Figures 4.4 and 4.5), as demonstrated by electron microscopy analysis, provided confirmation about MOS's capacity to more significantly improve host's intestinal health than VIRG.

We also revealed different mechanisms of MOS and VIRG actions against *S.* Enteritidis. MOS counteracted the pathogenicity effects of *S.* Enteritidis through increased secretions of neutral and acidic mucins into the intestinal lumen. Neutral and acidic mucins were profoundly secreted from the greater numbers of neutral (Figures 4.6 and 4.8) and acidic (Figures 4.7 and 4.9) goblet cells, respectively. Although the physiological relevance of these distinct mucin subtypes is still not clear, we previously discussed that mucins reduces *S.* Enteritidis colonization and invasion of intestinal epithelial cells through adherence at its mannose-specific lectin. Contrastingly, VIRG relied mostly on its strong bactericidal properties to clear the infection (Cocito, 1969) rather than involving mucins secretions. In fact, VIRG markedly reduced development of mucin-secreting goblet cells in both non-infected and *S.* Enteritidis -infected chicks. But, our observation that MOS also increased neutral and acidic mucin-secreting goblet cell numbers than VIRG in non-infected chicks confirmed findings of our first study (Chapter III) about MOS's inherent capacity to increase innate defense mechanisms against intestinal pathogens. Although the exact mechanism of MOS action on goblet cell development is still not clear, we believe it is linked with the antigenic properties of yeast cell walls.

In order to fully unveil the mucins protective or defensive mechanisms against intestinal *S.* Enteritidis, we next investigated MOS and VIRG effects on the two existing mucin types, namely secretory (gel-forming) and membrane-bound (not gel-forming) mucins (Montagne et al., 2004). To determine the mucins secreting capacity of goblet

cells, we measured MUC 2 gene expressions. For the first time, we have revealed that MOS's higher mucins-mediated defense mechanisms against *S.* Enteritidis was governed principally by increased goblet cell numbers rather than increased mucins-secreting capacity of goblet cells. The expressions of *MUC 2* were not different between infected chicks fed CTL, VIRG or MOS (Figure 4.10). It is known that membrane-bound mucins, as mediated by *MUC 1* expression, represent an important defense mechanism exerted by epithelial cells to limit its invasion by pathogens (McAuley et al., 2007; Furr et al., 2010). Therefore, our *MUC 1* down-regulation result due to MOS (Figure 4.11), in contrast to VIRG, indicated that MOS more significantly reduced epithelial cells damage by *S.* Enteritidis than VIRG. Very importantly, in this study, findings about *MUC 1* expression, *IL 12* expression and histological (light and electron microscopy) results were highly consistent.

It is well established that *S.* Enteritidis invades the intestinal epithelium by using its specialized Type III secretory system (T3SS) and disseminate through efferent lymphatics to the blood, thus causing extra-intestinal infections and systemic inflammation (Griffin and McSorley, 2011). Therefore, to investigate whether MOS, in contrast to VIRG, may beneficially modulate innate immunity and mitigate systemic inflammation, we adopted a *Salmonella* LPS-induced systemic inflammation model and microarray analysis approach (Chapter IV). We also explored nutrient metabolism in the liver and intestines as affected by MOS and VIRG during late inflammation, given that prolonged pro-inflammatory cytokines, especially IL 1 and IL 6, release causes profound nutrient mobilization, often leading to catabolism of skeletal muscles (Flores et al., 1989; Doyle et al., 2011) and adipose tissues (Feingold et al., 1992). That MOS increased innate immune responses than VIRG in non-challenged control hosts, as indicated by increased intestinal *IL 3* expression (Table 3.2), revealed that MOS exhibited inherent immune-stimulatory properties, which we, again, associated with the antigenic properties of yeast cell walls. Evidently, glucose mobilization occurred due to increased immune stimulation. However, whereas liver gluconeogenesis involving muscle catabolism has frequently been reported during intense inflammation (Flores et al., 1989), here we observed that gluconeogenesis, as mediated by *PEPCK* up-regulation (Table 3.2; Figure 3.7), occurred in the intestines only. Mobilized glucose was then rapidly metabolized via increased

glycolytic activities, as indicated by *ENO2* up-regulation, to meet host's elevated energy demands.

Interestingly, however, when MOS-fed birds were challenged with LPS, none of the pro-inflammatory cytokines were up-regulated (Table 3.3), thus revealing that MOS counteracted LPS's detrimental inflammatory effects. In support to our finding, we observed that no major nutrient mobilization activities occurred in these LPS-challenged birds fed MOS, and that energy requirements were sufficiently met by increased TCA cycle-derived energy, as indicated by *CS* up-regulation (Table 3.3; Figure 3.8). Contrastingly, VIRG failed to counteract or reduce LPS's inflammatory effects, as indicated by increased *IL 3* expression (Table 3.4), such that the host's higher energy demands triggered glucose mobilization through intestinal gluconeogenesis and increased liver glycolytic activities (Table 3.4; Figure 3.9). Intriguingly, during glucose mobilization, liver fatty acid synthesis simultaneously occurred as indicated by *FAS* up-regulation (Figure 3.5). To help explain these apparently conflicting findings, we had to consider LPS-induced inflammation during its intense phase. Results, so far discussed, demonstrate clearly that, prior to 24 h of LPS challenge, inflammatory responses were higher in VIRG- than MOS-fed birds. Obviously, the higher glucose mobilization and catabolism in VIRG hosts caused liver accumulation with glucose and its glucose metabolites, especially citrate. Our findings demonstrated that the exceptionally high liver citrate levels triggered *CS* down-regulation. Indeed, citrate is a well-known potent allosteric negative-feedback inhibitor of CS activity which majorly influences liver metabolic activities. Thereafter, the accumulated liver cytosolic citrate was rapidly converted into fatty acids, as evidenced by *ACLY*, *ME* and *FAS* up-regulations (Figure 3.9). ACLY, ME and FAS are well known as key lipogenic enzymes that convert cytoplasmic citrate into fatty acids (Kochan et al., 1997; Morral et al., 2007; Wang et al., 2009). Taken together, all of these findings revealed that dietary MOS terminated LPS-induced systemic inflammation earlier than VIRG. We firmly believe that MOS's inherent immune-stimulatory properties caused 'arming' of host's innate defense mechanisms that triggered more rapid clearance of the endotoxin. For the first time, we revealed occurrence of intestinal gluconeogenesis in chickens. Furthermore, this study provided strong evidence about cross-talks between intestinal mucosal immunity and

systemic immunity, considering that MOS and VIRG were administered through the diet only.

In summary, in this ambitious study, for the very first time, we have comprehensively studied and revealed the mechanisms by which MOS, in comparison with VIRG, more significantly improved innate defense mechanisms, including: i) increased intestinal development of neutral- and acidic-mucins secreting goblet cells, which ultimately secreted higher neutral and acidic mucins, respectively; ii) microflora enrichment with probiotic bacteria, especially bifidobacteria; iii) inherent stimulation of intestinal mucosal innate immunity. By virtue of these defense mechanisms, in contrast to VIRG, MOS: i) more significantly abrogated *S.* Enteritidis damage of intestinal epithelial cells; ii) caused earlier termination of *S.* Enteritidis -induced intestinal inflammation; iii) caused earlier termination of *Salmonella* LPS-induced systemic inflammation, leading to reduced glucose mobilization; iv) promoted longer and healthier intestinal villi in both non-infected and *S.* Enteritidis infected chicks. For the first time, we also revealed that MOS's higher mucins-mediated defense mechanisms was governed principally by increased goblet cell numbers rather than increased mucins-secreting capacity of goblet cells. Finally, based on all health benefits due to MOS herein reported, we demonstrated that administration of MOS at higher dosage than 0.2 % may not be needful for good intestinal health.

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## APPENDIX

**Appendix 1.** Schematic of the 2 x 2 factorial experimental design in an interwoven loop arrangement for each tissue (liver, intestine or breast meat). Diet x Injection is denoted as VC (antibiotic x control birds), VL (antibiotic x LPS birds), BC (prebiotic x control birds), and BL (prebiotic x LPS birds). Each arrow represents an array (total: 12) consisting of 2 aminoallyl labelled cDNA, either Cy-3 or Cy-5.