

MICROBIAL INFLUENCE ON INTESTINAL DEVELOPMENT
AND MODE OF ACTION OF MANNAN OLIGOSACCHARIDES IN BROILER CHICKEN

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Department of Animal and Poultry Science,
University of Saskatchewan,
Saskatoon, SK,
Canada

By
Niradha Withana Gamage

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by Professor Andrew Van Kessel, who supervised my thesis work or, in his absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon SK, Canada, S7N 5A8

ABSTRACT

The effect of intestinal microbiota and dietary supplementation of mannan oligosaccharides (MOS) on mucosal architecture and digestive physiology in broiler chicks was examined. In experiment 1, pre-sterilized eggs (Ross x Ross 308) were placed in three HEPA (high efficiency particulate air)-filtered isolator units at day 19 of incubation. Germ-free chicks in one isolator were conventionalized by exposure to cecal contents from a laying hen. Bacterial contamination occurred in one germ-free isolator such that these birds were monoassociated by a bacterium within the *Acinetobacter* spp. resulting in 3 categories of microbial status including germ-free (GF, n=10), conventionalized (CV, n=19) and monoassociated (Mono, n=13) birds. Dietary treatments assigned to each isolator consisted of a negative control (NC, 0 g/kg of MOS in the basal diet) and MOS (2 g/kg of MOS in the diet) resulting in a 2X3 factorial treatment arrangement. At 7 d of age, body weight was recorded and birds were killed to permit collection of visceral organs, intestinal tissues and cecal contents. Body weight, relative length of small intestinal segments and relative bursa weight were significantly increased in CV birds. These birds also had increased crypt depth and lamina propria area. Dietary MOS increased villus height and villus surface area in CV birds compared with GF and Mono birds. Transcripts for all housekeeping genes tested in ileal tissue were increased by MOS such that transcripts were normalized to unit mass of total RNA. In comparison to birds fed the NC diet, MOS significantly increased the abundance of proliferative cell nuclear antigen (PCNA), toll-like receptor (TLR)-4, avian β -defensin (GAL)-6, interleukin (IL)-8, peptide transporter 1 (PepT1) and sodium-dependent glucose transporter (SGLT)-1 transcripts in ileum per unit total RNA. However, the effect of microbial status on selected gene expression profiles was surprisingly limited. A second experiment was conducted to confirm the conventionalization protocol

produced a complex microbiota similar to conventionally reared birds. Twenty day-old broiler chicks (Ross x Ross 308) were assigned to one of two wire-floored battery cages provided the NC and MOS diets ad libitum and killed at 7 d of age. Terminal restriction fragment length polymorphism (TRFLP) analysis demonstrated that microbial diversity indices (Richness, Evenness, Shannon, and Simpson) were greater in conventionalized gnotobiotic birds compared to the conventionally reared birds confirming a successful conventionalization strategy in the gnotobiotic trial. These studies demonstrate that under good hygienic conditions, CV chicks thrive similar to GF animals. Based on responses to MOS observed in GF birds, evidence indicates that MOS, independent of changes in microbial composition, directly modifies host response parameters including innate immune activation, digestive and absorptive function.

Key words: chicken, microbiota, germ-free, small intestine, mannan oligosaccharides

ACKNOWLEDGEMENTS

It would not have been possible to complete this master's thesis without the guidance and help of the generous people around me, to only some of whom it is possible to give particular mention here.

I would like to gratefully and sincerely thank my supervisor, Professor Andrew Van Kessel for his guidance, understanding and patience during my graduate studies at University of Saskatchewan. His unsurpassed knowledge of gastrointestinal microbiology and mentorship were paramount in providing a well-rounded experience consistent my long-term career goals. Deepest gratitude is also due to the members of the advisory committee, Dr. Hank Classen, Dr. Murray Drew, Dr. Bernard Laarveld and Dr. Fiona Buchanan for the assistance they provided at all levels of the research project.

Very special thanks go out to Dr. Ursula Fernando for her assistance and guidance in getting my graduate career started on the right foot and providing me with the foundation for becoming a molecular biologist. Furthermore, I am heartily thankful to Dr. Rebecca Forder for her technical support in histomorphology. I am very grateful to the Alltech Inc. which provided the financial support for my research project.

I will always appreciate Jason Marshal, for his excellent technical assistance. I would also like to thank the team I worked with for the support and cooperation during the experiments. I can never forget the warm and friendly atmosphere of the entire group.

I would also like to thank my parents for their love and support through my entire life. I must acknowledge my loving husband, Shermey and my sons, Nethum and Mindiv for their commitments, encouragement, and patience throughout the entire process. Without their support I would not have finished this thesis. I will be grateful forever for your love.

DEDICATION

This thesis is dedicated to my beloved parents who have given me the opportunity of an education from the best institutions and also to my loving husband and my cute two sons for their unwavering love and support throughout my life

TABLE OF CONTENTS

PERMISSION TO USE.....	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS.....	vi
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS.....	x
1.0 GENERAL INTRODUCTION.....	1
2.0 LITERATURE REVIEW	6
2.1 Overview of the physiology of the avian intestine.....	6
2.1.1 Morphology and histology of the avian small intestine	7
2.1.2 Mucosal repair, restitution and apoptosis.....	10
2.1.3 Development of brush border enzymes	15
2.1.4 Overview of intestinal nutrient transport mechanisms	18
2.2 Intestinal Immunology and inflammation.....	21
2.2.1 Barrier function and innate immunity	22
2.2.2 Mechanisms and consequences of intestinal inflammation.....	29
2.3 Role of microbiota in the intestinal physiology of avian host.....	31
2.3.1 Effect of microbiota on nutrition and performance in chickens.....	33
2.3.2 Effect of microbiota on intestinal morphology and digestive function	36
2.3.4 Role of microbiota in host protein and energy nutrition	38
2.4 Mannan oligosaccharides as an antibiotic alternative in broiler chicken.....	40
2.4.1 Exclusion of pathogens.....	43
2.4.2 Effect of MOS on immune modulation and nutrient assimilation.....	47

3.0	INTESTINAL MORPHOLOGY, DIGESTIVE AND BARRIER FUNCTION IN CONVENTIONAL AND GNOTOBIOTIC BROILER CHICKENS FED DIETS WITH OR WITHOUT MANNAN OLIGO SACHARIDES	50
3.1	Introduction	50
3.2	Materials and methods	52
3.2.1	Birds and Diets	52
3.2.2	Confirmation of Microbial Status	55
3.2.3	Sample collection	56
3.2.4	Histomorphological parameters.....	57
3.2.5	Gene Expression Analysis by Real-Time quantitative PCR (qPCR)	57
3.2.6	Protein and Brush-border Enzyme Assays	59
3.2.7	Caspase-3 activity.....	60
3.2.8	TRFLP Analysis of microbial diversity.....	63
3.2.8	Statistical Analysis	65
3.3	Results	66
3.3.1	Confirmation of microbial status	66
3.3.2	Growth performance and gross morphology	66
3.3.3	Mucosal morphology in ileum.....	69
3.3.4	Expression of House Keeping Genes	71
3.3.5	Expression of PCNA and Caspase-3 activity	73
3.3.6	Immune modulation.....	75
3.3.7	Ileal brush- border membrane enzyme activity and nutrient absorption	75
3.3.8	Comparison of bacterial communities	81
3.4	Discussion	84
4.0	LIST OF REFERENCES	101

LIST OF TABLES

Table 3. Primers used for Real-time PCR	61
Table 3. (continued)	62
Table 4. Effect of microbial status and MOS supplementation on live body weight (BW), relative weight of selected visceral organs and relative length of small intestinal segments in gnotobiotic broiler chicks at 7 days of age.	68
Table 5. Effects of microbial status and MOS on histomorphological parameters of the ileum in gnotobiotic broiler chicks at 7 days of age.	69
Table 6. Effect of microbial status and MOS on expression of housekeeping genes in ileum and cecum in gnotobiotic broiler chicks at 7 days of age.....	72
Table 7. Effect of microbial status and MOS on PCNA transcript abundance and caspase-3 activity in ileum and cecum of gnotobiotic broiler chicks at 7 days of age.....	74
Table 8. Effects of microbial status and MOS on the transcript abundance of immune-related genes in ileum of gnotobiotic broiler chicks at 7 days of age.....	77
Table 9. Effects of microbial status and MOS on the transcript abundance of immune-related genes in cecum of gnotobiotic broiler chicks at 7 days of age.	78
Table 10. Effects of microbial status and MOS on brush border enzyme activity in ileum of gnotobiotic broiler chicks at 7 days of age.	79
Table 11. Effect of microbial status and MOS on the transcript abundance of brush border enzymes and nutrient transporters in ileum of gnotobiotic broiler chicks at 7 days of age.....	80
Table 12. Diversity indices calculated from TRFLP banding patterns in cecal contents taken from conventionalized isolator-reared birds conventionally reared birds	82

LIST OF FIGURES

- Figure 1.** Illustration of the interaction between microbial status and MOS supplementation on villus height (panel A) and villus surface area (panel B). Conventionalized (CV), monoassociated (Mono) and germ-free (GF) broilers were fed basal diet (NC) or the basal diet supplemented with mannan oligosaccharides (MOS). 70
- Figure 2.** Dendrogram comparing the bacterial profile between conventionalized isolator-reared birds (I) and conventionally reared birds (C) using TRFLP banding patterns. 83

LIST OF ABBREVIATIONS

AGP	antibiotic growth promotors
APN	aminopeptidase N
AMPs	antimicrobial peptides
AvBD	avian beta-defensins
BB	brush border
BW	body weight
Ca	calcium
CD	crypt depth
cDNA	complementary deoxyribonucleic acid
cm	centimeter
cpn60	chaperonin 60
Ct	threshold cycle
CV	conventional
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	grams
GAL6	avian β -defensin 6
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GF	germ-free
GIT	gastrointestinal tract
GLM	general linear models
HEPA	high efficiency particulate air
High iron diamine	HID
High iron diamine-alcian blue	HID-AB
IEC	intestinal epithelial cells
IgA	immunoglobulin A
IL-1 β	interleukin 1 β
IL-6	interleukin-6
IL8	interleukin 8
LP	lamina propria
LPA	lamina propria area
LPS	lipopolysaccharides
M	molar
Mg	magnesium
MGA	maltase glucoamylase
Min	minutes
mM	milimolar
μ l	microlitre
μ m	micrometre
μ M	micromolar
Mono	mono-associated
MOS	mannan oligosaccharides

MR	mannose receptor
mRNA	messenger ribonucleic acid
NK cells	natural killer cells
PAS	periodic acid-Schiff
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PepT1	peptide transporter 1
PRDX6	peroxiredoxin-6
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
SAS	statistical analysis software
SCFA	short chain fatty acid
SGLT1	sodium-dependent glucose transporter 1
SI	sucrose isomaltase
TLR	toll like receptor
TRFLP	terminal restriction fragment length polymorphism
UT	universal target
UV	ultra violet
VCR	villus crypt ratio
VH	villus height
VSA	villus surface area

1.0 GENERAL INTRODUCTION

The poultry industry has been one of the fastest growing food- producing animal industries in the world with the production of meat-type poultry experiencing tremendous growth over the past several decades (Windhorst, 2006). A primary emphasis of the commercial broiler industry has been to maximize the growth and meat yield of birds while maintaining their health at an optimal level. Recently, the significant influence of the intestinal microbiota on bird health and metabolism has been recognized, mediated either through competition with pathogens or through host sensing of microbial products affecting intestinal development, digestive and immune function. Therefore, increased attention has been given the development of dietary strategies to beneficially manipulate intestinal microbial composition or directly activate host sensing pathways leading to improved health and performance.

The relationship between the broiler chicken and the microbial community of the gastrointestinal system commences immediately after hatch and an environment is established where mutual benefits can accrue to both microbe and host. In chickens, the resident microbiota is established and stabilized by 2 to 3 weeks of age (Snel et al., 2002; Lu et al., 2003), and this complex process is influenced by various factors including the age of the bird, composition of the diet, and the use of additives such as enzymes, antibiotics and probiotics (Patterson and Burkholder, 2003; Xu et al., 2003; Gong et al., 2008). The most abundant and diverse microbial community of bacteria develops in the paired ceca; though, there are also abundant and diverse microorganisms in the crop, proventriculus, gizzard and small intestine. Interactions of the gut microbiota and their metabolites with the intestinal mucosa result in anatomical and physiological changes in the host digestive tract (Coates, 1980; Furuse and Okumura, 1994).

Gnotobiotic experiments are considered as a tool in the study of host microbial interactions. The microbial impact on the immune, nutritional and physiological processes of the gastro intestinal system (GIT) has been shown in comparative studies of germ-free (GF) and conventionalized (CV) animals. It has been reported that conventional chickens generally show reduced growth compared to the germ-free birds (Kussaibati et al., 1982; Furuse and Okumura, 1994) when readily digestible diets are provided. This has been explained as an increase in the maintenance protein and energy requirements of the conventional compared to germ-free bird associated with innate and adaptive immune mechanism activated to contain the normal microbiota within the GIT lumen. On the other hand, when diets contain a significant fiber component available to the bird only through microbial fermentation in the ceca, then the increased maintenance costs may be offset by increased energy extraction (Furuse and Okumura, 1994).

Commensal bacteria are microorganisms which enter the body of the host animal from the environment shortly after birth and colonize the mucous membranes and skin epithelia. They live in a symbiotic or mutually beneficial relationship with their host. The innate immune system has an ability to recognize and limit microbes early during infection. During microbial invasion, microorganism-associated molecular patterns (MAMPs) are detected by an array of pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs), leading to the activation of defense mechanisms that protect the host. The beneficial bacteria and their metabolites participate in the normal development and function of the mucosal immune system (Salminen et al., 1998; Rakoff-Nahoum et al., 2004; Mazmanian et al., 2005; Wagner, 2008). This is evident by the delayed immune cell development in the lamina propria and reduction in IgA producing cells in GF birds compared with their conventional counterparts (Thorbecke et al., 1957; Gordon

and Pesti, 1971; Honjo et al., 1993). Moreover, commensal bacteria compete with invading microorganisms for space and resources, thereby giving fewer opportunities for true pathogens to become established.

Intestinal mucus layers secreted by goblet cells consist mainly of secretory mucin glycoproteins (MUC2), which are variably modified to protect enterocytes from attachment by pathogens. Gut microbiota affect the functions and number of goblet cells (Deplancke and Gaskins, 2001; Moal and Servin, 2006) and numerous gnotobiotic studies on rodents have reported changes in mucus production and composition associated with the presence of intestinal microbiota (Kandori et al., 1996; Meslin et al., 1999; Sharma and Schumacher, 2001).

The optimum yield in meat-type birds is influenced by the efficient digestion and absorption of nutrients. Greater villus height leads to increased surface area and the potential for greater digestion and absorption of disaccharides and dipeptides are promoted. In chickens, it has been reported that there is rapid development of intestinal villi during the first 10 days post-hatch (Yamauchi and Isshiki, 1991). Conventional chickens have shown greater villi height in the small intestine compared to germ-free birds (Cook and Bird, 1973; Forder et al., 2007). Indigenous microbes may stimulate vascularization and villi development and thereby enhance the efficiency of digestion and absorption (Stappenbeck et al., 2002). Further, bacterial fermentation of non-digestible carbohydrates results in short chain fatty acids (SCFAs) which provide extra energy and increase intestinal tissue mass by accelerating enterocyte proliferation. However, this may be a unique character in birds since the other gnotobiotic studies on rodents (Abrams et al., 1963; Khoury et al., 1969; Meslin and Guenet, 1973) and pigs (Kenworth.R, 1967; Willing and Van Kessel, 2007) showed shorter villi in CV animals compared to their GF

counterparts explained by greater turnover and increased maintenance cost in the presence of microbiota.

It has been well documented that there is significant impact of diet on the composition and metabolic activity of the GIT microbiota (Wagner and Thomas, 1978; Jensen, 1998; Pluske et al., 1998). Supplementation of exogenous feed in the early post-hatch period may have the greatest impact on GI development (Uni et al., 1998a) and overall lifetime performance of chickens (Lilja, 1983; Geyra et al., 2001a). Subtherapeutic levels of antibiotics have been used to modify intestinal microbiota in agricultural animal production since mid -1940s (Dibner and Richards, 2005). Evidence indicates that their incorporation in feed has improved performance and health status in broiler chickens (Coates et al., 1955; Miles et al., 2006). However, recommendations to reduce or eliminate the use of antibiotics in animal feed have been discussed due to the emergence of antibiotic resistance in human pathogens (Swann, 1969; Greko, 2001; Roe and Pillai, 2003). Therefore, the antibiotic growth promoters (AGP) have been replaced by a wide variety of alternative feed additives to enhance the performance of farm animals including broilers.

Mannan oligosaccharides (MOS) are derived from the outer cell wall of *Saccharomyces cerevisiae* and have been introduced as a non-pharmaceutical alternative to AGP. Dietary MOS supplementation has been shown to improve growth performance (Hooge, 2004; Rosen, 2007) and lower pathogen shedding (Fernandez et al., 2000; Spring et al., 2000; Fernandez et al., 2002), although not consistently (Sarica et al., 2005; Yalcinkaya et al., 2008). The mechanisms by which MOS improves performance and/or reduces pathogen load are not fully understood, a fact that contributes to debate regarding efficacy. Mechanisms of action may include inhibitory

effects on pathogen colonization, immune modulation, improvement of intestinal morphology and expression of mucin and brush border enzymes (Ferket, 2004).

The aim of the present investigation was to determine the impact of microbial colonization on intestinal development in broiler chickens and to differentiate indirect effects of MOS mediated by changes in microbial composition versus direct effects observed in birds that lack a complex microbiota.

2.0 LITERATURE REVIEW

2.1 Overview of the physiology of the avian intestine.

The digestive system extracts nutrients and energy by processing the food which is ingested. Most of the adaptations of the digestive system found in different species have a close relationship with their dietary habits or environment (Stevens and Hume, 2004). The digestive system of birds differs notably from that of mammals and may be adaptations for lightening the body for flight. Major adaptations of the avian digestive system include a reduced buccal apparatus and the presence of unique structures such as the crop and gizzard (Dukes et al., 1993). The crop is specialized to temporarily store the food in transit to the lower alimentary tract and gizzard is modified to process unmasticated food and is considered as the functional analog of mammalian molars (Feduccia, 2011).

The avian small intestine possesses three anatomical divisions, the duodenum, jejunum and ileum, and is visibly similar to that of mammals. It has been reported that birds have significantly shorter small intestinal lengths and lower small intestine nominal surface area (the area over which nutrients are digested and absorbed) than nonflying mammals (Caviedes-Vidal et al., 2007). A reduction of intestinal volume and digesta mass may be an adaptation for lowering the energy cost of flight. The large intestine of birds comprises paired ceca and the rectum, of which the latter is relatively short and straight (Stevens and Hume, 2004). The physiology of chicken intestine plays a crucial role in digestion and absorption of nutrients, which of course are critical to support whole body metabolism. Chemical digestion and absorption of nutrients are primarily done in the small intestine. Also, the intestinal tract is responsible for a high proportion of protein turnover and energy utilization (Mcbride and Kelly,

1990). Fiber digestion by micro-organisms and absorption of the resulting volatile fatty acids primarily occurs in the paired ceca. The gastrointestinal tract is the most metabolically intense organ in the vertebrate body (Stevens and Hume, 2004).

The nature of the intestine is such that it represents the largest body surface area exposed to the external environment. Thus a second critical function of the gastrointestinal tract is acting as a physical, chemical and cellular barrier to enteric pathogens and toxins while providing a residence for the replication of commensal microbiota. This defence mechanism employed along the mucosal surfaces is essential to protect the host against enteric infections as well as to elicit tolerance to the presence of nutrient molecules. As such, the fine balance between tolerance and response acts directly or indirectly on digestion and absorption functions and consequently affects overall health of the bird.

2.1.1 Morphology and histology of the avian small intestine

The pattern of intestinal development and final structures within the mucosa has been observed to be different in poultry than in some mammalian species (Smith and Peacock, 1989). In birds, the initial development of GIT occurs during the incubation period (Romanoff, 1960). The rapid morphological and functional development in the GIT at the immediate post-hatch period (Uni et al., 1999) is influenced by the transition of nutrient source from lipid-rich yolk to exogenous feed (Moran, 1985) rich in carbohydrates and proteins (Sklan, 2001). During the first few days of post-hatch, the yolk sac plays an essential role in the gastro intestinal development, but it is exhausted within 4-14 days post-hatch (Murakami et al., 1988; Ferrer et al., 1995; Nitsan et al., 1991). The yolk sac represents 8% of body weight at hatch but declines to <1% by day 7 (Iji et al., 2001a). Research studies on intestinal growth have confirmed that the weight of

the small intestine increases more rapidly than body weight during the first week of life (Katanbaf et al., 1988; Sell et al., 1991; Sklan, 2001) and decreases in relationship to body weight by day 10 (Sell et al., 1991; Pinchasov and Noy, 1994).

The small intestine is anatomically subdivided to duodenum, jejunum and ileum. The duodenum is extended from the gizzard outlet to the pancreatic loop forming a narrow loop with the descending and ascending parts of the loop sandwiching the pancreas. In chickens, the ascending part of the duodenum receives digestive enzymes and bicarbonate from three pancreatic ducts (Baumel, 1993). Two bile ducts namely, the hepatoenteric duct and cysticoenteric duct carry bile from liver and gall bladder to the ascending limb of the duodenum, respectively. The jejunum is the longest segment of the small intestine extending from the end of the duodenum (pancreatic loop) to the Meckel's diverticulum. The chicken ileum is a short intestinal segment extending from Merckel's diverticulum to the cecal junction.

The intestinal wall is composed of four layers including the inner tunica mucosa, the tunica submucosa, the tunica muscularis and the outer tunica serosa. The tunica mucosa includes three sub layers, the inner epithelium, the middle lamina propria (LP) and outer muscularis mucosae. The mucosal projections can be either folds or villi depending on the species of bird. Mucosal folds are not formed in chicken (Gabella, 1985) but villi are present (Denbow, 2000). The villi are taller, more slender and more numerous than that of mammals. Also they do not possess lacteals but a well-defined network of blood capillaries is recognized (Dukes et al., 1993). The villus surface is covered with simple columnar epithelium composed of absorptive enterocytes, goblet cells and endocrine cells (Gusseklou, 2006; Kalita and Singh, 2014). Microvilli are the cylindrical protrusions of the luminal surface of individual enterocytes, which appear as a fuzzy striated border in light microscope.

It has been reported that there is dramatic changes in villus size and volume after hatching (Overton and Shoup, 1964; Uni et al., 1995; Uni et al., 1998a). Villus growth is almost complete by day 7 in duodenum. But the development continues beyond 14 days of age in jejunum and ileum (Uni et al., 1999). Increased villi number and size contributes to greater absorptive area per unit of intestine (Uni, 2006).

Intestinal glands or crypts of Lieberkuhn are tubular structures located at the bases of the villi. These crypts are populated with epithelial stem cells (which differentiate, proliferate and migrate to populate the villi and crypt base), goblet cells, endocrine cells and lymphocytes. Geyra et al. (2001b) reported that crypts were rudimentary in chicks at hatch and the numbers were increased by branching and fission until the number of the crypt per villus reached a plateau after 72 h post-hatch. When the chicks are two weeks of age, each villus is surrounded by three to four crypts (Uni, 2006).

In birds, the muscularis mucosa and LP are poorly developed (Denbow, 2000). Further, the submucosa of chicken is devoid of glands (Dukes et al., 1993). The gut associated lymphatic tissue (GALT) (Lillehoj and Trout, 1996) is a major component of mucosa associated lymphoid tissue (MALT) and mature during the first 2 weeks post-hatch in birds (Bar-Shira et al., 2003). The GALT in chicken consists of both organized lymphoid structures such as the bursa of Fabricius, cecal tonsils (CT), Peyer's patches (PP), Meckel's diverticulum and more diffusely scattered lymphocytes along the intraepithelium and LP (Burns and Maxwell, 1986; Lillehoj and Trout, 1996; Fasano and Shea-Donohue, 2005; Vaughn et al., 2006). Peyer's patches (PP) are large aggregates of lymphatic nodules located in the LP and submucosa of the chicken intestine (Casteleyn et al., 2010). They are very similar to the mammalian Peyer's patches (Befus et al., 1980). The primary and secondary lymphoid follicles of PP are mainly composed

of B lymphocytes and interfollicular regions are rich in T lymphocytes (Befus et al., 1980; Burns and Maxwell, 1986). Lamina propria is populated with fibroblasts, endothelial cells, lymphocytes, macrophages and IgA-secreting plasma cells. The chicken intestinal musculature is composed of four layers, (outer longitudinal, outer circular, inner circular and inner longitudinal), which are directly opposed to one another (Gabella, 1985). This is noticeably different than that of mammalian intestinal musculature which composed of only outer longitudinal layer and the inner circular layer.

2.1.2 Mucosal repair, restitution and apoptosis

Cell turnover refers to the total process of continual cell renewal including proliferation, migration and extrusion. The small intestine is the organ with the fastest cell turnover in the body. The mucosal lining of the intestinal tract consists of a rapidly proliferating and persistently renewing sheet of epithelial cells. In poultry, small intestinal enterocytes are non-polar, round cells at hatch and they increase in length with a defined brush border (microvilli) and distinct polarity from 24-48 h post-hatch (Uni, 2006). In mammals, it is generally believed that the cell mitosis is restricted to the stem cell zone located in the intestinal crypt (Cheng and Leblond, 1974; Quaroni, 1985a; Quaroni, 1985b). In contrast, Uni et al. (1998b) reported that cell proliferation occurs in both the crypt and along the entire length of the villus of the chicken small intestinal epithelium. Undifferentiated stem cells migrate along the crypt-villous axis, where they eventually lose their capacity to divide and differentiate into crypt or villous cells according to their functional specification along the crypt - villous vertical axis (i.e. absorptive enterocytes, mucus secreting Goblet cells and regulatory enteroendocrine cells). Finally, these epithelial cells

further migrate upward to the apex of the villi where they are exfoliated into the intestinal lumen after the induction of cell death (Mammen and Matthews, 2003; Traber et al., 1991).

A severe stress stimulus or a stimulus which persists for a prolonged period results in an irreversible injury in enterocytes. Cell death is the ultimate result of an irreversible cell injury. The process of cell death was identified in two main pathways namely, necrosis and apoptosis. Necrosis is always pathologic and it occurs when the cell is exposed to abnormal stresses such as ischemia and chemical injury. If there is severe damage to the membrane, then lysosomal enzymes enter the cytoplasm and digest the cell. The sequential biochemical and morphological changes in the cell ultimately result in cellular contents leaking out and necrosis. Apoptosis is a genetically controlled, tightly regulated intracellular suicide program occurring in a sequential manner without any accompanying inflammation in the tissues. Apoptosis is not always associated with cell injury as it serves many normal functions. Apoptosis inducing stimuli are recognized as a lack of growth or survival factors, lack of oxygen, physical and chemical injury, metabolic defects and binding of cell surface death receptors to special ligands (Ramachandran et al., 2000). In apoptosis, these stimuli directly damage DNA without causing complete loss of membrane integrity and dead cells are rapidly cleared by phagocytosis. Therefore, apoptosis does not induce an inflammatory response. However, the ultimate result of either apoptosis or necrosis is dependent on the intensity and duration of certain stimuli, the rapidity of the death process in the cell and derangement of the biochemical reactions in the injured cell (Abbas et al., 2005).

Intestinal epithelium repairs daily due to the mechanical damages caused by intestinal motility and physiologic trauma. The disruption is more extensive in the event of infection or by chemical or ischemic mucosal stress. Severe damage to intestinal integrity may facilitate systemic penetration of toxins, immunogens and factors causing generalized inflammatory

response and remote organ pathology (Mammen and Matthews, 2003). Therefore multiple mechanisms are involved in mucosal repair to protect the epithelial continuity in newly hatch chicks, once they are exposed to external stimuli including feed and microbes.

The process of intestinal mucosal repair is composed of restitution, proliferation, maturation, and differentiation of enterocytes. Intestinal epithelial restitution begins immediately after injury and the process of resealing the superficial wound results as a consequence of cell migration to the defective area, without the need of epithelial cell proliferation. During the process of restitution, epithelial cells which border the zone of injury are depolarized and dedifferentiated. Cells are flattened to make the morphologically distinct squamoid appearance and they begin to spread by extending pseudopod like structures called lamellipodia. Finally epithelial cells are re-differentiated and repolarized. The epithelial barrier function is restored by reestablishment of junctional complexes mainly tight junctions between intestinal cells (Lacy, 1988; Nusrat et al., 1992; Dignass, 2001; Mammen and Matthews, 2003). This whole process is termed epithelial restitution, which has been identified as the first step involved in the mechanism of mucosal repair of superficial injury in all regions of the intestinal tract (Mammen and Matthews, 2003). When restitution is completed, cell proliferation and migration are stimulated to restore the mucosal architecture and undifferentiated epithelial cells undergo maturation and differentiation to restore normal intestinal functions.

Villus height and crypt depth are the histological parameters commonly used to reflect cell proliferation in intestinal mucosa. It was reported that the longer villi are correlated with activation of cell mitosis (Samanya and Yamauchi, 2002) and it could also be a compensatory response to reduced nutrient availability (Dilworth et al., 2012). Increased crypt depth also indicates increased mitotic activity within the intestinal crypts that result in increased precursor

cells available to replace the villi cells (Fawcett and Jensh, 2002), suggesting increased mucosal turnover (Yason et al., 1987). However, it is suggested that an increased villus height/crypt depth ratio has been associated with increased digestion and absorption capacity in the small intestine (Pluske et al., 1996; Dilworth et al., 2012).

Proliferating cell nuclear antigen (PCNA) is a well-conserved cell cycle marker protein which is associated with DNA replication, DNA repair and cell cycle control (Strzalka and Ziemienowicz, 2011; Ivanov et al., 2006). Thus the PCNA positivity/abundance in intestinal tissues also considered as an indicator of cell proliferation (Huang et al., 2002; Wang et al., 2003; Willing and Van Kessel, 2007).

It has been reported that the rate of cell migration and proliferation, which is high in newly hatched chicks (Iji et al., 2001a), decreases rapidly when the rate of enterocyte maturation increases (Noy et al., 2001). Further, in chicks, the rapid growth of intestinal mucosa is also accomplished by an initial increased rate of enterocyte migration (Iji et al., 2001a). The migration of enterocytes from crypt to villus tip takes approximately 72 h in a 4-d-old chick while it slows to 96 h in older birds (Uni et al., 2000). The cells which migrate slowly from the synthesis zone are more mature and functionally advanced than the cells which migrate rapidly (Wild and Murray, 1992). The increased rate of cell migration in newly hatched chicks is associated with a shorter life-span for intestinal cells as well as the greater requirement for cell replacement. These processes may occur as a consequence of exposure to dietary components and microbes (Pluske et al., 1997) and this eventually increases the nutrient demand for the intestinal mucosa maintenance.

It has been described that the intestinal epithelium of conventionally-raised animals undergoes physiological inflammation in response to microbial and dietary antigens normally

present. Further, Rolls et al. (1978) reported that the intestinal microbiota in conventional chicks stimulated greater mitotic activity, faster migration and a rapid turnover of epithelial cells than their germ-free counterparts.

The process of intestinal mucosal repair is tightly regulated. A feedback loop between villus tip and crypt has been postulated to regulate the rate of cell loss at the villus tip and the cell production at the base of the crypt (Ramachandran et al., 2000; Watson and Pritchard, 2000), although, the nature of this regulation is not well delineated. Lynch et al. (1986) suggested the normal tissue mass is regulated by the presence of a growth factor and a death factor which regulate mitosis and apoptosis, respectively. Intestinal epithelial renewal is regulated by multiple peptide and non-peptide factors produced by different cell types such as neutrophils, macrophages, myoepithelial cells, platelets (Dignass, 2001).

Apoptosis follows multiple mechanically distinct pathways in intestinal epithelia dependant on the physical position of the intestinal cell along the crypt villous axis, the level of cell differentiation and the stimulation type involved (Watson and Pritchard, 2000).

Physiological occurrence and the anatomical location of apoptosis in the gastro intestinal tract are controversial. Some scientists have revealed that apoptosis may participate in the extrusion process at the villus tip and the apoptotic morphology is only seen once the shed cells are free in the lumen (Shibahara et al., 1995; Mayhew et al., 1999; Watson and Pritchard, 2000). Since most of the important signal transduction events in apoptosis take place in morphologically normal intestinal cells, it is difficult to interpret the exact position where the process of apoptosis begins along the crypt villus axis (Watson and Pritchard, 2000). Studies on chicken intestinal epithelium have suggested apoptotic cells were exfoliated from villus tip and the process may be mediated

by lymphocytes (Takeuchi et al., 1999). Increased villus length in the intestine could be a result of inhibition of apoptosis at the villus tip and increased proliferation in the crypt.

Caspases are cysteine-containing aspartate-specific proteases which activate during the execution phase of apoptosis. These enzymes have multiple cellular targets resulting in degradation of cellular components and activation of particular endonucleases that cleave nuclear DNA and nuclear and cytoplasmic proteins (Ramachandran et al., 2000). The cells destined to die lose the interaction with neighbour cells and become a target for phagocytosis especially by macrophages. Caspase-3, the principal effector enzyme in the apoptotic cascade is activated at the intestinal villus tip (Piguet et al., 1999).

2.1.3 Development of brush border enzymes

The intestinal brush border membrane is composed of a surface microvillus membrane and the underlying BB cytoskeleton. The luminal face of the microvilli membrane forms a carbohydrate-rich 'fuzzy coat' or glycocalyx and a part of it consists of relatively large glycoproteins (70-320 kDa) with specific BB enzyme activities (Holmes and Lobley, 1989). These BB border enzymes are composed of two or more subunits with a small anchoring segment (2-5 kDa) which attach these globular structures to the external face of the membrane (Semenza, 1986). Most of the BB enzymes which were studied (i.e. sucrase-isomaltase, aminopeptidase N (APN), dipeptidyl aminopeptidase IV (DPP IV), gamma -glutamyl transferase, are anchored to the membrane by an N-terminal hydrophobic peptide (Semenza, 1986). But, some BB enzymes have been shown to anchor to the membrane through diacylglycerol moiety of a phosphatidylinositol glycan attached covalently to the C-terminal sequence (Low et al., 1986; Low, 1987). These type of enzymes are alkaline phosphatase (Low,

1987), phosphodiesterase (Nakabayashi and Ikezawa, 1986) and trehalase (Takesue et al., 1986). However, the terminal digestion of dietary macromolecules by enzymes located on the BB membrane is essential in regulating nutrients available for absorption.

Starch is a main constituent in poultry feed which also acts as a major energy source in broiler chickens. Starch molecules are high molecular weight glucose polymers which cannot be absorbed directly requiring enzymatic activity the energy can be absorbed as glucose monomers. Pancreatic and intestinal carbohydrases play an important role in the digestion of ingested sugars since poultry saliva is deficient in amylase (Low and Zebrowska, 1986). Therefore, in birds, pancreatic α -amylase hydrolyzes dietary starch into oligosaccharides (Osman, 1982; Moran, 1985). The composition and the modes of action of the enzyme are similar to that of mammals (Moran, 1985). Glucose oligosaccharides (maltose, maltotriose, α -limit dextrins) and other disaccharides (sucrose, trehalose and lactose) are hydrolyzed at the BB border membrane by intestinal disaccharidases. These processes generate monosaccharides that are readily absorbable by enterocytes. The four groups of enzyme or enzyme complexes which hydrolysis disaccharides are; sucrose-isomaltase (also named palatinase), maltase-glucoamylase, lactase-phlorizin hydrolase and trehalase (Robayo-Torres et al., 2006). It has been reported that the jejunum and ileum are the major sites of disaccharidase activity (Iji et al., 2001b). Moreover, it was found that the disaccharidases in the small intestine of chicken are exclusively produced by the host, but lactase in the large intestine is probably of cecal bacterial origin (Siddons and Coates, 1972). Apart from its function in nutrient digestion, intestinal enzymes may also participate in nutrient transport, signal reception into cells and controlling cell growth and differentiation (Kenny, 1986).

The development of disaccharidase activity in the small intestine of poultry is identified as early as embryonic stage. A very slight sucrase activity was observed on d 9 of embryonic development in leghorn chickens (Brown and Moog, 1967) and the activity of maltase was identified to increase from d 13 to d 21 of embryonic development (Karrer and Parsons, 1968). The necessary disaccharidases are present in the intestine during the period of transition from yolk feeding to solid food post-hatch. Studies using Light Sussex × Rhode Island Red chickens have revealed that sucrase and maltase activity continued to increase up to d 43 after hatch (Siddons, 1969; Dautlick and Strittma, 1970). However, information on changes in intestinal disaccharidase activity during embryonic development and post-hatch in broiler chickens are sparse (Mahagna and Nir, 1996; Uni et al., 1998a). Lactase and trehalase activities are found only in traces in chickens (Siddons, 1969; Dautlick and Strittma, 1970; Chotinsky et al., 2001). Studies in broiler chickens by Uni et al. (1998a) revealed that sucrase and maltase activities in jejunal homogenates were high on d 1 and d 2 post-hatch but decreased thereafter to d 12. The reduced activity of both enzymes from d 7 to d 10 in broiler chickens are more pronounced than in egg-type chickens (Mahagna and Nir, 1996). The reason for the gradual decrease of sucrase and maltase activities may be explained by the variability of epithelial cell type along the crypt-villus axis with age (Holt et al., 1985). However the activity loss per cell or per unit of intestinal mucosa may be compensated by the increase in length of the intestine and mucosal surface area with age (Iji et al., 2001b). In addition, Siddons and Coates, (1972) reported that the production of small intestinal disaccharidases is not directly influenced by GIT microbiota in the chick.

It has been described that there are eight peptidases in the intestinal BB membrane namely, APN, aminopeptidase A, carboxypeptidase P, DPP IV, endopeptidase-24.11, angiotensin converting enzyme (ACE), aminopeptidase P, and gamma -glutamyl transferase (Bai

and Amidon, 1992). The protein fragments and polypeptides derived from the actions of gastric and pancreatic enzymes are further hydrolyzed to di/tri peptides and amino acids by endopeptidases and exopeptidases (Johnson, 2006). Aminopeptidases belong to the exopeptidases and are highly expressed at the small intestinal microvilli (Kim and Brophy, 1976; Kania et al., 1977; Kenny, 1986) and supply amino acids and peptides (Kim and Brophy, 1976) to the enterocytes. These enzymes are anchored to the cell membrane by an N-terminal hydrophobic sequence of 20 amino acids (Feracci and Maroux, 1980; Feracci et al., 1982; Maroux and Feracci, 1983) and have a very short cytoplasmic domain, thus nearly the entire molecule resides on apical surface of enterocytes (Semenza, 1986; Gal-Garber and Uni, 2000). Aminopeptidases have been identified and characterized in chicken intestinal tissues (Jamadar et al., 2003; Gilbert et al., 2007; Damle et al., 2010; Mane et al., 2010). Aminopeptidase N (APN) cleaves neutral and basic amino acids from the N-terminal end of peptides (Sanderink et al., 1988) and higher expression and activity of APN was observed in the ileum compared with the duodenum and jejunum of the chicken (Gal-Garber and Uni, 2000; Gilbert et al., 2007). The study of intestinal APN in broiler chicken is important since their rapid growth is partially depending on dietary amino acids. Sihm et al. (2006) reported that APN expression could be observed as early as 6 d of incubation in the chick's small intestine. However, the expression and activity of disaccharidases and peptidases are connected with the capacity of nutrient digestion and absorption in intestinal epithelium (Moreno et al., 1995).

2.1.4 Overview of intestinal nutrient transport mechanisms

Nutrients are absorbed by enterocytes from the intestinal lumen by active and passive transport mechanisms. Membrane-anchored transporters are responsible for most of the active transport across the BB membrane.

Proteins are digested by proteases and peptidases at the BB membrane and resulting peptides and free amino acids serve primarily as building blocks for structural and metabolic proteins. Numerous studies have revealed that peptides are absorbed faster and more efficiently than free amino acids in the small intestine (Daniel, 2004). The enterocyte apical membrane consists of an electrogenic proton peptide symporter designated as H⁺-dependent peptide transporter 1, PepT1 which has ability to uptake every possible di- and tripeptide released from breakdown of dietary proteins (Daniel, 2004). PepT1 may also have therapeutic importance in transporting peptide-like drugs ('peptidomimetics') such as β -lactam antibiotics, ACE, and anti-viral, and anti-cancer agents (Boyd and Meredith, 2006).

PepT1 has been cloned and characterized in different animal species including the chicken (Chen et al., 2002). The chicken PepT1 (cPepT1) is made up of 714 amino acids with 79.3 kDa total molecular weight (Chen et al., 2002). The cPepT1 exhibits several characteristics similar to mammalian PepT1 including 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 (Chen et al., 2002). The binding affinity and transport efficiency of PepT1 depend on the substrate size, charge, hydrophobicity, amino and carboxy termini modifications, side chain flexibility, presence of proline and stereospecificity (Amasheh et al., 1997). Di or tri peptides and other substrates of PepT1 are transported by a proton-coupled transport mechanism (Fei et al., 1994) dependent on pH gradient and negative intracellular membrane potential (Steel et al., 1997).

The highest PepT1 mRNA expression in chicken is observed in the small intestine with low expression in kidney and ceca (Chen et al., 1999; Chen et al., 2002). The intestinal PepT1 protein expression level may be evidence of maturity of enterocytes since the expression decreases from apex to the base of the villus (Ogihara et al., 1999). Further, PepT1 is not

expressed in the crypts in mice and rats (Ogihara et al., 1999). In chickens, PepT1 is developmentally regulated. PepT1 mRNA abundance increases 14 to 50 fold from embryonic day 16 to day of hatch (Chen et al., 2005). Several researchers have revealed that diet also has an impact on PepT1 gene expression (Erickson et al., 1995; Shiraga et al., 1999; Chen et al., 2005). In support, Shiraga et al. (1999) and Walker et al. (1998) showed that the intestinal PepT1 mRNA expression levels were up-regulated with the increased levels of dietary peptides.

Free amino acids (neutral, cationic, and anionic) are transported into the enterocytes via brush border amino acid transporters, which have varying substrate specificities (Kanai and Hediger, 2004; Palacin and Kanai, 2004; Verrey et al., 2004), but most of the time the specificities are overlapped (Palacin and Kanai, 2004). In addition, EAAT3 is considered a Na⁺-dependent amino acid exchanger on the apical membrane with a high affinity for anionic amino acids (Kanai and Hediger, 2004). Amino acid transporters have been reported in chick intestines from the late embryonic to early post hatch stages (Gilbert et al., 2007; Gilbert et al., 2008; Li et al., 2008). Further, the ileum has been reported as an important site for the assimilation of free amino acid in the growing chicks (Gilbert et al., 2007).

Glucose is the major monosaccharide available from the diets of farm animals and it is the primary energy source assimilated into the enterocytes by sodium-dependent glucose transporter 1 (SGLT1) (Hediger and Rhoads, 1994; Ferraris, 2001; Sklan et al., 2003; Wright and Turk, 2004). SGLT1 is composed of 14 transmembrane domains, and has a predicted weight is approximately 73 kD (Wright and Turk, 2004). This nutrient transporter is highly expressed in the small intestinal BB membrane (Hediger and Rhoads, 1994; Wright and Turk, 2004). It has been reported that SGLT1 protein present in several species including the chicken (Gal-Garber and Uni, 2000; Gal-Garber et al., 2000). Studies on cellular distribution of SGLT1 in chickens

revealed that the specific nutrient transporter is expressed throughout the length of the villus, but similar to Pept1, not in the crypts of enterocytes (Barfull et al., 2002). However, studying the expression of intestinal SGLT1 is essential as it is the only known intestinal glucose transporter in chickens (Wright and Turk, 2004) and glucose plays a significant role in overall energy homeostasis.

SGLT 1 co-transporters one molecule of Na^+ and one molecule of glucose into enterocytes (Hediger and Rhoads, 1994; Wright and Turk, 2004). A transepithelial osmotic gradient is generated for Na^+ by transportation of Na^+ from cytosol into the extracellular fluid by Na^+/K^+ ATPase at the basolateral membrane of enterocytes. Glucose absorption also facilitates paracellular fluid absorption across the BB membrane. The glucose transported to the cytosol by SGLT1 leaves the cell at the basolateral surface via facilitated transport through glucose transporter 2 (GLUT2). This contributes to an osmotic gradient between the paracellular space and the intestinal lumen driving paracellular water absorption (Wright and Loo, 2000; Wright and Turk, 2004). The expression of SGLT1 in the chicken is regulated by different factors including dietary composition (Gilbert et al., 2008; Liu et al., 2008a) and intestinal development stage (Uni et al., 2003; Gilbert et al., 2007). It has been found that elevated dietary sugar concentrations increase SGLT expression (Miyamoto et al., 1993).

2.2 Intestinal Immunology and inflammation

The intestinal epithelium represents an extensive surface area which mediates a critical interface between the animal host and a wide variety of antigens such as dietary substances, microorganisms, and exogenous toxins. Therefore, a mucosal defense mechanism is essential in the intestine to prevent penetration of harmful compounds and pathogenic agents while permitting the exchange of nutrients between the gut lumen, intestinal epithelium and the

systemic circulation. The gastrointestinal immune defense system in chicken is classically broken down into innate and adaptive immunity. The innate immune system provides an immediate non-specific response against the invading microorganisms and also sends messages to activate T lymphocytes to generate the antigen specific adaptive immune response (Yuan and Walker, 2004). The adaptive immune system takes days to weeks to elicit its maximum response by T and B lymphocytes, which results in antigen-specific humoral and cellular responses and immunologic memory (Yuan and Walker, 2004; Rakoff-Nahoum and Bousvaros, 2010).

The immune system plays an important role in commercial birds reared in intensive conditions due to their vulnerability to rapid spread of infectious agents and disease outbreaks (Sharma, 2003). If pathogens enter to intestinal tissues, the initial response is an inflammatory response, which results in behavioral, immunologic, vascular and metabolic responses in the host (Korver and Klasing, 2004). The energy expenditure for the immune activation to the GI antigens may negatively impact feed efficiency and divert nutrients away from animal production (Collett, 2004; Korver, 2006). However, these responses ultimately result in loss of skeletal muscle, decreased appetite, reduced growth rate, morbidity and in some situations mortality (Korver and Klasing, 2004).

2.2.1 Barrier function and innate immunity

Intestinal barrier function is described as the maintenance of host integrity by effective monitoring of the mucosal surface to prevent the access or penetration of potential harmful agents such as microorganisms, toxins and antigens to the underlying tissues (Grootjans et al., 2010). A continuous monolayer of epithelial cells in the intestinal mucosa serves as a selective physical barrier, separating the contents of luminal environment from the layers of tissue

comprising the interior milieu (Gordon, 1989; Gasbarrini and Montalto, 1999). The epithelial cells are joined firmly together by tight junctions (Madara et al., 1990; Fasano and Shea-Donohue, 2005). Further, these highly regulated gates respond to the activities in the lumen and to the signals and messages from LP and the epithelium itself (Perdue, 1999). In addition, intestinal epithelial cells (IEC) secrete a variety of compounds that participate in the innate immune defense such as mucins, antimicrobial peptides and cytokines. Mucins are synthesized and secreted by Goblet cells through baseline secretion and active exocytosis (Perez-Vilar and Hill, 1999), and act as a physical barrier against bacterial translocation. Also, mucins play an important role in epithelial growth and repair (Perdue, 1999).

Antimicrobial peptides (AMPs) are an important component of the innate immune system and are divided into two main families, defensins and cathelicidins (Ganz, 2003). It has been reported that the tissues of chicken express both defensins (Sugiarto and Yu, 2004; Wehkamp et al., 2004; Bar-Shira and Friedman, 2006; van Dijk et al., 2007) and the cathelicidins (Lynn et al., 2004; van Dijk et al., 2005; Xiao et al., 2006; Goitsuka et al., 2007) as in mammals.

Defensins are cysteine -rich cationic peptides which constitute an integral component of the innate immune system. In mammals, two main classes of defensins are distinguished according to the positions of their disulfide bonds. They are alpha-defensins and beta-defensins (Oswald, 2006; Menendez and Finlay, 2007). The α -defensins have only been identified in humans, monkeys and rodents, and are expressed in different cell types, including granule-containing granulocytic leukocytes and intestinal Paneth cells. The epithelial cells lining the respiratory, gastrointestinal and urogenital tracts synthesize β -defensins (Fellermann and Stange, 2001). Further, a third class of defensins named tetra-defensins, which is composed of a different disulfide motif, has been identified only in rhesus monkeys (Tang et al., 1999; Ganz, 2003).

Defensins possess a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, protozoans, fungi and some enveloped viruses (Ganz, 2003). Although the actual mechanism of antimicrobial activity is not clear, many researchers have proposed that the dimer formed by binding of cationic sites of the β defensins to the negatively charged bacterial membrane may alter transmembrane potential and membrane permeability, which leads to cell death (Evans and Harmon, 1995; Sugiarto and Yu, 2004; Oswald, 2006; Achanta et al., 2012). Thus they play a crucial role in host defense and disease resistance. It has been reported that they enhance phagocytosis, neutrophil recruitment, production of pro-inflammatory cytokines, suppress anti-inflammatory mediators and regulate complement activation. Also they can be chemokines and adjuvants (Yang et al., 2002).

Beta-defensins are the only defensins expressed in avian species (Lynn et al., 2007). As Paneth cells appear to be scarce in birds (Bezuidenhout and Vanaswegen, 1990), macrophages, epithelial cells and heterophils have been shown to be the main source of avian defensins (Brockus et al., 1998; Harmon, 1998; Bar-Shira and Friedman, 2005). Beta-defensins in chickens are also called gallinacins. A total of 14 different β defensins were identified in chicken genome and these genes are designated as avian beta-defensin (AvBD) 1-14 (Lynn et al., 2007). A study on the immune protection in the digestive tract during the first week of life in chicken reveals that there is elevated expression of β defensin mRNAs at the day of hatch throughout the intestine and it is subsequently decreased during the first week of post-hatch (Bar-Shira and Friedman, 2006). Infection with bacteria or their components has been shown to induce the expression of AvBD genes in chickens (Yoshimura et al., 2006; Milona et al., 2007; Subedi et al., 2007). Further, it has been reported that the reaction of AvBD to bacterial membrane

components leads the production of proinflammatory cytokines such as interleukin-1 β , interleukin-6 and tumor necrosis factor alpha (Hasenstein et al., 2006).

The intestinal epithelial lining is highly populated by intraepithelial leukocytes including natural killer (NK) cells. NK cells have been described as large granular lymphocytes, with both cytotoxicity and cytokine-producing effector functions (Trinchieri, 1989). Thus, they play an important role in first line of defense against invasive pathogens in the intestinal tissue (Lodoen and Lanier, 2006). In chicken, NK cells are abundant in intestinal epithelium, but are less detectable in peripheral blood lymphocytes and spleen (Goebel et al., 2001). Also, it has been reported that NK cell expression in chickens was minimal in chicks during the first few weeks after hatching but increased as the birds became older (Sharma and Coulson, 1979).

Cytokines are small peptide molecules, which are produced by immune cells (lymphocytes, macrophages, dendritic cells etc.) as well as by IEC. The production of cytokines is stimulated by invasion of enteropathogens or by simply binding of microbes to epithelial cells. Some cytokines which are constitutively expressed by the intestinal epithelial cells, such as TGF- α , IL-1, IL-10, IL-15, and IL-18 play a role in the basal influx of immune cells into the intestinal mucosa, epithelial cell growth and in homeostasis (Stadnyk, 2002). Further, the normal intestinal epithelial cells express some other cytokines such as IL-1 α or β , IL-6, IL-8, and TNF- α , MCP-1, CCL20, and GM-CSF and are markedly up-regulated in response to microbial infection (Jung et al., 1995; Stadnyk, 2002). These cytokines induce physical changes in the host animal clinically characterised by fever, anorexia, negative nitrogen balance and catabolism of muscle cells (Kraft et al., 1992; Ingenbleek and Young, 1994; Van Miert, 1995; Langhans, 1996). Further, the cytokines travel through the blood to the liver where they trigger hepatocytes to synthesize and secrete acute phase proteins. The interaction of microbes with the epithelial cell apical membrane

may result in phosphorylation of critical enzymes (myosin light-chain kinase, protein kinase C) and rearrangements of F-actin and associated proteins. This increases the permeability of tight junctions (Yuhan et al., 1997; Philpott et al., 1998). Further, it is documented that the cytokines increase intestinal epithelial permeability and thus, alter its barrier properties (McKay and Baird, 1999; Perdue, 1999).

The gut associated lymphatic tissue provides safe passage of paracellular macromolecules while preventing harmful antigens from entering the systemic circulation and inducing systemic tolerance (Fasano and Shea-Donohue, 2005). It provides the first line of defense against bacterial, viral and parasitic antigens introduced via GIT (Rothwell et al., 1995; Lillehoj and Trout, 1996; Mast and Goddeeris, 1999; Muir et al., 2000). The studies by Lillehoj and Chung (1992), Van Immerseel et al. (2002) and Bar-Shira et al. (2003) revealed that there was an infiltration of granulocyte and T-lymphocyte to cecal lamina propria in 7d old chicks. Further, some gnotobiotic studies revealed delayed immune cell development in the lamina propria in germ-free birds compared with conventionally reared animals (Umesaki et al., 1993; Rothkotter et al., 1994; Umesaki et al., 1999; Gaskins, 2003). Innate immune response is very significant in young chicks since the acquired immune system is not fully developed until they are one week old (Bar-Shira et al., 2003). Pattern recognition receptors (PRRs) are transmembrane or intracytoplasmic receptors which have an ability to bind specific microbial ligands designated pathogen-associated molecular patterns (PAMPs) (Werling and Coffey, 2007; Meade et al., 2009; Santaolalla and Abreu, 2012). There are four distinct classes of PRR families have been identified including, transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) such as the mannose receptors (MRs), cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs).

These PRRs sense the presence of distinct microbial ligands such as lipopolysaccharides (LPS), peptidoglycans, flagellin, bacterial DNA; viral double stranded RNA and probably the compounds in the cell wall of yeasts.

The most studied PRRs are TLRs that are type I transmembrane glycoproteins consisting of an extracellular N-terminal domain which has leucin-rich repeats (LRR) and one or two cysteine-rich regions, a transmembrane domain and a conserved intracellular Toll/interleukin-1 receptor (TIR) domain (Collier-Hyams and Neish, 2005; MacDonald and Monteleone, 2005; Cario, 2008; Brownlie and Allan, 2011). A series of studies has demonstrated that intestinal epithelial Toll-like receptors (TLRs) elicit an immune response to maintain barrier function (Cario, 2008). Different types of TLRs have been described in mammals and birds (Rock et al., 1998; Luo and Zheng, 2000) and according to the literature, a wide class of macromolecules acts as TLR ligands including proteins, lipids, and nucleic acids, such as lipopolysaccharide (LPS), lipoteichoic acids, lipoproteins, peptidoglycans, zymosan, flagellin, dsRNA, and unmethylated CpG (Aderem and Ulevitch, 2000; Aderem, 2001). Therefore, TLRs are important in identification and discrimination of distinct microbial components (Akira et al., 2006). For example, TLR2 has been shown to signal the presence of lipopeptides from Gram positive bacteria or molecules on the bacterial and yeast cell wall, whereas TLR4 is the predominant receptor for LPS from Gram-negative organisms (de Paiva et al., 2011). These TLR2 and TLR4 have been the most extensively studied TLRs to date. The LRR domain recognizes PAMPs and activates signal-transducing proteins that initiate a cascade of signals (Akira and Takeda, 2004). It is speculated that PRR-induced signal transduction pathways result in the synthesis of a broad range of molecules, including cytokines and chemokines (Akira et al., 2006).

Two predominant intracellular TLR pathways have been identified in the TLR signaling cascade, namely, the MyD88-dependent and the TRIF-dependent pathway (MyD88-independent pathway). The MyD88-dependent pathway uses the adapter molecule MyD88 and leads to early activation of NF- κ B which leads to the transcription of proinflammatory genes, such as IL-1, TNF- α , IL-6 and IL-8 (Yuan and Walker, 2004; Collier-Hyams and Neish, 2005; Turin and Riva, 2008) from monocytes, macrophages, and dendritic and endothelial cells (Aderem and Ulevitch, 2000; Kadowaki et al., 2001; Medzhitov, 2001). The latter pathway signals through Toll-IL-1R domain-containing adaptor-inducing IFN- β and produce cytokines such as IFN- β (Yamamoto et al., 2002; Oshiumi et al., 2003). Also, this pathway may activate through NF- κ B in a delayed fashion and leads to the production of TNF and other inflammatory cytokines (Covert et al., 2005). All the TLRs, except TLR3 and TLR4, signal exclusively through the MyD88-dependent pathway. TLR4 activates both pathways while TLR3 identifies viral dsRNA and induces inflammatory cytokines such as TNF and IL-6 exclusively through the MyD88-independent pathway (Kawai et al., 1999; Hoebe et al., 2003). However, cytokines produced by both pathways, trigger secondary pathways which alarm the host about the invading pathogens and lead to the immune activation (Zhong et al., 2006).

There are ten TLR genes (TLR1A, 1B, 2A, 2B, 3, 4, 5, 7, 15, and 21) that have been identified in chickens (Boyd et al., 2001; Yilmaz et al., 2005; Temperley et al., 2008; Nguyen et al., 2011). TLR2 and TLR4 have been characterized at the molecular and functional level in chicken (Boyd et al., 2001; Fuku et al., 2001; Leveque et al., 2003). In mammals, TLRs have been found in apical and basolateral surfaces of enterocytes as well as intracellularly. Apart from IECs, other cell types in the intestine, including macrophages, dendritic cells, B cells, T cells and stromal cells, can express TLRs (Abreu, 2010). The expression of TLRs by IECs is generally low

in the normal intestinal tissues, but it will increase during intestinal inflammation (Cario and Podolsky, 2000; Naik et al., 2001; Bogunovic et al., 2007). For example, it has been reported that the level of TLR2 and TLR4 expression is low in normal adult colon, but TLR4 found to be highly expressed on the apical side of colonic IECs from patients with active Crohn's disease (Cario and Podolsky, 2000). However, cellular localization of TLRs has not been reported on chicken intestine.

2.2.2 Mechanisms and consequences of intestinal inflammation

It is obvious that gastrointestinal tract is susceptible to inflammatory responses due to its continuous exposure to antigenic, mitogenic, and toxic stimuli. These stimuli, arising from food, the environment and the resident microbiota, contribute to disturbances in immune or epithelial homeostasis and lead to gut inflammation. In fact, intestinal mucosa displays a state of "physiological inflammation" even under normal conditions. This is evident by profuse leukocytes present in the intraepithelial and subepithelial tissues and a basal level of expression of pro and anti-inflammatory mediators.

Inflammation is the most common type of response that occurs in the body as a result of the defense mechanism against constant and massive environmental challenges. The outcome of intestinal inflammation could be categorized as acute, if there is resolution and return of tissue function, or as chronic if there is persistent tissue dysfunction. Inflammatory response, irrespective of whether it is acute or chronic; results as a consequence of the combined action of triggers/initiators (e.g. PAMPs or microbial-associated molecular patterns), sensors (e.g. TLRs), mediators (e.g., cytokines and chemokines) and effectors (e.g. macrophages, NK cells and heterophils) (Sartor, 2002; Rakoff-Nahoum and Bousvaros, 2010; Newton and Dixit, 2012;

Santaolalla and Abreu, 2012). Invasion of microbial pathogens, leads to a cascade of immune responses starting with recognition of PAMPs by pattern recognition receptors (PRRs, e.g. toll-like receptors). This initiates the activation of immune cells such as; neutrophils, macrophages and T helper 1 cells (T_{H1}) in bacterial and fungal infections, eosinophils, mast cells, and T helper 2 cells (T_{H2}) in parasitic infections, cytotoxic T cells in viral infections (Sartor, 2002; Newton and Dixit, 2012). Epithelial cells and antigen presenting cells (APCs) respond to the tissue injury by secreting chemokines, cytokines and leukotriens. Antigen-specific immunoglobulins released by plasma cells facilitate pathogen clearance. Therefore both innate and adaptive immune mechanisms play significant roles to minimize pathogen invasion and tissue damage. All compartments of the intestinal tract are susceptible for acute inflammation due to pathogenic infections, luminal toxins and ischemia. Acute inflammation lasts hours to days depending on the type and severity of tissue damage and the affected tissue responds by vascular dilation, endothelial activation and neutrophil activation. The inflammatory process is subsequently downregulated and the injury may be healed with resolution and return of tissue function. If intestinal tissue shows defective mucosal barrier functions (either congenital or acquired) or dysregulated immune responses, the tissue injury further progress to chronic inflammatory state.

Different groups of inflammatory cytokines are involved in acute and chronic inflammation. Interleukin-1(IL-1), TNF-alpha, IL-6, IL-11, and IL-8 are responsible in acute inflammation while the cytokines involved in chronic inflammation can be subdivided into cytokines mediating humoral responses such as IL-4, IL-5, IL-6, IL-7, and IL-13, and those mediating cellular responses such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, transforming growth factor-beta, and tumor necrosis factors alpha and beta. Analysis of gene expression data and histo-morphological parameters can be used to determine the inflammatory

response of the intestine. A study of newly hatched chicks shows an increased pro-inflammatory cytokine, IL-1 β and chemokine, IL-8 expression, which in turn reveals the response of gut immune system to feed and the commensal bacteria immediately after hatching (Bar-Shira and Friedman, 2006). Also, there are several reports on inflammatory responses in the intestinal tissues after bacterial challenges in chicken. It has been reported that up-regulation of mRNA expression in cytokines and TLRs in chickens infected with H9N2 influenza virus (Nguyen et al., 2011). Another study by showed the expression of IL-1 β , IL-6 and interferon- γ mRNA were increased 10 d post- challenge with *Salmonella typhimurium* infected broiler birds (Fasina et al., 2008). Further, the changes in innate immune gene expression including TLR2, TLR4, IL6 and IL8 were observed in chickens after in vivo challenge with commensal (*Campylobacter jejuni*) and pathogenic (*Salmonella enterica*) bacteria (Shaughnessy et al., 2008; Shaughnessy et al., 2009).

2.3 Role of microbiota in the intestinal physiology of avian host

The microbial community of the intestinal tract is composed of unicellular microorganisms such as bacteria, fungi and protozoa (Gabriel et al., 2006). A large number of studies have revealed that the digestive flora of birds is different from that of monogastric mammals (Smith, 1965; Lei et al., 2012) and the reason for this variance may be due to the distinct anatomy and physiology of avian GIT. In chicken, the dominant bacterial activity is found in the crop and ceca and less activity is found in small intestine (Gabriel et al., 2006).

The gastrointestinal tract of the bird is theoretically sterile and the gut epithelium is relatively undifferentiated at the time of hatch prior to invasion by organisms present in the feed, water and the surrounding environment. The diversity of the GIT microbial community in the

chicken depends on numerous factors including age, breed, specific location in the GIT (e.g. small intestine, ileum, cecum), diet and geographic location (Apajalahti et al., 2001; Apajalahti et al., 2002; Apajalahti et al., 2004). The establishment of the microbiota is affected by the conditions at post-hatch period and the microbial profile becomes more complex and stable as chickens age (van der Wielen et al., 2002; Lu et al., 2003; Lan et al., 2005) .

In poultry, the crop, duodenum and ileum are predominantly colonized by *Enterococci* and *Lactobacilli* during the first week of life whereas the ceca are mainly colonized by coliforms, *Enterococci* and *Lactobacilli* (Barnes et al., 1972; Mead and Adams, 1975; van der Wielen et al., 2000; Snel et al., 2002). After the first week of life, lactobacilli become the majority in the small intestine and *Escherichia coli*, *Clostridium* and *Bacteroides* species reside in high numbers in ceca (Lev and Briggs, 1956; Mead and Adams, 1975; Amit-Romach et al., 2004; Dumonceaux et al. 2006b). The intestinal microbiota is fully established and stable by 2 to 3 weeks of age in broiler chicken (Barnes et al., 1972; van der Wielen et al., 2000; Snel et al., 2002).

Studies have demonstrated that the diet plays a significant role in the determination of microbial ecology of the avian gut (Jensen, 1993; Apajalahti, 2004). So, the chemical composition of the digesta favors the growth of specific microbes (Apajalahti et al., 2004). Studies on birds fed with different dietary components such as fiber components (Amerah et al., 2009; Choct et al., 1996) and feed additives such as antibiotics (Dumonceaux et al. 2006b), prebiotics, probiotics (Fuller, 1989; Ohimain and Ofongo, 2012) and enzymes (Choct, 2009) have been shown to influence gut microbial population and activity. The interaction between the dietary composition and gut microorganisms regulates the intestinal development, mucosal architecture and mucus composition of the gut (Apajalahti et al., 2004).

2.3.1 Effect of microbiota on nutrition and performance in chickens

Intestinal microbiota play a significant role in nutrition, health, and growth performance of the host animal (Barrow, 1992) by regulating gut morphology, nutrition, pathogenesis of intestinal diseases and immune related responses. During the early post-hatch period, the microorganisms which colonize the gut form a synergistic relationship with their poultry host. The digestive flora is found in the gut lumen, submerged in the mucus layer or forming a cell layer by adhering to the digestive mucosa (Fuller, 1984). Gastro intestinal microbiota can be categorized into two main groups, potential pathogenic organisms and beneficial commensals, according to their activity in the gut. However, the composition and activity of gut microbiota, may affect either positively or negatively on the health and growth of birds.

There are several metabolic activities performed by micro-organisms in the chicken GIT. Among them some activities are beneficial to the host, such as synthesis of nutrients, uptake or modification of nutrients (Maisonnier et al., 2003), production of digestive enzymes and detoxification of food constituents or endogenous products. Others are detrimental such as production of noxious metabolites and release of toxins. Commensals are normal inhabitants of GIT, which may be involved in the provision of nutrients to the host, immune stimulation and competitive exclusion of harmful organisms by inhibiting their growth and establishment (Jeurissen et al., 2002; Tellez et al., 2006; Dibner et al., 2008). Competitive exclusion (CE) is a phenomenon which limits the colonization of pathogenic bacteria by certain beneficial commensal bacteria that reside in the gastro-intestinal tract (Nurmi and Rantala, 1973). Competitive or antagonistic effects of CE bacteria against opportunistic pathogens are mediated by several mechanisms including the production of antimicrobial metabolites (e.g. bacteriocins, SCFA, hydrogen peroxide and other metabolites of oxygen), occupying or modifying the

receptors which bind pathogens or their toxins, or competing for essential nutrients (Gabriel et al., 2006).

Poultry do not produce enzymes which digest non-starch polysaccharide (NSP) fraction of dietary fibre. However, the cecal microbiota use NSPs as their main sources of carbon and energy and are able to hydrolyse these undigested carbohydrates to short chain fatty acids (SCFA) and some gases in chickens (Pinchasov and Elmaliah, 1994; Jorgensen et al., 1996; Jamroz et al., 1998; Jamroz et al., 2002). The SCFA produced as a result of microbial fermentation are primarily comprised of acetate, butyrate and propionate (Jamroz et al., 1998). It has been reported that the concentration of SCFAs increase from very low levels at day 1 chicks to high levels at day 15, after which they then stabilize (Van der Wielen et al., 2000). Short-chain fatty acids elicit beneficial effects in the avian host such as providing extra energy (better feed conversion ratio), accelerating intestinal epithelial cell proliferation (changing intestinal morphology/increasing intestinal tissue weight) and inhibiting the growth of the pathogenic bacteria (lowering gut pH) (Jozefiak et al., 2004; Tellez et al., 2006; Adil and Magray, 2012). A previous study by Jozefiak et al., 2004 has provided evidence that SCFAs provide up to 8 % of the energy requirement in chicken.

Gut microbiome of poultry are capable of synthesizing vitamins (mainly vitamin B), but these vitamins are excreted with feces since they are not absorbed by cecum (Coates, 1980). Coprophagic birds may have the benefit of bacterial vitamin synthesis. In addition, cecal bacteria catabolize uric acid to ammonia, which can be absorbed by the host to synthesize some amino acids (Vispo and Karasov, 1997). Some dietary nitrogen is incorporated into the cellular protein of bacteria, thus gut bacteria themselves may be a source of amino acids (Metges, 2000). However, the majority of bacterial proteins are lost with bird feces since the cecum does not

have the ability to digest or absorb these proteins. Therefore, these bacterial proteins can be only utilized by coprophagic birds (Koutsos and Arias, 2006; Vispo and Karasov, 1997). Further, the gut microbiota contributes to the absorption of some minerals in the ceca such as sodium whilst they have some negative effects on the absorption of calcium and magnesium (Braun, 2003; Jozefiak et al., 2004; Adil and Magray, 2012).

Adverse effects of the gastrointestinal microorganisms affect animal production with respect to the growth of animals and the quality of their products. The digestive microbiota competes with the host for the dietary ingredients, particularly the poorly digestible feedstuffs. When the intestinal tract is invaded by pathogenic bacteria, they provoke a harmful effect in the host, by creating local or systemic infections, intestinal degeneration and toxin production. Activation of innate immunity due to microbial invasion occurs at a very high metabolic cost and results in a reduction in feed intake and nutrient utilization, thereby repartitioning nutrients away from growth and towards the immune response (Johnson, 1997). Ultimately, pathogenic invasion may cause either low-grade damage (e.g. Decreased body weight gain, poor feed conversion efficiency) or severe enteric damage, notably overt disease and high mortality (Yegani and Korver, 2008). Moreover, digestive microbiota is also able to alter the composition and organoleptic quality of meat (Gabriel et al., 2006).

The intestinal microbiota has an ability to alter the functional physiology of the avian host. Gnotobiotic techniques have made it possible to identify the effect of GIT microbes on nutritional and physiological characteristics in chickens. The term “gnotobiotic” is described as an animal or system in which all existing life forms are known. With respect of the microbial inhabitants, the gnotobiotic animals can be classified into germ-free or animals associated with a known number of microbial strains, *i.e.* mono-associated, conventionalized or specific pathogen

free (Coates, 1973; Furuse and Okumura, 1994). The results of the gnotobiotic studies cannot always be solely applied to the animals in normal conventional conditions, since the biological features of host and microbes may vary from real rearing conditions to gnotobiotic systems.

Studies using gnotobiotic chickens can be used to investigate the effect of gut microbiota on nutrient metabolism and performance characteristics in the host. Variable responses of brush border disaccharidase activities to germ-free conditions in different animal species have been reported. Higher disaccharidase activities in the small intestine of GF rats (Reddy and Wostmann, 1966) and GF pigs (Willing and Van Kessel, 2009) were recorded compared to their CV counterparts and explained by the presence of more mature enterocytes due to slow rate of cell turnover in GF animals. However, in gnotobiotic studies using chicks, there was no direct effect of microbial status on disaccharidase production observed in the small intestine (Siddons and Coates, 1972).

2.3.2 Effect of microbiota on intestinal morphology and digestive function

The gastro-intestinal microbiota results in anatomical and physiological changes in the intestine due to its interaction with the mucosa and the production of some metabolites (Coates, 1980; Furuse and Okumura, 1994). The absolute and relative lengths (length/body weight) of the small intestine in CV chicks are higher compared to their GF counterparts (Furuse and Yokota, 1984b). Also, it has been reported that the small intestine and cecum of GF birds had a reduced weight and a thinner wall compared to their conventional counterparts (Furuse and Okumura, 1994; Gabriel et al., 2006). This may be mainly associated with increased cellularity in the lamina propria (Miniats and Valli, 1973) and the thickening of the submucosa and muscular layers (Shurson et al., 1990; Furuse and Okumura, 1994; Gaskins, 1997).

An increase in villus height has been reported with microbial colonization in the broiler chicken. In conventional birds, intestinal villi are higher compared with their germfree counterparts (Cook and Bird, 1973; Gabriel et al., 2003; Gabriel et al., 2006). Similarly; conventional chicks have been shown to have longer villi than the birds with a low bacterial load (Forder et al., 2007). Further, the dietary addition of probiotics have also shown increased villus height in broiler chicken (Chichlowski et al., 2007; Samli et al., 2007; Awad et al., 2010). Commensal bacteria play important roles in the metabolism of several nutrients which are unable to be digested by the host. Further, it has been reported that luminal and systemic SCFA induce mucosal proliferation by increasing plasma glucagon-like peptide-2, ileal pro-glucagon mRNA, and glucose transporter -2 expression and protein (Adil and Magray, 2012). Thus, SCFA produced by microbial activity have a direct impact on mucosal proliferation and villus morphology, thereby increasing the intestinal absorptive surface area (Galfi and Bokori, 1990; Sakata and Inagaki, 2001). However, (Gabriel et al., 2006) reported that the intestinal microvilli are less regular and the surface area of microvilli per surface unit is less in CV birds than in GF birds. The authors also stated that CV chicks have deeper crypts and a higher number of dividing cells than the GF birds. Further, it has been reported that the transit rate of intestinal epithelial cells is lower in GF chickens than in the CV state (Rolls et al., 1978). These reports suggest that the increased microbial activity at the level of the brush border may increase both the damage to enterocytes and the need for cell renewal in the gut. Therefore, in the presence of microbiota, enterocytes reach the top of the villi more rapidly and are less mature. As a result, the total activity of digestive enzymes may decrease in CV animals (Corring et al., 1981; Gabriel et al., 2006). But the presence of microbiota has been reported to not show a significant difference in the intestinal disaccharidase activities in chickens (Siddons, 1969; Siddons and Coates, 1972).

GF chicks do not exhibit enlarged ceca as in GF rabbits and rodents and there is no difference in the transit time of food through the gut compared to CV birds (Ford, 1971).

Bacteria extensively modify endogenous and exogenous cholesterol in the intestinal tract and biliary bile acid pattern. Primary bile acids are subjected to deconjugation, dehydrogenation, dehydroxylation, and sulfation reactions by intestinal microbes based on the nature of the secondary bile acids (Midtvedt, 1974). Gnotobiotic animal studies have revealed a higher total body pool of cholesterol in GF animals compared to their CV counterparts (Kellogg and Wostmann, 1969; Wostmann, 1973; Claus et al., 2008). Thus, it is evident that in the GF state, there is an increase in the efficiency of bile acid reabsorption, related to either lack of microbial conversion in the lower gut or to a decreased intestinal excretion in the absence of an intestinal microbiota (Abrams and Bishop, 1967). Further, high concentrations of bile acids in the small intestine increase the absorption of Ca and Mg (Wostmann, 1981). Fat digestion and absorption are influenced by the bile acids and their salts secreted into the gut. Boyd and Edwards, 1967 reported that the GF chickens utilize dietary fats somewhat better than their CV counterpart. Catabolism of bile acids by a variety of intestinal bacteria reduces digestion of fat and fat soluble vitamins (Engberg et al., 2000), reduces absorption of lipids (Eyssen, 1973) and produces toxic degradation products which lead to depressed growth performance (Baron and Hylemon, 1997) and an increased incidence of disease.

2.3.4 Role of microbiota in host protein and energy nutrition

Feed occupies the largest cost factor in broiler production with the cost of ingredients providing energy a key consideration. The net energy utilization in chicken is influenced by the energy requirement of the gut. Host-related factors which affect the energy requirement in the

intestines are identified as gut structure (intestinal growth and maintenance, overall surface area of the gut), rate of passage of digesta, capacity to digest and absorb nutrients, barrier function and the activity of gut microbiota (Hughes, 2003). Utilization of dietary energy and the increased intestinal protein synthesis by intestinal bacteria increase the energy requirement for the maintenance (Furuse and Yokota, 1984b; Furuse and Okumura, 1994). Dietary factors which increase the gut microbial activity may decrease energy utilization (Choct, 1999). Further, the energy requirement is increased due to the energy spend for detoxification of various substances produced by intestinal bacteria.

Microorganisms reside in the GIT play an important role in nitrogen economy in birds. They (directly or indirectly) influence the protein metabolism in the gut to a larger extent compared with that in other organs (Muramatsu et al., 1987). These organisms reduce the proteins present in the digesta since they utilize the endogenous proteins (e.g. proteins produced from mucus, cellular debris, and bacterial biomass) and dietary proteins not hydrolysed by the host (Gabriel et al., 2006). However, it has been reported that GF and CV chickens have similar proteases in the small intestine (Salter and Coates, 1971). Therefore, the nitrogenous compounds of the digesta in crop, proventriculus, gizzard, duodenum, jejunum and ileum had little difference between GF and CV chicks. In contrast, Furuse and Okumura (1994), found considerable differences in the nitrogen content of digesta of the lower gut contents and excreta of GF and CV; the authors suggested that additional protein digestion was mediated by microbial proteolytic enzymes in CV hind gut.

Gut microbiota are able to break down nitrogenous compounds (dietary and urinary) into SCFA and ammonia. Although this fermentation is observed throughout the avian gut (from crop to cecum), this is primarily seen in the cecum, which is densely populated with bacteria. Short

chain fatty acids are an energy source for enterocytes and the host (Jozefiak et al., 2004), while ammonia is a source of nitrogen in microbial protein synthesis (Stevens and Hume, 1998; Jozefiak et al., 2004). Alternatively, some bacteria ferment amino acids, producing high concentration of ammonia that may reduce animal growth (Pond and Yen, 1987; Veldman and Van der Aar, 1997), partly due to an increase in enterocyte turnover (Visek, 1978a). Proteolytic bacteria in the hind gut produce amino acids by microbial digestion of endogenous and microbial protein in chicken. Parsons et al., 1983 found that CV chickens excrete higher amounts of endogenous amino acids compared to GF birds when high a fiber diet was fed. Thus, the author indicates that there is substantial amino acid synthesis in the gut in the presence of microbiota. Further, it was found that the rate of protein fractional synthesis in the liver and gut is higher in CV chicks than in GF chicks, but the effect was not significant (Muramatsu et al., 1987). Protein fermentation by intestinal microbes depends on the composition of the diet. Protein supplements which have poor digestibility induce increased microbial fermentation compared with highly digestible protein diets (Williams, 1995).

2.4 Mannan oligosaccharides as an antibiotic alternative in broiler chicken

The poultry industry has made significant advances in the areas of nutrition, management, and genetics to maximize the efficiency of bird performance including feed utilization, growth rate, and meat yield. More recently, the industry has become more focused on improving bird health and welfare, food safety and environmental issues. Antibiotics have been extensively used as a growth promoter in poultry feed, mainly to stabilize intestinal microbiota, enhance general performance and to control intestinal pathological conditions (Gaskins et al., 2002; Dibner and Richards, 2005; Page, 2006). The direct effect of AGP on intestinal microbiota has been

suggested to reduce the competition for vital nutrients between the bird and the microbiota (Ferket, 1991) and to minimize the growth-depressing metabolites (e.g. ammonia and bile degradation products) produced by microbes, (Vissek, 1978b; Anderson et al., 1999). Additionally, AGP are thought to enhance nutrient digestibility and absorption, due to the thinning of gut wall and villus lamina propria (Jukes et al., 1956; Franti et al., 1972; Anderson et al., 1999). The reduced gut wall thickness may be partly related to reduced mucosa cell proliferation resulting from a reduction in luminal short chain fatty acids generated by microbial fermentation (Frankel et al., 1994). Also, AGP have been associated with a reduction in opportunistic pathogens and subclinical infections, thus reducing the metabolic cost of the innate immune stimulation. Although a direct anti-inflammatory effect of AGP has recently been hypothesized (Niewold, 2007), the mode of action of AGP has largely been considered to be related to effects on the intestinal microbial population (Castanon, 2007), as antibiotics in feed do not have growth-promoting effects in GF animals (Coates et al., 1955; Coates et al., 1963).

The development of antibiotic resistance in bacteria has been identified as a consequence of subtherapeutic levels of antibiotics in animal feed. Therefore, the usage of AGP is of special concern by many scientists, consumers and government regulators with respect of its threat to human health (Smith et al., 2003; Nollet, 2005). As a result, the European Union banned all AGP in animal production on January 1, 2006 (Cervantes, 2006; Michard, 2008) and tighter regulation is also expected in Canada and the US (Diarra and Malouin, 2014). The increasing regulatory control on antibiotic use to address the growing concern over widespread antibiotic resistance has driven the need to find alternatives to AGP to maintain the welfare of birds and the efficiency of poultry production.

Mannan oligosaccharide (MOS) are one of the most extensively investigated alternatives to antibiotic growth promoters in commercial broiler chickens (Spring, 1999; Hooge, 2003).

Mannan oligosaccharide is derived from the outer cell wall layer of the yeast *Saccharomyces cerevisiae* var. *boulardii*. The composition of the cell wall consists of mannose, proteins, glucans and phosphate radicals (Lyons, 1994; Klis, F. M., 2002).

Numerous studies have been conducted to investigate the effect of MOS on growth performance in broilers under different management conditions with various genetic lines, diets, experimental periods, and inclusion levels (Hooge, 2004; Yalcinkaya et al., 2008; Bozkurt et al., 2009; Yang et al., 2008b). Several researchers have found that the addition of MOS in broiler feed results in improved growth performance (Hooge, 2004; Rosen, 2007). The meta-analysis of some of the global broiler chicken pen trials (1993-2003) revealed that MOS significantly increased body weight by +1.61%, reduced feed conversion ratio (FCR) by -1.99%, and reduced mortality by -21.4% compared to the negative control diets (Hooge, 2004). More recent studies have also reported performance enhancement with MOS supplementation (Pelicano et al., 2004; Yang et al., 2008a; Bozkurt et al., 2009). Yang et al. (2008a) reported that the positive effect of MOS was more profound in the birds fed with a wheat-based diet than the corn-based diet although the mechanism was not clear.

However, studies of the effect of MOS on broiler performance have produced inconsistent results. Contradictory to above findings, some studies have reported no benefit of MOS supplementation in the diet on broiler performance. In two reports, the BWG and FCR were not significantly influenced by MOS in broilers by the age of 42 days (Yalcinkaya et al., 2008; Sarica et al., 2005). A common concern by the scientific community is the lack of

reporting of experiments where no improvement is found, however, the extent to which this phenomenon applies is not quantifiable.

Several reports suggest three possible modes of action by which broiler performance is improved by dietary MOS supplementation (Oyofa et al., 1989a; Spring et al., 2000; Ferket, 2004; Hooge, 2004). They are: 1) control of pathogenic/potential pathogenic bacteria by a “receptor analog” mechanism where MOS adsorb pathogenic bacteria containing type 1 fimbriae (mannose-sensitive lectin) and distract the pathogens away from intestinal lining, 2.) immune modulation by acting as a non-pathogenic microbial antigen and thereby stimulating gut associated and systemic immunity, and 3.) modulation of intestinal morphology and digestive function (e.g. increases intestinal villi height, uniformity, integrity, mucin expression and brush border enzymes) as indirect outcomes of 1 and 2 above.

2.4.1 Exclusion of pathogens

Mannan oligosaccharide supplementation may improve broiler performance through modulation of the intestinal microbial populations and exclusion of pathogens. It has been demonstrated that pathogens possessing type 1 fimbriae are much more virulent than non-fimbriaed bacteria (Duguid et al., 1976; Connell et al., 1996; Gunther et al., 2002). Type 1 fimbriae are expressed in *Escherichia coli*, *Salmonella* as well as most other species of bacteria in the Enterobacteriaceae family (Duguid and Old, 1980; Jones et al., 1995; Duncan et al., 2005). Mannose-specific type-1 fimbriae present in bacteria allow them to attach to the receptors containing D-mannose (Eshdat et al., 1978) in the intestinal epithelial lining. Attachment of pathogenic microbes to the enterocyte cell wall is a prerequisite for bacterial colonization and the onset of infections (Gibbons and Vanhoute, 1975). However, the attachment of Gram negative

bacteria can be inhibited by blocking their type-1 fimbriae with mannose or similar sugars. Thus, dietary inclusion of MOS may allow enteric pathogens to attach to the mannan compounds in the gut lumen instead of the epithelia, thereby reducing pathogen colonization and subsequent infections.

Reduction of *Salmonella* infection by MOS has been demonstrated in a number of studies (Fernandez et al., 2000; Spring et al., 2000; Fernandez et al., 2002). Spring et al. (2000) indicated that MOS decreased the number of salmonella positive chickens from 89.8% to 55.7% upon challenge with *Salmonella* Typhimurium and *Salmonella* Dublin. Similarly, some other *in vivo* and *in vitro* studies reported that mannose decreased the adherence and colonization of *Salmonella* Typhimurium in the intestine of young chicks (Oyofe et al., 1989a; Oyofe et al., 1989b; Oyofe et al., 1989c). A reduction of mucosa-associated coliforms was also observed in the birds supplemented with MOS as early as 7 days of age (Yang et al., 2008a). Similar findings were observed by Iji et al. (2001c) and Santin et al. (2001) and the authors suggested that the reduced coliforms may contribute to the observed improvement in gut morphology and function. Ileal luminal coliform counts have also been shown to be reduced by MOS (Jamroz et al., 2003; Yang et al., 2008b).

Pathogenic bacteria may cause direct damage to the intestinal epithelium as well as elicit immune responses in birds. Immune response can manifest as fever and reduced appetite mediated by acute phase cytokines (Johnson, 1997; Spurlock, 1997). Further, cytokines modulate nutrient metabolism including glucose, lipids, amino acids and minerals by coordinating muscle, adipose, bone and hepatic metabolism in favour of providing these nutrients in support of the immune response. Therefore, infection burdens are associated with growth retardation due to

decreased food intake, impaired nutrient absorption from intestinal damage and increased metabolic requirements to support repair and immune response.

Prebiotics are defined as non-digestible feed ingredients which elicit a beneficial effect in gut health by selectively enhancing the metabolic activity or growth of beneficial intestinal microorganisms (e.g. bifidobacteria and lactobacilli) (Gibson and Roberfroid, 1995; Cummings and Macfarlane, 2002; Ohimain and Ofongo, 2012). Mannan oligosaccharides have been characterized as prebiotics for poultry based on the reports (Denev et al., 2005; Baurhoo et al., 2007a; Baurhoo et al., 2007b; Baurhoo et al., 2009) that dietary inclusion of MOS caused a significantly increased intestinal lactobacilli and bifidobacteria population than the broilers fed control diet. Although the exact mechanism is unclear, it was hypothesized that the decreasing number of viable Gram-negative bacteria (type 1 fimbriae-bearing) due to dietary MOS may increase the Gram-positive organisms in the gut. Lactobacilli and bifidobacteria are Gram-positive bacteria that include no pathogenic species and are suggested to maintain the integrity and health of the chicken gut by several modes of action including competing against potential pathogens for epithelial binding sites and nutrients, secreting bacteriocins (Gibson and Wang, 1994; Kawai et al., 2004) and modifying the synthesis and secretion of mucin in the gut (Smirnov et al., 2005). Moreover, lactate produced by lactobacilli and bifidobacteria during carbohydrate fermentation, may decrease the growth of the pathogenic bacteria such as *Salmonella* and *E. coli*, (Barrow, 1992). However, in broilers, the effect of MOS on intestinal lactobacilli and bifidobacteria is not consistent as reported throughout the literature (Spring et al., 2000; Fernandez et al., 2002; Biggs et al., 2007).

Increased villus height has been observed in several studies in which broiler chickens were fed MOS-supplemented diets (Loddi et al., 2002; Baurhoo et al., 2007b; Yang et al., 2007a;

Baurhoo et al., 2009). An association between increased intestinal villus height and increased colonization of lactobacilli and bifidobacteria in MOS-fed broilers was identified in studies conducted by Baurhoo et al. (2007b) and Baurhoo et al. (2009). The effect of beneficial bacteria such as *Lactobacillus* sp. on increased villus height to crypt depth ratio in the duodenum and ileum has been reported in chicks (Awad et al., 2010). Additionally, it was found that the birds given MOS had shallow crypts compared to controls (Savage et al., 1997; Yang et al., 2008a) consistent with less turnover rate of the intestinal epithelium. An increased villus height: crypt depth ratio observed in birds supplemented with MOS (Ferket et al., 2002) may indicate reduced damage of enterocytes and therefore the need for cell renewal in the gut. As a result, there is less nutrient demand for maintenance of intestinal mucosa, which in turn favors faster growth rate of the whole animal.

The specific activities of digestive enzymes and gut morphology are indications of the rate of epithelial cell turnover and thus the maturity and functional capacity of enterocytes. It has been reported that specific activities of brush border enzymes are enhanced by MOS (Iji et al., 2001c; Sklan, 2001; Yang et al., 2008a). Significant increases in the specific activities of sucrase, maltase, and leucine aminopeptidase were detected in the jejunum but not ileum of broiler chickens up to an inclusion level of 3g kg⁻¹ MOS (Iji et al., 2001c).

MOS supplementation has also been reported to reduce some pathogens which do not possess type 1 fimbriae (*i.e.* coccidia, *Clostridium perfringens* and *Campylobacter*), but the mechanism remains unknown (Novak and Troche, 2007). Feed supplemented with MOS has been found to decrease *Campylobacter* colonization in broiler chickens (Shane, 2001; Anderson et al., 2005). Also, it has been documented that feeding birds with MOS may prevent colonization by *C. perfringens* (Hofacre et al., 2003; Abudabos and Yehia, 2013). Although

MOS does not bind to these bacterial species, Ferket, (2004) suggested that those bacterial counts were reduced by MOS, possibly by enhancing the mucin barrier or stimulating gut associated immunity.

2.4.2 Effect of MOS on immune modulation and nutrient assimilation

The innate immune system relies on a large family of pattern recognition receptors (PRRs), which recognize and respond to the certain molecular structures conserved among invading pathogens, which are called pathogen associated molecular patterns (PAMPs) and endogenous molecules released from damaged cells, known as damage-associated molecular patterns (DAMPs). For example, TLR4 specifically recognizes lipopolysaccharide (LPS), which is a major component of the outer membrane of Gram-negative bacteria, and mannose receptors are capable of recognizing a broad range of Gram-negative and Gram-positive bacteria, yeasts, parasites, and mycobacteria (Stahl and Ezekowitz, 1998; Raetz and Whitfield, 2002). Several reports have indicated that MOS is immunomodulator in poultry (Shashidhara and Devegowda, 2003; Singboottra et al., 2003; Kocher et al., 2004; Baurhoo et al., 2007b). The immunomodulatory effect of MOS has been associated with its mannan molecule, which may serve as a high affinity ligand for host PRRs such as mannose receptors (Tizard et al., 1989; Davis et al., 2002b) and TLR4 (Sheng et al., 2006). Also, the microbial populations altered by MOS may result changes in the abundance patterns of PAMPs affecting activation of PRRs.

Although little is known about the effects of MOS on cytokine production, Che et al. (2008) and Che et al. (2012) reported that MOS may have direct effects on cytokine secretions of phagocytes. It was suggested that MOS has a unique character of immune modulation by enhancing the protective antibody response for disease prevention while suppressing the acute phase inflammatory response (Ferket et al., 2002). For instance, Ferket et al. (2002) showed

decreased fever response at 8 h post-injection LPS from Salmonella Typhimurium strain SL 684 in birds fed MOS. Hung et al. (2008) also found reduced fever at 2 h after injection of LPS in pigs supplemented with dietary MOS. Further, a study by Che et al. (2011) reported that rectal temperatures of MOS-fed pigs declined markedly by d 7 post-infection with porcine reproductive and respiratory syndrome virus. Also, a similar study showed that dietary MOS increased serum interleukin IL-10, an immunosuppressive anti-inflammatory cytokine.

In contrast to suppressed inflammation, a study with male turkeys showed increased production of IgA with MOS (Savage et al., 1996). Also, Cetin et al. (2005) reported significant increases in the serum IgG and IgM levels in turkeys fed MOS. An experiment on broiler breeders by Shashidhara and Devegowda (2003) found that the birds fed MOS had a higher antibody response after vaccination with bursal disease virus. Here, the MOS supplementation positively affected the maternal antibody titers in the progeny.

The release of bioactive compounds or the indirect activation of immune system by intestinal bacteria may influence goblet cell dynamics (Bienenstock and Befus, 1980). Thus, mucus production is enhanced by increasing the number of goblet cells in the intestinal mucosa in the presence of microbial antigens (Edens et al., 1997; Ferket et al., 2002). Mucins, which are produced by goblet cells, are key components of the first line of defense against intestinal pathogens. Increased mucin production (Chee et al., 2010; Smirnov et al., 2005; Uni and Smirnov, 2006) and goblet cell number per villus (Baurhoo et al., 2007a; Baurhoo et al., 2007b; Solis de los Santos et al., 2007) have been reported as a result of feeding MOS. Mucins may reduce the colonization of pathogenic microorganisms (Blomberg et al., 1993) by trapping invading organisms and eliminating them from the intestine (Belley et al., 1999). Specific mannosyl receptors in the oligosaccharide units of mucins may also competitively bind to type 1

fimbriae of Gram-negative bacteria (Sajjan and Forstner, 1990) preventing their attachment to epithelial cells. Also, it is reported that some commensal bacteria, namely lactobacilli and bifidobacteria, stimulate mucin synthesis and secretion to help eliminate intestinal pathogens (Smirnov et al., 2005). In an *in vitro* study, MUC2 and MUC3 mRNA expression were increased by the probiotic *Lactobacillus plantarum* in intestinal epithelial cells associated with the inhibition of adherence of *E. coli* (Mack et al., 1999). So, the growth of beneficial bacteria favored by MOS (Baurhoo et al., 2007b), may indirectly increase mucin production in broiler chickens. Stimulation of mucin production and secretion may represent an important defense mechanism of dietary MOS against intestinal pathogens.

3.0 INTESTINAL MORPHOLOGY, DIGESTIVE AND BARRIER FUNCTION IN CONVENTIONAL AND GNOTOBIOTIC BROILER CHICKENS FED DIETS WITH OR WITHOUT MANNAN OLIGO SACHARIDES

3.1 Introduction

The composition and metabolic activity of GIT microbiota is increasingly recognized as having important influences on the host, affecting major functions of the intestinal tract including nutrient assimilation and protection against pathogens (Klasing et al., 1999; Dawson, 2001; Choct, 2009; Adil and Magray, 2012). There are many factors which alter the composition of the intestinal bacterial community in the avian host, such as diet (Apajalahti et al., 2001; Knarreborg et al., 2002), age (Knarreborg et al., 2002; van der Wielen et al., 2002; Zhu et al., 2002), antibiotic administration (Knarreborg et al., 2002; Dumonceaux et al., 2006b; Wise and Siragusa, 2007) and pathogenic infections (Kimura et al., 1976; Johansen et al., 2006). Unfortunately, the abundant and diverse array of gastrointestinal bacteria, and the dynamic nature of the community structure and metabolic activity pose a significant challenge to elucidate specific relationships linking health of the host animal with environmental factors and the abundance of specific members of the microbial community.

Both benefits and costs to the bird are associated with the activity of GIT microbiota. The commensal microbiota established in the GIT hamper colonization of pathogenic organisms by a phenomenon called competitive exclusion (Nurmi and Rantala, 1973) employing a variety of mechanisms, such as producing antimicrobial metabolites (Fuller, 1984; Piard and Desmazeaud, 1991; Mulder et al., 1997), modifying the receptors used by adverse bacteria or their toxins (Rolfe, 1991) and competing with pathogens for the use of essential nutrients (Rolfe, 1991). Further, some bacteria metabolize nutrients that the host cannot digest and convert to end

products such as SCFA, which provide extra energy and promote gut epithelial cell proliferation (Jozefiak et al., 2004; Adil and Magray, 2012), or some may release enterotoxic bacterial metabolites such as ammonia (Furuse and Okumura, 1994). Further, the host recognizes evolutionarily conserved structures on pathogens, through pattern recognition receptors (PRRs), that trigger proinflammatory and antimicrobial defenses designed to limit pathogens, but which can also reduce appetite, increase metabolic rate (Johnson, 1997; Klasing, 2007) and impair nutrient assimilation (Nabuurs et al., 1993; Gabriel et al., 2006).

Mannan oligosaccharides (MOS) derived from the cell wall of yeast, *Saccharomyces cerevisiae*, have been investigated and commercially employed as alternatives to antibiotic growth promoters in poultry production (Spring, 1999; Hooge, 2003). Possible modes of action in which broiler performance is improved by dietary MOS supplementation include: 1.) modulation of microbial populations by agglutination of pathogens bearing type 1 fimbriae, 2.) modulation of immune responses and stimulation of gut associated and systemic immunity, and 3.) modulation of intestinal morphology and digestive functions (Spring et al., 2000; Iji et al., 2001c; Loddi et al., 2002). Whether observed improvements in bird health and performance are mediated by MOS-induced changes in GIT microbiota, which subsequently modulate responses in host defense and nutrient assimilation, or whether MOS directly modulates host defense and nutrient assimilation, for example, through activation of PRR, is unclear.

We hypothesized that the reported beneficial effects of MOS on broiler chicken health and performance are mediated by direct effects of MOS on the host following interaction with pathogen recognition receptors. Therefore, the experiments were designed to elucidate the interaction between the three components, namely MOS, the broiler chicken and the resident microbiota by employing a gnotobiotic rearing protocol. The objectives of the study were to

investigate 1.) the microbial influence on intestinal development in broiler chickens and 2.) to differentiate effects of MOS mediated by changes in microbial composition versus effects directly mediated by MOS interaction with avian tissues. The effects of dietary MOS under different microbial conditions are reported on bird performance and organ weight, ileal morphology and markers (gene expression, enzymatic activity) of cell turnover (PCNA, Caspase-3), barrier function (TLR2, TLR4, Gal-6, IL8) and nutrient assimilation (SGLT-1, PepT-1, sucrase, maltase and glucoamylase).

3.2 Materials and methods

3.2.1 Birds and Diets

3.2.1.1 Gnotobiotic study

Germ-free chickens were reared according to methods previously developed in our laboratory (Drew et al., 2003). Briefly, 144 fertile eggs (Ross x Ross 308) were sterilized by immersion for 14 min in 0.3% hypochlorite solution prior to incubation. At day 19 of incubation, eggs were sprayed with warm (35°C) chlorine dioxide based sterilant (Clidox[®]-S, Pharmacal Research Laboratories Inc., Naugatuck, CT) and placed in four sterile HEPA-filtered gnotobiotic isolators (36 eggs in each) maintained at 55-60 % humidity and 32-34°C. Birds in two isolators were conventionalized at one day of age by placing cecal contents collected from a healthy adult laying hen in the water supply (1 g of cecal material from a cage-housed, mid-cycle laying hen mixed with 1 mL of sterile peptone and 2 mL of cysteine hydrochloride).

Hatched birds in each gnotobiotic isolator were randomly assigned to one of two dietary treatments (Table1), namely a negative control (NC, basal diet without any dietary additives) and

MOS supplementation (MOS, 2 g Bio-Mos/kg feed, Alltech Inc., and Nicholasville, KY, USA). The experimental starter diet was formulated to meet or exceed the nutrient requirements of broilers (NRC, 1994). The vitamin premix was included at two times the commercial recommended inclusion in order to account for inactivation during sterilization by gamma irradiation (50 kGy). The room temperature was maintained at 34⁰C and continuous light was provided for the chicks throughout the experiment. All chicks had *ad libitum* access to sterilized feed and water for the duration of the study.

Incubation of peri-cloacal swabs indicated microbial contamination of one germ free isolator on day 2 post hatch. Also, a poor hatch rate was experienced necessitating the combining of two conventionalized isolators, and the reorganization of treatment assignments and number of birds per treatment as in Table 2. At 7 days of age, all birds were weighed and euthanized by cervical dislocation to permit collection of tissues and intestinal contents for analysis. The experiment was approved by the University of Saskatchewan Animal Research Ethics Board (Protocol number 20080026) and complied with guidelines of the Canadian Council of Animal Care.

Table 1. Ingredients and chemical composition of experimental diets containing mannan oligosaccharides (MOS) fed to broiler chickens from 1 to 7 days of age.

Item	Treatment	
	Negative control	MOS
Ingredients (%)		
Corn	53.3	53.3
Soybean meal (48%)	37.6	37.6
Canola oil	3.25	3.25
Dicalcium phosphate	1.92	1.92
Limestone	1.56	1.56
Salt	0.35	0.35
DSM broiler premix ¹	0.50	0.50
FCL vitamin premix V8V ²	0.10	0.10
Choline chloride	0.07	0.07
DL-Methionine	0.28	0.28
L-Threonine	0.02	0.02
L-Lysine HCL	0.17	0.17
Probond (pea starch) ³	0.26	0.26
Sodium bicarbonate	0.22	0.22
Celite ⁴	0.40	0.20
MOS ⁵	-	0.20
Total	100	100
Calculated composition (% , unless otherwise indicated)		
AME (kcal/kg)	3050	3050
Crude protein	22	22
Chloride	0.28	0.28
Non-phytate phosphorus	0.50	0.50
Total phosphorus	0.79	0.79
Sodium	0.21	0.21
Arginine	1.51	1.51
Isoleucine	0.95	0.95
Lysine	1.38	1.38
Methionine	0.62	0.62
Threonine	0.88	0.88
Tryptophan	0.31	0.31

¹ DSM Nutritional Products Canada Inc. High River, Alberta. Provided per kg of diet: 11,000 IU vitamin A; 2,200 IU vitamin D; 30 IU vitamin E; 2 mg menadione; 1.5 mg thiamine; 6 mg riboflavin; 60 mg niacin; 4 mg pyridoxine; 0.02 mg vitamin B12; 10 mg pantothenic acid; 0.6 mg folic acid; 0.15 mg biotin; 80 mg iron; 80 mg zinc; 80 mg manganese; 0.8mg iodine; 10 mg copper; 0.3 mg selenium; 0.625 mg antioxidant; 500 mg calcium carbonate.

² Landmark Feeds, Landmark, Otterburne, Rosenort, Winnipeg. Provided per kg of diet: 14,500 IU vitamin A; 4,700 IU vitamin D; 45 IU vitamin E; 2.2 mg vitamin K; 3 mg thiamine; 10 mg riboflavin; 100 mg niacin; 5 mg pyridoxine; 0.02 mg vitamin B12; 20 mg pantothenic acid; 1.7 mg folic acid; 0.25 mg biotin.

³ Parrheim Foods, Saskatoon, Saskatchewan, Canada.

⁴ Celite Corporation, Lompoc, California.

⁵ Alltech Inc., Nicholasville, KY, USA.

Table 2. Microbial status and number of birds fed each diet in different isolators of the gnotobiotic trial

Isolator	Microbial Status	Diet	Number of birds
1	Germ free	Control diet	5
		MOS diet	5
2	Mono-associated ¹	Control diet	6
		MOS diet	7
3	Conventionalized	Control diet	8
		MOS diet	11

¹Isolator originally assigned as germ-free; reassigned as mono-associated following post experiment analysis of microbial status.

3.2.1.2 Conventional study

Twenty broiler chickens (Ross x Ross 308) were placed in one of two wire mesh cages (10 birds/pen) and fed a corn soybean meal based diet (Table 1) supplemented with or without MOS (Alltech Inc., 2g/kg). Chicks were reared in a conventionally ventilated animal room with continuous light and the temperature was maintained at 34°C. Also, birds were provided *ad libitum* access to feed and water. At 7 days of age, all birds were killed by cervical dislocation to permit collection tissues and intestinal contents.

3.2.2 Confirmation of Microbial Status

The cloaca of several birds in each isolator was swabbed using a sterilized cotton swab throughout the experiment. Swabs were placed in 5 mL of brain heart infusion broth (Difco Laboratories, Sparks, MD) with 0.5% cysteine hydrochloride and incubated within the isolators at 32°C to screen for bacterial contamination based on visible detection of microbial growth. After euthanasia, the content of one cecum per bird was aseptically placed in 15 mL sterile plastic tubes containing 1 mL of 0.1% sterile peptone buffer with 5 g/L of cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO). The samples were serially diluted and plated on blood agar base containing 5% defibrinated sheep blood (VWR Int., Mississauga, ON, Canada). Plates

were cultured under anaerobic (AnaeroPack, Mitsubishi Gas Chemical Company Inc., Japan) and aerobic conditions at 37°C for 24 hours. Following culture colonies were assessed for morphological variation and enumerated to permit calculation of colony forming units (cfu) per gram contents. Where colony growth was inconsistent with germ-free status, colonies were isolated and identified by amplification of the *cpn60* universal target (UT) gene and query of the sequence in the cpnDB (<http://cpndb.cbr.nrc.ca>; Hill et al., 2002; Hill et al., 2004) using FASTA (Pearson and Lipman, 1988).

3.2.3 Sample collection

Following cervical dislocation, the small intestine was excised from the carcass via a midline incision and was divided into three segments including the duodenum (from the outlet of gizzard to the end of pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to ileo-cecal junction). Each small intestinal segment was dissected and the length was measured and recorded. Tissue sections (1 cm long) from the mid-point of each small intestinal location and the remaining cecum were enclosed in tissue cassettes (Fisher Scientific Company, Ottawa, Ontario) and placed in 10% buffered formalin solution for gut morphological measurements. Also, 3-5 cm of tissue from each segment of small intestine and cecum were immediately snap-frozen in liquid nitrogen and stored at -80 °C for analysis of mRNA transcript abundance, protein content and enzyme activity. The wet weight of the liver, spleen, and bursa of Fabricius were also recorded. Intestinal contents were expelled from the ileum and cecum using gentle pressure and frozen at -20°C for molecular microbial analysis.

3.2.4 Histomorphological parameters

The tissues of intestinal segments (fixed in 10% buffered formaldehyde), were subjected to a process that consisted of serial dehydration, clearing and impregnation with paraffin wax. Processed tissues were further embedded in paraffin and longitudinal sections (7 μm) were cut and mounted individually onto Poly-L-Lysine coated Superfrost[®] slides. Sections were then deparaffinized using CitriSolv clearing agent (Fisher Scientific, Pittsburg, PA) and hydrated in preparation for staining with haematoxylin and eosin.

The image analysis program VideoPro[®] (Version 6.210, Leading Edge Pty Ltd., Australia) was employed to measure a variety of parameters for each of the stained sections in which 10 villi/section were measured. VideoPro[®] was used to compute measurements of villous height (VH), crypt depth (CD), total villous surface area (VSA) and lamina propria area (LPA). Villus to crypt ratio (VCR) was obtained by VH/CD.

3.2.5 Gene Expression Analysis by Real-Time quantitative PCR (qPCR)

Frozen ileal and cecal tissues were crushed under liquid nitrogen prior to isolation of total RNA using RNeasy[®] Mini kit (QIAGEN[®], Hilden, Germany). Samples were treated with RNase-Free DNase (QIAGEN[®], Hilden, Germany) to minimize the contamination with genomic DNA. Reverse transcription (RT) of total RNA (2 μg) to single-stranded complementary DNA (cDNA) was performed using high capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, USA) according to instructions. Quantitative Real-Time PCR (qPCR) was conducted using gene-specific primers (Table 3) with a Bio-rad CFX96 Real-Time PCR detection system on a C1000 thermal cycler (BioRad, Guénette, Canada). Primers were designed using Beacon Designer 4 (Premier Biosoft International, Palo Alto, CA) software based on published cDNA sequences or were adopted from previous reports (Table 3). Primers were

validated by query against GenBank using Primer Blast, confirmation of a single amplicon of expected molecular weight following agarose gel electrophoresis and formation of a single peak dissociation curve following qPCR. Further, the primers were verified by sequencing purified amplicons (National Research Council, Plant Biotechnology Institute, Saskatoon, SK) and query of the sequence in the Genbank database using BLAST® (National Center for Biotechnology Information, Bethesda, MD) (Altschul et al., 1990).

Reaction mixtures for qPCR were prepared in Hard-Shell 96-well PCR plates (BioRad, Guénette, Canada) in duplicate and were composed of 12.5 µL of iQ SYBR green SuperMix (BioRad), 600 nM of each primer specific for each gene, 1-2 µL of the cDNA and adjusted to 25 µL with UV-exposed DNA grade water (Fisher Scientific, New Jersey, USA). All polymerase chain reactions were conducted in duplicate under the following conditions: 95°C for 3 min and 35 cycles of 95°C for 40 s, primer specific annealing temperature (Table 3) for 40 s and 72°C for 40 s.

Standard curves were created by pooling cDNA from each sample and preparing a 5-fold dilution series. Corresponding relative arbitrary values were assigned to each cDNA dilution. For each gene target, threshold cycle values (Ct) were plotted against the corresponding relative arbitrary value for each dilution (Bio-Rad CFX Manager software 1.6). Interpolation in the standard curve of the mean Ct values obtained for test cDNA samples yielded arbitrary values corresponding to specific transcript abundance.

All selected “housekeeping genes” investigated to normalize expression data were significantly affected by dietary supplementation with MOS. Therefore gene expression was reported using two different approaches. Firstly, since cDNA was prepared from the same amount of total RNA using identical protocols for all samples, values are reported without

further normalization based on housekeeping gene abundance. Despite the effect of treatment, for comparison, expression data was also normalized by dividing mean arbitrary values by mean values for selected housekeeping genes, namely Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and peroxiredoxin 6 (PRDX6).

3.2.6 Protein and Brush-border Enzyme Assays

Ileal tissue (frozen at -80°C) from each bird was ground under liquid nitrogen using mortar and pestle. The ground tissue (20mg) was homogenized in 1 mL of 1% Triton X-100 (in phosphate buffered saline, pH 7.4) by needle and syringe homogenization. Homogenized samples were centrifuged at 1500g for 5 min and the supernatant was separated.

The activities of sucrase and maltase in the ileum tissue were analyzed as described by (Dahlqvist, 1964) with some modifications. Briefly, 40 μL of tissue homogenate was added to the same volume of 0.1 M substrate solution (sucrose or maltose) in a 96 well plate. The reaction mixtures were incubated at room temperature for 30 minutes. Then the liberated glucose concentration was determined spectrophotometrically at 505 nm using Wako Autokit Glucose assay (Wako Diagnostics, VA) according to manufacturer's instructions. Sucrose was used as a substrate for sucrase activity and maltose was used to measure the maltase activity, representing the combined activities of sucrase-isomaltase and maltase-glucoamylase, respectively (Bjornvad et al., 2005; Lieberman et al., 2013).

APN activity was assayed by the modified method of Maroux et al. (1973) based on the hydrolysis of the substrate L-alanine-4-nitroanilide to L-alanine and p-nitroaniline. Homogenized tissue samples (10 μL) were added to 200 μL of 10 mM L-alanine-4-nitroanilide in 50 mM TRIS HCl, pH 7.3) and incubated at 37°C for 5 minutes. Standards were prepared using 6.25 to 200 μM 4-nitroaniline and the blank in 200 μL substrate plus 10 μL of boiled (denatured)

homogenate. The color development of the reaction and standards were measured at 405 nm using Spectramax (Molecular Devices Corp., Sunnyvale, CA). Interpolation of OD at 405 nm for samples against the standard curve yielded μM product liberated during the incubation.

Protein content in homogenized tissue samples was determined using the Bio-Rad Protein assay (Bio-Rad Laboratories) according to the method of Bradford (1976) using bovine serum albumin as the protein standard. All brush border enzyme activities were expressed as micromoles of substrate released per minute per gram of protein.

3.2.7 Caspase-3 activity

The EnzCheck® Caspase-3 Assay Kit#1 (Molecular Probes, Inc., Eugene, OR) was used to measure caspase-3 activity in tissue homogenates based on the proteolytic cleavage of the peptide Asp-Glu-Val-Asp (DEVD). Homogenized intestinal tissue (5 mg) was suspended in 200 μL of $1 \times$ Cell Lysis Buffer (10 mM TRIS, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% TRITON™ X-100), incubated on ice for 30 minutes and centrifuged at $2300 \times g$ for 5 min to pellet the cellular debris. Duplicate aliquots (50 μL) of the supernatant were transferred to individual wells in a 96 well plate. A reaction blank was prepared containing 50 μL Cell Lysis Buffer. A 2X substrate solution was prepared into a final concentration of 0.2 mM of Z-DEVD-AMC (where Z represents a benzyloxycarbonyl group and AMC represents 7-Amino-4-methylcoumarin) and 50 μL of it was added to each sample and control. After that the microplate was covered and incubated at room temperature for 10 minutes. A standard curve was prepared by adding 100 μL of AMC (ranging 10-100 μM) into empty microtitre plate wells. Fluorescence emission was measured at 441 nm following excitation at 342 nm using a Fluoroskan Ascent fluorometer (Thermo Labsystems, Helsinki, Finland) and caspase-3 activity expressed as $\mu\text{mol/g}$ tissue/minute.

Table 1. Primers used for Real-time PCR

Gene	Gene bank Accession no.*	Description	Real-time PCR sense/antisense	Annealing temperature °C
GAPDH	NM_204305	Glycerldehyde-phosphate dehydrogenase	GTGAAAGTCGGAGTCAACGGA/ AAGGGATCATTGATGGCCAC	61
PRDX6	NM_001039329.1	Peroxiredoxin-6	CGCTGATAAGGACCGAGAG/ AAAGGGATGTGGAGGATTGG	57
18S	AF173612.1	18S rRNA	GCCTGCGGCTTAATTTGACT/ TAAGAACGGCCATGCACCA	54
β-actin	NM_205518.1	Beta-actin	CATTGCTGACAGGATGCAGAAG ATAGAGCCTCCAATCCAGACAGA	52
MUC2	XM_421035	Mucin 2	CCTGTGCAGACCAAGCAGAAA/ CCTCTGAGTTTTTCAGCAAAGAACAC	57
TLR2	NM_204278.1	Toll-like receptor 2	GGCTGTGAACCTGAGAACC/ CTGATGACTGCTGAGAATACG	55
TLR4	NM_001030693	Toll-like receptor 4	ATCACTTCTGTCCTGTCTCC/ CTGTTGCCACTCCTTATCTTG	53
IL-8 ^a	AJ009800	Interleukin-8	ATGAACGGCAAGCTTGGAGCT/ TCACAGTGGTGCATCAGAATTGA	61
Gal6	NM_001001193.1	Galinacin-6	AGACAGAAGGCAGCGGTGAT/ AAGAGGACAGACAGCAGG	54
PCNA	NM_204170	Proliferating cell nuclear antigen	GGGTTCGGGCGGCATCAG/ TCTTCATTTCCAGCACACTTCAG	57

Table 2. (continued)

Gene	Gene bank Accession no.*	Description	Real-time PCR sense/antisense	Annealing temperature °C
APN	NM_204861.1	Aminopeptidase-N	GTCCAACAGAGCCACTTCC/ CGTCCACCAGCCAATACC	54
SI	Y08960.1	Sucrase-isomaltase	TCAGAATCAGACACCCAATCG GGCAACCTTCACATCATAACAAC	48
MGA	XM_422811.2	Maltase-glucoamylasae	GGCAGTTGATAGGCAGTTCC/ GTGGCTGGCTGTTGAATAGG	53
SGLT-1	AJ236903.1	Sodium glucose co-transporter1	GTCTACCTGTCAATCCTTTCAC/ GGCATCATACCCTCCAACC	52
PepT-1	AY129615.1	Peptide Transporter-1	ATGTTCCCTTGCTGGTCTGG/ TGCGTATTGCTGCTTATTGAG	52

* NCBI GeneBank Accession number.

^aThe primers were adopted from Laurent et al. (2001).

3.2.8 TRFLP Analysis of microbial diversity

Frozen cecal samples which were at -20°C were thawed in a 50°C water bath and homogenized before a sub-sample for DNA extraction was taken. Genomic DNA was extracted from 0.35 g cecal digesta using a protocol described previously (Dumonceaux et al., 2006a). The DNA concentration of each sample was quantified using UV absorbance (NanoDrop® ND-1000 spectrophotometer; NanoDrop Technologies, Inc., Wilmington, USA). The DNA concentrations were adjusted to 2-5 ng/μL with double-distilled water and 1:100 dilutions of them were used as template in PCR reactions. A fragment of the 16S rRNA gene was amplified from the isolated DNA using the universal forward primer Bact-8F(5'-AGAGTTTGATCCTGGCTCAG-3') labeled at the 5' end with 6-carboxyfluorescein, and the reverse primer 926r (5'-GTCAATTCC TTTRAGTTT-3') (Dicksved et al., 2007). The PCR reaction contained 5 μl of 10x PCR reaction buffer (Invitrogen, Burlington, ON), 1.5 mM MgCl₂, 0.24 μM of each primer, 0.2 mM each of a deoxynucleoside triphosphate (dNTP)mixture, 2.5 U of Taq polymerase (Invitrogen, Burlington, ON), and UV-exposed DNA-grade water, (Fisher Scientific) was added until the volume was 50 μL. The amplification program was performed as an initial denaturing step of 94°C for 5 min followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 60 s and a final extension of 7 min at 72°C.

PCR product size and specificity were checked by electrophoresis of an aliquot in a 1.5% agarose gel stained with ethidium bromide. Remaining PCR product was purified using QIAquick PCR purification kit (Qiagen, Ontario, Canada) according to the manufacturer's protocol. DNA concentration of purified product was measured by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc).

For the TRFLP analysis, 200 ng of the purified PCR product was digested at 37°C overnight using 15 U of MspI (Fermentas, Burlington, Canada), and 2 µL of 10X reaction buffer in a total volume of 20 µL. Digested PCR product (2 µL) was then mixed with 9 µL of formamide and 0.5 µL of internal standard (ABI GeneScan 600 LIZ size standard) and the reaction mixture was denatured at 95°C for 5 min. This was followed by immediately cooling on ice for 2 min. The lengths of the fluorescently-labelled terminal restriction fragments (TRFs) were analyzed by the ABI PRISM 3130 genetic analyser (Applied Biosystems, USA) in gene scan mode and the T-RFLP profiles were analyzed using GeneMapper software (Version 3.7; Applied Biosystems, USA). The sizes of the fluorescently labeled fragments (in base pairs) were determined by comparison with the internal standard (ABI). Therefore, the data of the analysed samples consisted of size (base pairs) and peak area for each TRF. Peaks less than 4 base pairs apart were binned and normalized using the sum of total peak area. Relative peak areas of each TRF were obtained by dividing the area of the peak of interest by the total area of peaks within the lower threshold at 20 bp and an upper threshold at 600 bp. Peaks less than 1% of the total area were excluded from further analysis. The profiles of TRF relative peak area for each sample were imported into Bionumerics software (version 5.1, Applied Maths, Austin, TX, USA) and unweighted pair-group method with arithmetic mean (UPGMA) was used to cluster profiles using the dice similarity coefficient with area sensitivity.

The Shannon-Weiner diversity index (Shannon, 1948) was calculated using the formula shown (Equation 1.1) below in which p_i represents the proportion of a species i present in a sample of n different species. The value for the p_i was calculated as the proportion of an individual peak area relative to the sum of all peak areas.

$$\text{Shannon Index (H)} = - \sum_{i=1}^n P_i \ln P_i \dots\dots\dots \text{Equation 1.1}$$

The Simpson's index of diversity (Simpson, 1949) was calculated using Equation 1.2.

$$\text{Simpson's index of diversity (D)} = 1/\sum (P_i)^2 \dots\dots\dots \text{Equation 1.2}$$

Evenness (Margalef, 1957) was calculated using Shannon-Weiner index based on Equation 1.3, where $H_{\max} = \log_2(S)$.

$$\text{Evenness (E)} = H/H_{\max} \dots\dots\dots \text{Equation 1.3}$$

H = Shannon Index of general diversity; S= Number of species.

3.2.8 Statistical Analysis

After adjustments for contamination and the poor hatch rate the experiment was a 3 X 2 factorial arrangement. Sources of variation in the statistical model included fixed main effects of microbial status (conventionalized, mono-associated, and germ-free), diet (with or without MOS) and their interaction. The PROC GLM procedure of SAS (SAS Institute, Cary, NC; Version 9.2) was used for statistical analysis of all parameters using bird as the experimental unit. Differences were considered significant at $P < 0.05$. Values reported are means and pooled standard error of means (SEM).

3.3 Results

3.3.1 Confirmation of microbial status

Colony counts and morphology for cecal contents from birds in the conventionalized isolator (Isolator 3, Table 2) were consistent with a diverse and abundant conventional microbiota. Further, no colony growth was observed for birds reared in Isolator 1 consistent with germ-free status. In Isolator 2 (Table 2), bacterial growth was observed on culture of cecal contents on aerobic blood agar. Total aerobic counts ranged from 10^1 - 10^3 cfu/g cecal contents and no growth was observed in anaerobic agar. Colony morphology was uniform and Gram-staining of several colonies indicated single cell morphology and type. The nucleotide sequence of the cpn60 UT amplified from selected colonies (n=10) were identical having the highest sequence identity (87%) within cpnDB to a member of the *Acinetobacter* species. Bacteria in the genus *Acinetobacter* are strictly aerobic, Gram-negative proteobacteria ubiquitous in nature and commonly found on or in soil, water, animals and humans (Vanechoutte, et al., 2011). The microbial status of this isolator was therefore designated as monoassociated and the experiment analyzed as a 3X2 factorial arrangement including microbial status; germfree (GF), mono-associated (Mono) and conventionalized (CV) and dietary supplementation (NC or MOS).

3.3.2 Growth performance and gross morphology

The effects of microbial status and dietary MOS supplementation on body weight (BW), internal organ (liver, spleen and bursa of Fabricus) weight and small intestinal segment (duodenum, jejunum and ileum) length in broiler chicks at day 7 are summarized in Table 4. Initial body weight of day 1 old chicks could not be measured under the sterile hatch conditions

employed. Microbial status significantly affected the final body weight of chicks.

Monoassociated chicks were heavier than the CV birds ($P < 0.05$) whereas GF birds were intermediate.

Relative bursa weight was greater ($P < 0.05$) in CV and Mono birds compared to germ-free (GF) birds. Relative weight of liver and spleen was not affected ($P > 0.05$) by microbial status.

Relative length of duodenum and ileum was greater ($P < 0.05$) in CV chicks compared with GF chicks whereas these segments were intermediate in relative length in mono-associated birds.

Dietary supplementation with MOS tended ($P = 0.08$) to increase the relative bursa weight, however, no other effects of MOS on gross parameters were observed.

Table 3. Effect of microbial status and MOS supplementation on live body weight (BW), relative weight of selected visceral organs and relative length of small intestinal segments in gnotobiotic broiler chicks at 7 days of age.

Treatment	BW (g)	Relative weight of organs*			Relative length of SI segments‡		
		Liver (%)	Spleen (%)	Bursa (%)	Duodenum (cm/g)	Jejunum (cm/g)	Ileum (cm/g)
Microbial status¹							
CV	129 ^b	3.96	0.06	0.14 ^a	0.11 ^a	0.28	0.23 ^a
Mono	147 ^a	3.94	0.09	0.12 ^a	0.10 ^{ab}	0.25	0.21 ^b
GF	144 ^{ab}	4.05	0.05	0.08 ^b	0.09 ^b	0.25	0.20 ^b
Diet²							
NC	142	4.08	0.08	0.10	0.09	0.27	0.21
MOS	138	3.89	0.06	0.12	0.10	0.25	0.22
Pooled SEM	3.18	0.08	0.01	0.01	0.002	0.006	0.005
P-value							
Microbial status	0.04	0.88	0.35	0.00	0.00	0.09	0.01
Diet	0.49	0.29	0.42	0.08	0.06	0.22	0.17
Microbial status × Diet	0.47	0.58	0.24	0.19	0.54	0.89	0.65

* Relative weight of visceral organs represents the organ weight expressed as a percentage of body weight.

‡ Relative intestinal lengths represent the length of the duodenum, jejunum and ileum as a percentage of body weight.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values in a column not sharing a common superscript differ (P<0.05).

3.3.3 Mucosal morphology in ileum

Ileal mucosal morphology measured on day 7 is shown in Table 5. There was a significant interaction of microbial status by diet for VH and VSA (Table 5; Figure 1; $P<0.05$) such that MOS supplementation increased these parameters in CV and monoassociated birds whereas MOS reduced these parameters in GF birds. The main effect of microbial status was significant for CD, VCR, and LPA. Crypt depth was reduced in GF compared to CV chickens with Mono birds demonstrating an intermediate value (Table 5; $P<0.05$). Accordingly, VCR was highest in GF and lowest in CV ($P<0.05$). The LPA value was lower ($P<0.05$) in GF versus CV and Mono, which were similar. MOS supplementation did not affect CD, VCR or LPA.

Table 4. Effects of microbial status and MOS on histomorphological parameters of the ileum in gnotobiotic broiler chicks at 7 days of age.

Treatment	Histomorphometric parameter¶				
	VH µm	CD µm	VCR	VSA µm ²	LPA µm ²
Microbial status¹					
CV	347	95 ^a	3.83 ^b	42 600	13 300 ^a
Mono	345	91 ^{ab}	4.01 ^{ab}	42 400	13 100 ^a
GF	333	83 ^b	4.23 ^a	37 900	9 900 ^b
Diet²					
NC	340	90	4.06	40 600	12 300
MOS	343	89	3.98	41 400	11 800
Pooled SEM	3.38	1.31	0.06	768	310
P-value					
Microbial status	0.28	0.00	0.03	0.05	0.00
Diet	0.67	0.83	0.53	0.63	0.37
Microbial status×Diet	0.00	0.17	0.07	0.02	0.17

¶ VH, Villus height; CD, Crypt depth ;VCR, Villus crypt ratio; VSA, Villus surface area; LPA, Lamina propria area and LP/SA, Lamina propria area to villus surface area ratio.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values in a column not sharing a common superscript differ ($P<0.05$).

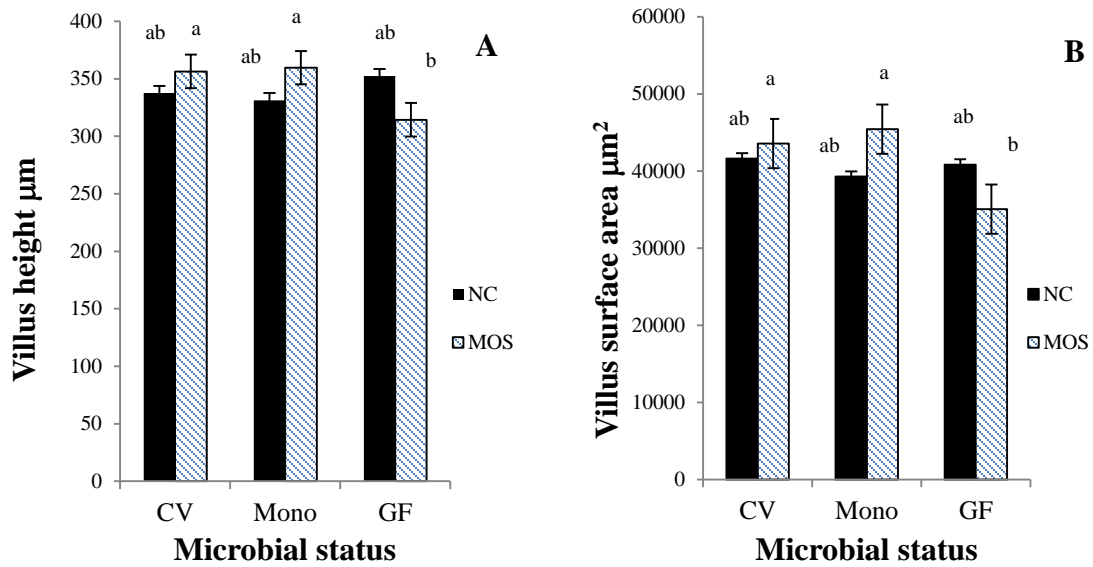


Figure 1. Illustration of the interaction between microbial status and MOS supplementation on villus height (panel A) and villus surface area (panel B). Conventionalized (CV), monoassociated (Mono) and germ-free (GF) broilers were fed basal diet (NC) or the basal diet supplemented with mannan oligosaccharides (MOS).

^{a-b} Bars not sharing common letters in a single plot are significantly different ($P < 0.05$).

3.3.4 Expression of House Keeping Genes

A number of housekeeping genes were evaluated for normalization of expression of genes of interest. Four housekeeping genes in ileal tissue, including GAPDH, PRDX6, 18S ribosomal RNA subunit, and β -actin, were evaluated (Table 6). When selected house keeping genes were expressed as arbitrary units per μg total RNA (used for transcription of cDNA), no significant effect of microbial status was observed. However, for all four housekeeping genes examined, dietary supplementation of MOS significantly ($P < 0.05$) increased ileal expression. This result was observed using 3 different preparations of cDNA prepared from independent RNA extractions in qPCR assays conducted by different individuals (data not shown). In contrast, expressions of GAPDH and PRDX6 in cecal tissues were not significantly affected by either microbial status or MOS supplementation when expressed per μg total RNA (Table 6).

Table 5. Effect of microbial status and MOS on expression of housekeeping genes in ileum and cecum in gnotobiotic broiler chicks at 7 days of age.

Treatment	Gene of interest [¶]					
	Ileum			Cecum		
	GAPDH*	PRDX6*	18S*	Beta- actin*	GAPDH*	PRDX6*
Microbial status¹						
CV	132	108	99	147	246	947
Mono	136	134	91	130	238	785
GF	176	191	105	164	258	794
Diet²						
NC	84 ^b	55 ^b	72 ^b	85 ^b	235	775
MOS	213 ^a	234 ^a	125 ^a	209 ^a	260	909
Pooled SEM	21.8	28.8	8.34	14.1	10.5	57.4
P-value						
Microbial status	0.65	0.48	0.80	0.47	0.76	0.43
Diet	0.00	0.00	0.00	0.00	0.27	0.28
Microbial status ×Diet	0.29	0.49	0.98	0.60	0.40	0.96

[¶] GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PRDX6, Peroxiredoxin-6; 18S, 18S ribosomal RNA.

*Means are expressed as arbitrary units per µg total RNA.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values within a column for each main effect not sharing a common superscript are significantly different P<0.05).

3.3.5 Expression of PCNA and Caspase-3 activity

Expression of PCNA and activity of caspase-3 were measured as indicators of mucosal proliferation and apoptosis, respectively (Table 7). Abundance of PCNA mRNA in ileum and cecum was not influenced by microbial status when expressed per μg total RNA. But PCNA transcript abundance in ileum was significantly increased in CV and Mono compared to GF birds when it was normalized to mean expression of GAPDH and PRDX6. Observations on the effect of dietary supplementation with MOS on PCNA expression were consistent between ileum and cecum. Supplementation with MOS significantly increased ($P < 0.05$) PCNA expression per unit total RNA in both locations, however, after normalization to mean GAPDH/PRDX6 expression, no effect of MOS on PCNA was observed. Caspase-3 activity in ileum was numerically higher in CV and Mono birds compared with GF birds, but differences were not statistically significant ($P = 0.173$). Similarly, no significant differences of Caspase-3 activity in cecum were observed.

Table 6. Effect of microbial status and MOS on PCNA transcript abundance and caspase-3 activity in ileum and cecum of gnotobiotic broiler chicks at 7 days of age.

Treatment	Ileum			Cecum		
	PCNA* per unit RNA	PCNA‡ per unit HK genes	Caspase-3 μmol/ g protein/min	PCNA* per unit RNA	PCNA‡ per unit HK genes	Caspase-3 μmol/ g protein/min
Microbial status¹						
CV	100	903 ^a	6.23	871	145	8.89
Mono	93	785 ^a	6.59	746	156	6.69
GF	81	418 ^b	2.73	824	180	7.03
Diet²						
NC	50 ^b	711	5.11	708 ^b	154	6.98
MOS	134 ^a	694	5.25	920 ^a	167	8.10
Pooled SEM	12.8	58	0.85	45.3	10.8	0.63
P-value						
Microbial status	0.79	0.00	0.17	0.44	0.47	0.30
Diet	0.00	0.87	0.94	0.02	0.59	0.41
Microbial status ×Diet	0.44	0.32	0.53	0.13	0.27	0.49

*Means are expressed as arbitrary units per μg total RNA.

‡Means are expressed as arbitrary units per μg total RNA for gene of interest divided by the mean arbitrary units per μg total RNA for housekeeping (HK) genes GAPDH and PRDX6.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values within a column for each main effect not sharing a common superscript are significantly different (p<0.05).

3.3.6 Immune modulation

Expression of TLR2, TLR4, IL8 and Gal6 was measured to assess treatment responses associated with microbial sensing and immune response. In the ileum and cecum, microbial status did not significantly affect the mRNA abundance of TLR2, TLR4, IL8 and Gal6 (Table 8; Table 9: $P > 0.05$) regardless of normalization to total RNA or housekeeping gene abundance. In the ileum, dietary supplementation with MOS significantly ($P < 0.5$) increased TLR4, IL-8 and Gal6 and tended ($P < 0.10$) to increase TLR2 when expressed per unit total RNA. However, the effect of MOS was not significant when the transcript abundance of these genes were expressed per unit of housekeeping genes. In cecum, supplementation with MOS did not affect expression of TLR2, TLR4 or IL8, when expressed per unit total RNA or normalized to housekeeping genes. In the case of Gal6, increased ($P < 0.05$) expression was observed in response to MOS supplementation when expressed per unit total RNA, but not when normalized to expression of housekeeping genes.

3.3.7 Ileal brush- border membrane enzyme activity and nutrient absorption

Neither microbial status nor diet significantly affected the enzyme activity of sucrase, maltase and APN in the ileum (Table 10). Also, microbial status did not affect the mRNA abundance of selected brush border enzymes (MGA and APN) or nutrient transporters (SGLT-1 and PepT-1) in the present study ($P > 0.05$). Sucrase-isomaltase transcript abundance was below the level of quantification (a threshold cycle of less than 35) using the primers identified in Table 10.

However, chicks fed a diet supplemented with MOS had significantly ($P < 0.05$) higher quantities of mRNA for APN, SGLT-1 and PepT-1 but not MGA, compared with chicks fed with control diet when expressed per unit total RNA. Maltase glucoamylase and APN transcript abundance were significantly increased in CV birds compared to GF birds after normalization to per unit of housekeeping genes. Also MGA was increased in MOS fed birds compared to the birds fed control diet when it is normalized to housekeeping genes.

Table 7. Effects of microbial status and MOS on the transcript abundance of immune-related genes in ileum of gnotobiotic broiler chicks at 7 days of age.

Treatment	Gene of interest [¶]							
	Per unit total RNA*				Per unit HK gene [‡]			
	TLR2	TLR4	IL8	Gal6	TLR2	TLR4	IL8	Gal6
Microbial status¹								
CV	164	326	875	106	102	407	143	89
Mono	136	282	525	135	92	332	35	126
GF	137	381	591	124	55	253	28	59
Diet²								
NC	67	200 ^b	384 ^b	52 ^b	69	378	84	88
MOS	225	459 ^a	944 ^a	191 ^a	97	283	54	95
Pooled SEM	38.3	36.1	120	21.1	17	48	30	14
P-value								
Microbial status	0.94	0.49	0.38	0.80	0.57	0.50	0.23	0.30
Diet	0.06	0.00	0.04	0.00	0.48	0.38	0.68	0.84
Microbial status ×Diet	0.87	0.06	0.37	0.63	0.30	0.43	0.52	0.19

[¶] TLR2, Toll like receptor 2; TLR4, Toll like receptor 4; IL8, Interleukin 8; Gal6, Gallinacin 6.

*Means are expressed as arbitrary units per µg total RNA.

[‡]Means are expressed as arbitrary units per µg total RNA for gene of interest divided by the mean arbitrary units per µg total RNA for housekeeping (HK) genes GAPDH and PRDX6.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values within a column for each main effect not sharing a common superscript are significantly different (p<0.05).

Table 8. Effects of microbial status and MOS on the transcript abundance of immune-related genes in cecum of gnotobiotic broiler chicks at 7 days of age.

Treatment	Gene of interest [¶]							
	Per unit total RNA*				Per unit HK gene [‡]			
	TLR2	TLR4	IL8	Gal6	TLR2	TLR4	IL8	Gal6
Microbial status¹								
CV	436	635	886	88	75	105	160	159
Mono	461	604	527	77	94	122	98	163
GF	493	593	603	99	105	138	96	217
Diet²								
NC	397	525	772	70 ^b	85	120	141	161
MOS	529	696	572	106 ^a	97	124	95	199
Pooled SEM	35.4	54.1	80.3	5.51	6.24	8.48	17.3	12.9
P-value								
Microbial status	0.81	0.94	0.14	0.22	0.15	0.36	0.19	0.18
Diet	0.08	0.14	0.26	0.00	0.32	0.81	0.20	0.15
Microbial status ×Diet	0.48	0.10	0.83	0.40	0.31	0.36	0.28	0.43

[¶] TLR2, Toll like receptor 2; TLR4, Toll like receptor 4; IL8, Interleukin 8; Gal6, Gallinacin 6.

* Means are expressed as arbitrary units per µg total RNA.

[‡] Means are expressed as arbitrary units per µg total RNA for gene of interest divided by the mean arbitrary units per µg total RNA for housekeeping (HK) genes GAPDH and PRDX6.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values within a column for each main effect not sharing a common superscript are significantly different (p<0.05).

Table 9. Effects of microbial status and MOS on brush border enzyme activity in ileum of gnotobiotic broiler chicks at 7 days of age.

Treatment	Enzyme activity ($\mu\text{mol/g protein/min}$)		
	Sucrase	Maltase	APN
Microbial status¹			
CV	59	152	33
Mono	59	139	34
GF	61	173	33
Diet²			
NC	60	148	33
MOS	60	161	33
Pooled SEM	2.6	6.11	1.1
P-value			
Microbial status	0.96	0.12	0.93
Diet	0.99	0.28	0.98
Microbial status \times Diet	0.59	0.62	0.32

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

Table 10. Effect of microbial status and MOS on the transcript abundance of brush border enzymes and nutrient transporters in ileum of gnotobiotic broiler chicks at 7 days of age.

Treatment	Gene of interest [¶]							
	(per unit total RNA)*				(per unit HK gene) [‡]			
	MGA	APN	SGLT-1	PepT-1	MGA	APN	SGLT-1	PepT-1
Microbial status¹								
CV	71	134	39	143	584 ^a	124 ^a	337	163
Mono	38	118	39	151	369 ^{ab}	80 ^{ab}	369	143
GF	51	132	64	175	256 ^b	71 ^b	251	85
Diet²								
NC	45	56 ^b	24 ^b	82 ^b	515 ^a	89	344	124
MOS	61	201 ^a	71 ^a	230 ^a	291 ^b	94	293	136
Pooled SEM	11.5	20.3	11.1	26.5	50.3	7.72	29.1	19.9
P-value								
Microbial status	0.50	0.92	0.59	0.87	0.01	0.01	0.35	0.37
Diet	0.56	0.00	0.04	0.01	0.01	0.79	0.41	0.79
Microbial status ×Diet	0.60	0.32	0.36	0.10	0.16	0.56	0.23	0.25

[¶]MGA, maltase glucoamylase and APN, aminopeptidase-N; SGLT-1, sodium-glucose cotransporter1; PepT-1, peptide transporter1.

* Means are expressed as arbitrary units per µg total RNA.

[‡] Means are expressed as arbitrary units per µg total RNA for gene of interest divided by the mean arbitrary units per µg total RNA for housekeeping (HK) genes GAPDH and PRDX6.

¹ CV, conventionalized (n=19); Mono, mono-associated (n=13); GF, germ-free (n=10).

² NC, negative control (n=19); MOS, 2 g/kg Bio-Mos (n=23).

^{a,b} Mean values within a column for each main effect not sharing a common superscript are significantly different (p<0.05).

3.3.8 Comparison of bacterial communities

TRFLP analysis of the cecal microbial profile was conducted to compare community composition between conventionalized isolator-reared birds and birds reared in battery cages in a conventional environment. Clustering of TRFLP banding patterns identified a distinct microbial profile in conventionalized isolator-reared birds and conventionally reared birds (Figure 2). Conventionalized-isolator-reared birds formed a single cluster, whereas conventional birds formed two distinct clusters although one of these contained only 3 birds. No evidence of clustering of TRFLP profile was evident associated with MOS supplementation. Surprisingly, all indices indicated that microbial communities in conventionalized isolator-reared birds were more diverse than in conventionally reared birds (Table 13). Microbial diversity indices were not affected by MOS supplementation.

Table 112. Diversity indices calculated from TRFLP banding patterns in cecal contents taken from conventionalized isolator-reared birds conventionally reared birds

Treatment	Diversity Index			
	Richness	Evenness	Shannon	Simpson
Experimental environment¹				
Isolator	18	0.61	2.51	0.89
Conventional	13	0.53	1.98	0.77
Diet²				
NC	15	0.57	2.28	0.84
MOS	16	0.57	2.20	0.83
Pooled SEM	0.52	0.01	0.07	0.02
P-value				
Microbial status	0.00	0.00	0.00	0.00
Diet	0.14	0.77	0.46	0.80
Microbial status×Diet	0.33	0.89	0.87	0.96

¹ Two Experimental environments included Isolator: Conventionalized birds reared in an isolator, Conventional: conventional birds reared in a conventional environment

² Two experimental diets: NC, negative control; MOS, 2 g/kg Bio-Mos.

^{a,b} Mean values within a column not sharing a common superscript are significantly different (p<0.05).

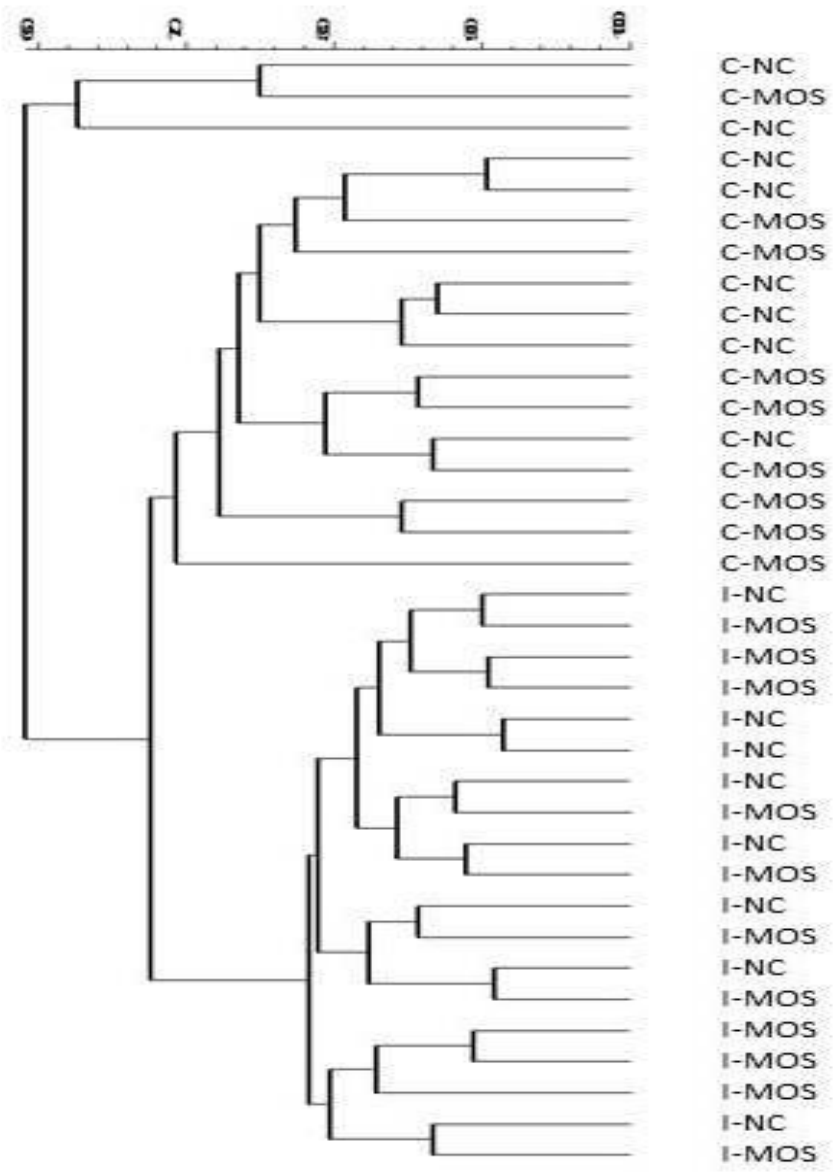


Figure 2. Dendrogram comparing the bacterial profile between conventionalized isolator-reared birds (I) and conventionally reared birds (C) using TRFLP banding patterns. Profiles from birds fed the control diet and MOS supplemented diets are labelled ‘NC’ and ‘MOS’ respectively.

3.4 Discussion

In poultry, the intestinal microbial community which is established during the first few weeks post-hatch undergoes major changes demonstrating increased diversity with age (Knarreborg et al., 2002; Lu et al., 2003). Microbial succession stimulates the development of the gut including modulating the acquired immune system (Falk et al., 1998; Macpherson and Harris, 2004; Rakoff-Nahoum et al., 2004), the integrity of the intestinal barrier (Sommer and Baeckhed, 2013) and the assimilation of nutrients (Smith et al., 2007; Musso et al., 2011; Krajmalnik-Brown et al., 2012). A number of mechanisms permit sensing of microbiota by the host and changes to GI function. These include host sampling of bacterial antigens, recognition of microbiota-associated molecular patterns by host receptors, recognition and metabolism of bioactive fermentation products and effector molecules derived from microbes (Willing and Van Kessel, 2010). Interestingly, in the present study, comparison of germ-free and conventionalized birds did not identify marked physiological responses to microbial colonization as reported in mammals (Abrams et al., 1963; Kawai and Morotomi, 1978; Shirkey et al., 2006).

Mannan oligosaccharides (MOS) derived from outer cell wall of *Saccharomyces cerevisiae*, has been studied as an alternative to AGP in farm animal production. Dietary addition of MOS has been shown to elicit specific changes in the GIT including microbial composition, morphology, nutrient digestibility, and immune response in different species including broilers. Results of the current work suggest MOS actions in the host are mediated in part by direct effects on the mucosa rather than modulation in intestinal microbial community composition.

Although necessitated by microbial contamination and an unexpectedly poor hatch rate, two major compromises are evident in the revised experimental design and suggest caution in data interpretation. Firstly, our observations are limited to a relatively small number of birds and,

although data was confirmed as normally distributed, statistical robustness was limited. Further, the effect of microbial status was confounded by environment in each isolator such that isolator-to-isolator variation was confounded with variation due to microbial status. Indeed, statistical principals would normally require that isolator be considered the experimental unit; however, replication of isolator as the experimental unit would not be economically or logistically possible for the current model.

Although a small number of birds were used, and body weight at hatch could not be recorded, bodyweight at 7 days of age was greater in both monoassociated and germ-free birds compared with CV birds. Increased rate of gain has been observed previously in gnotobiotic chickens (Furuse and Yokota, 1984b) and in gnotobiotic pigs (Shirkey et al., 2006). The performance advantage in germ-free animals has been assigned to reduced energy requirement for maintenance and/or higher nutrient uptake (Furuse and Okumura, 1994). Thus, the GF host is anticipated to have lower requirements associated with maintaining gut barrier function and detoxification of fermentation products (Furuse and Okumura, 1994; Salter et al., 1974), as well as potentially increased digestive and absorptive capacity associated with lower epithelial turnover (Corring et al., 1981; Gabriel et al., 2006). Reduced relative organ size observed in germ-free birds in this study supports the hypothesis of reduced maintenance nutrient requirements, but surprisingly, despite these significant differences in organ size, there was very little evidence in the present study of marked physiological changes in the GIT mucosa.

The liver, spleen, bursa of fabricius and intestines are vital organs in the inflammatory immune response (Roura et al., 1992) and are responsible for acute phase protein synthesis, lymphocyte activation, antibody production, and antigen sampling (Abbas et al., 2000). Therefore, it is expected that lymphoid organs tend to be smaller in germ free animals than in

conventional animals (Gordon et al., 1966; Olson and Wostmann, 1966; Bauer, 1968). The elevated size of the bursa in CV birds is consistent with markedly higher immune stimulation in the presence of commensal antigens in the intestine of CV birds which may indirectly induce the activity of the bursa (Ratcliffe, 2006). Gastrointestinal microbiota and their fermentation products also influence the size of the liver (Jozefiak et al., 2006), which was evident by a previous report of reduced liver weight in germ free vs. conventional chickens (Muramatsu et al., 1983). There was no significant difference for relative weight of spleen between CV and GF chicks in agreement with others (Thorbecke et al., 1957; Hegde et al., 1982) despite the role of this organ in immune response.

The relative lengths of the small intestinal segments were decreased in GF and monoassociated birds compared with their CV counterpart. In agreement with our findings, Furuse and Yokota (1984a) reported that the absolute and relative lengths of the small intestine in germ free chickens were lower than those of comparable conventional animals. Also, some studies have revealed that the presence of antibiotics in animal feed reduces the length of the small intestine (Gordon and Brucknerkardoss, 1961; Miles et al., 2006). However, the exact mechanism by which gut microbiota influence intestinal length is unknown. Shirkey et al. (2006) hypothesized that the increased intestinal length in conventional pigs may be a compensatory response to increase the absorptive capacity to increase intestinal surface area and/or to increase the competition with microbiota for nutrient absorption. Further, the production of glucagon-like peptide-2 (GLP-2), a trophic factor specific for the bowel (Drucker et al., 1996) stimulated by the microbial fermentation product, butyrate (Tappenden et al., 2003), may contribute to the increased length in CV gut (Lovshin et al., 2000).

Compared with mammals, morphometric changes in the intestinal mucosa are remarkably small in the germ-free chicken. Gnotobiotic studies in mice (Abrams et al., 1963; Khoury et al., 1969), rats (Meslin and Guenet, 1973) and pigs (Kenworth.R, 1967; Coates, 1980; Willing and Van Kessel, 2007) report dramatic differences in mucosal morphology including villus height, crypt depth and width. Only a small although significant decrease in CD and increase in VCR were observed in GF (not monoassociated birds) compared with CV birds in this study. No change in villus height was observed. These observations are generally consistent with previous reports comparing germ-free and conventional birds (Cook and Bird, 1973; Rolls et al., 1978).

A unique feature of poultry is that enterocyte proliferation occurs in both crypts and along the length of the villi (Uni et al., 1998b; Geyra et al., 2001b; Noy et al., 2001) in contrast to mammals where cell proliferation is restricted to the crypts. Our finding of reduced crypt depth and higher VCR in GF chicks suggest reduced cell turnover in GF animals compared to CV animals similar to observed in mammals. The abundance of PCNA transcripts represents the total mitotic cells present in the mucosa, including the epithelial population and lamina propria cells. Changes in ileal mucosal morphometry, which suggested increased epithelial turnover, were partly supported by increased PCNA transcript abundance in CV chicks when normalized to GAPDH and PRDX6 expression. This is in agreement with previous reports in chickens (Cook and Bird, 1973), mice (Abrams et al., 1963) and pigs (Abrams et al., 1963; Willing and Van Kessel, 2007) where conventional animals have shown increased intestinal epithelial proliferation compared to their germ-free counterparts.

The increased enterocyte proliferation may be a response mechanism to replace the mature enterocytes which were lost in the presence of intestinal microbiota. Bacteria may alter the enterocyte replacement rate by different mechanisms inducing the expression of

enterotrophic factors (Sakata, 1987; Siggers et al., 2008), the stimulation of local physiological inflammatory response (Shirkey et al., 2006) which in turn stimulates cell proliferation (Corredor et al., 2003) or by releasing enterotoxic bacterial metabolites such as ammonia (Visek, 1978a; Suzuki et al., 2002). Also the resident bacteria positively influence epithelial cell differentiation and proliferation in the intestine through the production of SCFA (Shanahan, 2002). It is however, curious that the low microbial load associated with mono-association did not result in morphological change or reduced PCNA expression. Further, while we did not collect morphological data on the cecum, this area of high microbial colonization under conventional conditions, did not demonstrate altered expression of PCNA.

Neither the ileum nor cecum showed evidence of alteration in apoptotic activity as assessed by the activity of caspase-3. Caspases play a vital role in the execution-phase of cell apoptosis (Nicholson and Thornberry, 1997), thus they contribute to the maintenance of homeostasis in the intestine. Numerous studies have confirmed the ability of intestinal bacteria to activate the caspase cascade (Rose et al., 1998; Akhter et al., 2009; Rolli et al., 2010; Behar et al., 2011). The lack of effect of microbial status on caspase-3 activity is consistent with the unremarkable changes in mucosal morphology observed in the chicken and the implication that the avian mucosa is less responsive to a commensal microbiota. On the other hand caspase may be a less reliable marker in the bird due to several mechanisms including a 1) phagocytosis mechanism of neighboring parenchymal cells which clear the apoptotic cells quickly (Coles et al., 1993) and/or 2) the possibility of a caspase-independent death program(s) in enterocytes (McCarthy et al., 1997; Miller et al., 1997; Sarin et al., 1997; Lavoie et al., 1998; Weil et al., 1998). There is increasing evidence that caspase-3 performs a significant role in mediating non-apoptotic functions including modulation of intestinal cellular growth (Lamkanfi et al., 2007; Yan et al.,

2001). Further, it has been reported that certain microbiota in CV birds may inhibit enterocyte apoptosis (Hausmann, 2010; Ohland and MacNaughton, 2010).

The development of immune competence in birds during the immediate post-hatch period is very critical, since chicks are immediately exposed to feed and microbial antigens. The studies using germ-free animal models and bursectomized or bursal duct-ligated chickens, reported that gut microbiota is essential for the GALT development (Thorbecke et al., 1957; Hegde et al., 1982; Ekino et al., 1985) .

The lamina propria can provide some insight into the intestinal immune status of the animal. The physiological inflammation due to colonizing gut microbiota has been determined by cellular infiltrates in the intestinal tract during the immediate post-hatch period (Lillehoj and Chung, 1992; Van Immerseel et al., 2002; Bar-Shira et al., 2003). Further, the delayed immune cell development, including lymphocytes in the lamina propria, was reported in germ-free birds compared with conventionally reared animals (Berg and Savage, 1975; Rothkotter et al., 1994; Umesaki et al., 1999; Gaskins, 2003). Our finding of greater LPA in CV birds compared to the GF and Mono birds is consistent with the notion that increased immune cell infiltration and thickening of lamina propria occurs in the presence of GIT microbiota. The lamina propria area was markedly reduced in GF chickens, probably reflecting reduced leukocyte infiltration. This response was only observed compared with GF and not monoassociated chickens, which were somewhat surprising. However, the increase in lamina propria area was not correlated with any changes in mucosal inflammatory cytokine expression.

The innate immune system of the GIT has a crucial role in sensing of invading pathogens and mounting of specific responses that eliminate or limit the infection effectively. Intestinal epithelial cells respond to signals from both apical and luminal compartments and are able to

discriminate between antigens on pathogens and those found on commensals or dietary elements that constitute antigenic material. Toll like receptors are the primary sensors of innate immune response which recognize PAMPs and subsequently initiate innate cellular responses and adaptive immune response to various pathogens. TLR2 has been shown to signal the presence of lipopeptides from Gram-positive bacteria or molecules on the bacterial and yeast cell wall, whereas TLR4 is the predominant receptor for LPS from Gram-negative organisms (de Paiva et al., 2011). Although various TLRs differ in their pathogen recognition, stimulation of their common signaling pathway results in inflammatory cytokine production and can stimulate β -defensin expression by intestinal epithelial cells. The first line defense against invading pathogens and the antimicrobial activity executed by non-oxidative mechanisms make β -defensins a key component of innate immunity in avian species (Sahl et al., 2005). Surprisingly, none of the four immune-related genes tested increased in expression in response to microbial colonization in the avian gut at day 7 post-hatch.

The TLR expression data in our study is in agreement with some gnotobiotic pig studies, including a recent study by George et al. (2012), which showed that there is no significant changes in both TLR2 and TLR4 among normal-fed, antibiotic-fed and gnotobiotic animal groups. In addition, a study by Willing and Van Kessel, 2007 also reported that TLR4 was not significantly different between CV and GF animals.

Our results for immune related genes could be explained by the concept of intestinal homeostasis, a situation which pro-inflammatory response is either not generated or is rapidly controlled in the presence of normal flora. Intestinal homeostasis in chicken is assumed to be similar to that of mammals, although it has not been fully described (Brisbin et al., 2008). There are a number of mechanisms proposed for the mammalian gut to limit the response to

commensals. The presence of TLRs on intracellular and basolateral surfaces of IECs, but not on apical surface is assumed to be one of the important mechanisms in modulation of inflammatory response against resident bacteria. However expression pattern of TLRs in chickens have not been examined at the cellular level. Also, it is speculated that some cytokines such as transforming growth factor (TGF) – β (Monteleone et al., 2004) and interleukin (IL)-10 (Steidler et al., 2001) regulate the response of TLR-mediated pro-inflammatory signals in mammals. A similar anti-inflammatory function is evident in chicken intestinal cells producing TGF- β 4 which is assumed to be involved in immune homeostasis (Withanage et al., 2005). In addition, enterocytes constitutively and inducibly expressed high levels of Toll-interacting protein (Tollip), an intracellular protein that inhibits TLRsignaling (Otte et al., 2004; Kalliomaki et al., 2012; Zhu et al., 2012). Further, Tollip is directly associated with TLR2 and TLR4 (Zhang and Ghosh, 2002) and overexpression of Tollip inhibits TLR activation in intestinal cells after stimulation with LPS or lipotechoic acid (Otte et al., 2004). Although Tollip has been found in chicken, the role of the TLR signaling pathway in chicken enterocytes has received little attention. Another TLR suppressor molecule named single immunoglobulin IL-1R related molecule (SIGIRR) was found in surface enterocytes to regulate the intestinal immune system balance between host mucosal defense and tolerance toward the resident microflora (Garlanda et al., 2004).

Digestion and absorption of nutrients at an optimal level are vital for the growth, development and health of animals. In general, it is believed that germ-free animals have some characteristics (slower small intestine cell renewal, slower gastric emptying and intestinal transit time) which should lead to better utilization of the ingested diet (Wostmann, 1981). Brush border enzymes are differently expressed along the crypt-villus axis, which is evident by mature

enterocytes near the villus tip which have higher hydrolase enzyme activity compared with immature crypt cells with lower activity (Fan et al., 2001).

Consistent with rather unremarkable changes in intestinal morphology and markers of cell proliferation and apoptosis in germ-free and conventional chickens, few changes in expression and activity of brush border hydrolases were observed. Indeed, only the normalized expression of maltase glucoamylase and aminopeptidase N were altered in GF and monoassociated birds. Oddly, expression of these hydrolases was reduced in germ-free birds without change in corresponding activity. We have previously noted a paradoxical reduction in expression of brush border hydrolases but increased activity in germ-free compared with conventional pigs (Willing and Van Kessel, 2009; Willing and Van Kessel, 2010). The response may reflect reduced microbial inactivation of brush border hydrolases in the germ-free state, contributing to a longer half-life on the enterocyte surface and thus reduced demand for transcription and synthesis. This is consistent with another study on chicks, which concluded that the presence of microbiota has no direct effect on small intestinal disaccharidase production (Siddons and Coates, 1972). However, these findings are in contrast to the findings of GF rats (Reddy et al., 1968; Kawai and Morotomi, 1978) and mice (Yolton et al., 1971) which had higher brush border enzymes including lactase, maltase, sucrase and alkaline phosphatase.

Interpretation of small intestinal absorptive activity is not easy since previous reports obtained in germ-free and conventional animals of the same species are contradictory. Some studies report faster passive and carrier-facilitated absorption in GF small intestine (Heneghan, 1963; Herskovi et al., 1967; Ford and Coates, 1971), while others found that there was no such clear-cut difference (Cole and Boyd, 1967; Wiech et al., 1967; Tennant et al., 1971). Intestinal nutrient transporters play a significant role in nutrient utilization and changes in intestinal

morphology observed here could indicate altered expression of nutrient transporters associated with changes in enterocyte maturity. Glucose, the major monosaccharide derived from most practical diets of farm animals, is absorbed through the brush border membrane by SGLT-1 (Wright, 1993). Another transport system in the intestine designated as PepT1, has an ability to handle every possible di- and tripeptide derived from dietary proteins (Adibi, 1997; Daniel, 2004). Although we found no change in SGLT-1 or PepT1 expression, up-regulation of SGLT-1 mRNA and protein in the small intestinal epithelium was found in GF compared with CV mice (Swartz et al., 2012). In contrast, (Hooper et al., 2001) reported that mouse intestinal colonization with gut microbiota induces the expression of SGLT1 in the ileum. Published data on the effects of microbial status on intestinal nutrient transporters in chickens are scarce. A study by (Hardin et al., 2000), observed an increased jejunal glucose transport following exposure to proinflammatory cytokines, but this was not associated with changes in the brush border expression of SGLT-1.

The effect of microbial status on the gene expression data was markedly less than anticipated based on previous observations in pig experiments conducted in our lab. One possible explanation that we assumed was the conventionalization of GF birds in the isolators produced microbiota with lower diversity compared with real conventional birds. Thus, a small conventional trial was conducted for comparative purposes. The results of the diversity indices and cluster analysis based on the TRFLP profiles of the 16SrRNA verified that there were two distinct bacterial populations among birds in the two trials. Further, the isolator reared birds were more diverse than the conventionally reared birds indicating our conventionalization model produced birds with microbiota at least as diverse as a conventional system. Interestingly, microbial composition among isolator reared birds was more similar than among conventionally

reared birds, consistent with the use of the single cecal inoculant in the isolators and the control of subsequent introduction of new environmental strains. Similar type of results were observed in two groups of pigs reared in outdoor (extensive) and indoor (intensive) environmental conditions (Mulder et al., 2009; Schmidt et al., 2011). These studies showed reduced microbial diversity in outdoor animals compared to indoor and isolator housed groups.

Further, it is difficult to ensure that germ free animals are free from all exogenous antigenic stimuli since most diets contain dead organisms or other antigenic materials which provoke an immune response (Wostmann, 1971). Therefore, one other plausible explanation for the less remarkable effect of microbial status on the observed intestinal parameters may be the substantial antigenic, including microbial activity likely present in the diet. This might contrast studies in pre-weaned mammals (e.g. the pig) fed milk-based diets.

Stably expressed housekeeping genes are used as internal standards to correct for variation in RNA loading amount and quality. Unfortunately, housekeeping gene expression can vary considerably in some experimental contexts (Thellin et al., 1999; Bustin, 2000; Suzuki et al., 2000; Warrington et al., 2000). In our study, four housekeeping genes were tested for suitability to normalize expression data including GAPDH, PRDX6, 18S and beta-actin. Surprisingly, all housekeeping genes were up-regulated by dietary MOS in ileum tissue but not cecal tissue. MOS upregulation of ileal housekeeping genes was confirmed in separate preparations of total RNA, reverse transcribed cDNA and by independent technical hands. Significant influence of diet on the expression of mRNA for GAPDH and β -actin was also noted by Gilbert et al. (2008). According to their study, the expression of GAPDH and β -actin were greater in the chicks consuming corn gluten meal compared to the chicks consuming equal amounts of soybean meal. Further, it has been reported that nutritional manipulations can alter

GAPDH transcription in chicks (Mozdziak et al., 2003) and mouse (Sanllorenti et al., 1992) and rats (Yamada et al., 1997). While the challenges of selecting appropriate housekeeping genes is well described (Vandesompele, et al., 2002), the consistent upregulation by MOS in ileum only in the current study is perplexing. Whether this represents a general upregulation in ileal gene expression mediated by MOS or a biological or technical anomaly is not clear. Unfortunately, the finding complicates interpretation of expression data for genes of interest which have been presented both as corrected for mean expression for GAPDH and PRDX6 and per unit total RNA (Bustin, 2000) .

Mannan oligosaccharide is an alternative antibiotic growth promotor that may either modify microbial composition or modify GI development directly by activating microbial sensing pathways. Further, MOS is reported to competitively bind to the type 1 fimbriae of pathogenic and opportunistic pathogenic bacteria and prevent them from attaching to the gut mucosa (Loddi et al., 2002). This may be one mechanism by which MOS improves resistance to enteric disease and it may save energy for body growth. In addition to directly inhibiting colonization of enteric pathogens (Oyofe et al., 1989b; Spring et al., 2000; Valancony et al., 2001), other mechanisms of action of MOS include enhancing immunity (Ferket et al., 2002; Humphrey et al., 2002), improving the brush border mucin barrier (Iji et al., 2001c; Loddi et al., 2002), increasing the integrity of gut mucosa (Sonmez and Eren, 1999; Ferket et al., 2002) and reducing enterocyte turnover (Kumprecht and Zobac, 1997; Hooge, 2004; Rosen, 2007).

Dietary inclusion of MOS did not improve body weight of the chicks in the current study; however, the number of birds used was very small such that no conclusion can be drawn. Published reports on the utilization of MOS as a growth promoter in broiler diets are inconsistent (Hooge, 2004; Rosen, 2007; Yalcinkaya et al., 2008). Discrepancies in growth performance in

broilers are probably due to the different management conditions applied in each experiment including environment and diet composition. In our study, broiler chicks were reared in a very clean environment, exposed only to cecal material from a healthy bird. Thus, mechanisms associated with pathogen exclusion may not have contributed to growth benefit in this system (Podmaniczky et al., 2006).

The effect of MOS on GIT development and the weight of visceral organs are not well elucidated. Iji et al., (2001c) reported that the weight of visceral organs (small intestine, pancreas and proventriculus/gizzard) of chicks did not differ between different inclusion levels of MOS (1, 3, 5 g/kg diet). Also, Yang et al. (2005) and Mohamed et al. (2008) reported that MOS treatments had no effect on the relative weight of small intestine, liver, gizzard, spleen and bursa. In agreement with those findings, we found that the selected relative organ weights of chicks were not influenced statistically by dietary MOS.

The effect of dietary MOS supplementation on intestinal morphology was very limited. Only villus height was affected where MOS marginally increased height in the presence of microbiota (CV or Mono) but marginally reduced height in the absence of microbiota (GF). MOS has been reported to increase villus height in the small intestinal tissues of conventional broilers in several studies (Iji et al., 2001c; Baurhoo et al., 2007b; Yang et al., 2007a; Baurhoo et al., 2009). In the case of germ-free birds, MOS may have interacted with a mucosa that otherwise received little microbial stimulation, activating responses leading to a reduction in villus height.

Increased VH correlates with enhancing digestive and absorptive capacity associated with more mature and presumably increased functional enterocytes (Stappenbeck et al., 2002). Effects of MOS on brush border enzyme activities in the ileum were investigated in our study. The specific activities of maltase and sucrase were not significantly affected by dietary addition of

MOS, which is in agreement with studies in ileum (Iji et al., 2001c) and jejunum (Yang et al., 2007; Yang et al., 2008) of broilers fed MOS. This may be supported by another study by (Yang et al., 2008) that showed the lack of difference in heat production of broilers given MOS. The processes of digestion or secretion, absorption and nutrient metabolism are considered as most significant sources of heat increment in the gut. The absence of heat increment with MOS indicated the lack of elevated digestion and absorption in birds fed MOS (Yang et al., 2008). However, Iji et al. (2001c) has reported higher specific activities of maltase and leucine aminopeptidase in the jejunum of 28-day-old broiler chicken fed MOS at 3.0 gkg⁻¹ diet. The authors speculated that the discrepancy between the jejunal and ileal data may be due to the relatively higher microbial digestion of oligosaccharides in ileum than in jejunum. Interpretation of gene expression data for genes associated with nutrient assimilation in response to MOS in the present study is very difficult given the MOS-associated increase in expression of house-keeping genes in ileum. Our results showed that MOS significantly increased the non-normalized transcription abundance in APN, SGLT1 and PepT1 which was lost when normalizing to HK genes. However, MOS-associated up-regulation was only evident in MGA when mRNA abundance is expressed per unit of housekeeping genes. Given the lack of changes in specific activity of mucosal hydrolases and the disagreement between gene expression response depending on normalizing method, conservative interpretation of our results suggest limited effects of MOS on nutrient digestion and absorption under conventional or germ-free conditions.

The immune modulatory effect of MOS has been elucidated in different farm animal species including poultry (Cotter et al., 2000; Shashidhara and Devegowda, 2003; Cheled-Shoval et al., 2011; Yitbarek et al., 2012), swine (Podzorski et al., 1990; Newman and Newman, 2001; O'Quinn et al., 2001; Davis et al., 2002a) and cattle (Franklin et al., 2002; Franklin et al., 2005).

Mannose binding proteins are present in the blood serum which binds to the mannose containing structures of various viruses and bacteria invading the host (Newman, 1994).

While there was no evidence an effect of MOS on microbial sensing through TLRs or induction of inflammatory response in the cecum, gene expression values in ileum for TLRs, IL-8 and Gal6, were all upregulated when normalized to total RNA independent of microbial status. Normalization to HK genes negated this effect. The findings in the current study in elevated ileal TLR4 expression in the MOS group are in agreement with the reports from Cheled-Shoval et al. (2011) and Yitbarek et al. (2012). Higher expression of TLR4 has been observed in both ileum and cecum of *Clostridium perfringens*-challenged broiler chickens supplemented with MOS (Yitbarek et al., 2012). In ovo feeding of MOS to embryos 3d before hatch has been associated with increase intestinal TLR4 expression at day of hatch (Cheled-Shoval et al., 2011). Although TLR4 has been recognized as a principal receptor for LPS of Gram negative bacteria (Kannaki et al., 2010), it has been further implicated in identification of mannan components of *Saccharomyces cerevisiae* and *Candida albicans* (Tada et al., 2002). In addition, Sharma et al., (2010) reported that mannan components act as MAMPs (ligands) which especially activate TLR2 and TLR4. It has been reported that activation of TLR4 signaling is linked with induction of proinflammatory cytokine production (Hoshino et al., 1999; Hirschfeld et al., 2000). The IL8 transcript abundance was greater in the ileum of MOS-fed broilers, thereby agreeing with the finding of increased TLR4 in the present study. Moreover, a previous study has shown that TLRs induced the expression of IL8 by chicken monocytes (He et al., 2011).

Given the absence of an effect of MOS on immune-related genes in cecum, perhaps a MOS mediated increase in ileum is less likely. Regardless, if an increase in immunity-related gene

expression is considered, it occurred in both conventional and germ-free birds, suggesting direct action of MOS on the mucosa rather than mediation by changes in microbial composition.

Interestingly, no effect of MOS was observed on TRFLP profile in the current study, whether fed in a conventional or an isolator environment. Diversity indices were not increased with MOS supplementation in both experiments. Therefore, the results of the present study do not support an impact of MOS intestinal microbial composition as a mechanism of action. However, a study conducted by Dimitroglou et al. (2010) revealed that MOS affected the intestinal microbial species richness and diversity in fish fed diets containing fishmeal. Further, several studies of poultry have demonstrated changes in microbial composition associated with MOS supplementation (Spring et al., 2000; Jamroz et al., 2003; Yang et al., 2008a,b).

In conclusion, gross and morphometric changes observed in germ-free and monoassociated birds were consistent with reduced energy requirements for maintenance in germ-free birds compared with their conventional counterparts. However, the present experiment demonstrated that intestinal microbiota demonstrate a remarkably limited effect on the gross morphology and histology of broiler chicks at 7 days of age in contrast to more dramatic effects reported in mammalian species. However, this finding is not inconsistent with other reports in birds. Microbial inoculation of isolator reared ex-germfree birds showed very limited activation of genes associated with immune or digestive function further underlying the contrast with reports of the marked influence of microbiota in mammals. It is unclear why the response to microbial colonization seems to be much more muted in the bird compared to mammals although there have been some reports that modern broilers have genetically lower immune responsiveness (Siegel et al., 1984; Siegel et al., 1989; Miller et al., 1992; Qureshi and Havenstein, 1994).

Observed physiological responses to MOS supplementation were apparent in both conventionalized and gnotobiotic (germ-free and monoassociated) birds. Significant interactions between MOS supplementation and microbial status were observed in villous morphology, but these also support direct effects of MOS, a microbial product, which produced a different response when other products (stimuli) of microbial origin were limited. Although difficult to interpret, gene expression data suggest MOS did alter gene expression. Further, no effect on MOS on the intestinal microbial population was observed using TRFLP. Therefore, we conclude that mannan oligosaccharides in broiler feed directly modify innate immune defenses and enterocyte functionality without bacterial mediation.

4.0 LIST OF REFERENCES

- Abbas, A. K., A. H. Lichtman, and J. S. Pober. 2000. Cellular and molecular immunology. 4th ed. Philadelphia, W.B. Saunders.
- Abbas, A. K., N. Fausto, V. Kumar, S. L. Robbins, and R. S. Cotran. 2005. Robbins and cotran pathologic basis of disease. 7th ed. Philadelphia, Saunders.
- Abrams, G. D., and J. E. Bishop. 1967. Effect of normal microbial flora on gastrointestinal motility. *Proceedings of the Society for Experimental Biology and Medicine*. 126: 301-304
- Abrams, G. D., H. Sprinz, and H. Bauer. 1963. Influence of normal flora on mucosal morphology and cellular renewal in ileum - a comparison of germ-free and conventional mice. *Laboratory Investigation*. 12: 355-364.
- Abreu, M. T. 2010. Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. *Nature Reviews Immunology*. 10: 131-143.
- Abudabos, A. M., and H. M. Yehia. 2013. Effect of dietary mannan oligosaccharide from *saccharomyces cerevisiae* on live performance of broilers under *clostridium perfringens* challenge. *Italian Journal of Animal Science*. 12: 231-235.
- Achanta, M., L. T. Sunkara, G. Dai, Y. R. Bommineni, W. Jiang, and G. Zhang. 2012. Tissue expression and developmental regulation of chicken cathelicidin antimicrobial peptides. *Journal of Animal Science and Biotechnology*. 3:15. doi:10.1186/2049-1891-3-15.
- Aderem, A. 2001. Role of toll-like receptors in inflammatory response in macrophages. *Critical Care Medicine* 29: S16-S18.
- Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature*. 406: 782-787.
- Adibi, S. A. 1997. The oligopeptide transporter (pept-1) in human intestine: Biology and function. *Gastroenterology*. 113: 332-340.
- Adil, S., and S. N. Magray. 2012. Impact and manipulation of gut microflora in poultry: A review. *Journal of Animal and Veterinary Advances*. 11: 873-877.
- Akhter, A., M. A. Gavrilin, L. Frantz, S. Washington, C. Ditty, D. Limoli et al. 2009. Caspase-7 activation by the Nlrc4/Ipaf inflammasome restricts legionella pneumophila infection. *Plos Pathogens*. 5: e1000361.
- Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nature Reviews Immunology*. 4: 499-511.

- Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell*. 124: 783-801.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology*. 215: 403-410.
- Amasheh, S., U. Wenzel, M. Boll, D. Dorn, W. M. Weber, W. Clauss et al. 1997. Transport of charged dipeptides by the intestinal H⁺/peptide symporter PepT1 expressed in *Xenopus laevis* oocytes. *Journal of Membrane Biology*. 155: 247-256.
- Amerah, A. M., V. Ravindran, and R. G. Lentle. 2009. Influence of insoluble fiber and whole wheat inclusion on the performance, digestive tract development and ileal microbiota profile of broiler chickens. *British Poultry Science*. 50: 366-375
- Amit-Romach, E., D. Sklan, and Z. Uni. 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Science*. 83: 1093-1098.
- Anderson, D. B., V. J. McCracken, R. I. Aminov, J. M. Simpson, R. I. Mackie, M. W. A. Verstegen et al. 1999. Gut microbiology and growth-promoting antibiotics in swine. *Pig News and Information*. 20: 115-122.
- Apajalahti, J. 2004. Structure and dietary modulation of intestinal microbial communities. Proceedings of the 2nd mid-Atlantic Nutrition Conference. University of Maryland, College Park, MD. 69-76.
- Apajalahti, J. H. A., A. Kettunen, M. R. Bedford, and W. E. Holben. 2001. Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. *Applied Environmental Microbiology*. 67: 5656-5667.
- Apajalahti, J. H. A., H. Kettunen, A. Kettunen, W. E. Holben, P. H. Nurminen, N. Rautonen, M. Mutanen. 2002. Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Applied Environmental Microbiology* 68: 4986-4995. Apajalahti JH1, Kettunen H, Kettunen A, Holben WE, Nurminen PH, Rautonen N, Mutanen M.
- Apajalahti, J., A. Kettunen, and H. Graham. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*. 60: 223-232.
- Awad, W. A., K. Ghareeb, and J. Boehm. 2010. Effect of addition of a probiotic micro-organism to broiler diet on intestinal mucosal architecture and electrophysiological parameters. *Journal of Animal Physiology and Animal Nutrition*. 94: 486-494.
- Bai, J. P. F., and G. L. Amidon. 1992. Structural specificity of mucosal-cell transport and metabolism of peptide drugs - implication for oral peptide drug delivery. *Pharm. Res*. 9: 969-978.

- Barfull, A., C. Garriga, M. Mitjans, and J. M. Planas. 2002. Ontogenetic expression and regulation of Na⁺-D-glucose cotransporter in jejunum of domestic chicken. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 282: G559-G564.
- Barnes, E. M., D. A. Barnum, E. G. Harry, and G. C. Mead. 1972. Intestinal flora of chicken in period 2 to 6 weeks of age, with particular reference to anaerobic bacteria. *British Poultry Science*. 13: 311-326.
- Baron, S. F., and P. B. Hylemon. 1997. Biotransformation of Bile Acids, Cholesterol, and Steroid Hormones. Pages 470-510 in *Gastrointestinal Microbiology, Volume 1*. Mackie, R. I., B. A. White, and R. E. Isaacson, eds. Springer Science & Business Media, New York, NY.
- Barrow, P. A. 1992. Probiotics for Chickens. Pages 255-257 in *Probiotics :The Scientific Basis*. Fuller, R., ed. 1st ed. Chapman & Hall, London; New York.
- Bauer, H. 1968. Cellular Defense Mechanisms. Pages 210-226 in *The Germfree Animal in Research*. Coates, M. E., H. A. Gordon, and B. S. Wostmann, eds. Academic, New York.
- Baumel, J. J. 1993. *Handbook of avian anatomy :Nomina anatomica avium*. 2nd ed. Cambridge, Mass., Nuttall Ornithological Club.
- Bedford, M. 2000. Removal of antibiotic growth promoters from poultry diets: Implications and strategies to minimise subsequent problems. *Worlds Poultry Science Journal*. 56: 347-365.
- Befus, A. D., N. Johnston, G. A. Leslie, and J. Bienenstock. 1980. Gut-associated lymphoid-tissue in the chicken .1. morphology, ontogeny, and some functional-characteristics of peyers patches. *Journal of Immunology*. 125: 2626-2632.
- Behar, S. M., C. J. Martin, M. G. Booty, T. Nishimura, X. Zhao, H. Gan et al. 2011. Apoptosis is an innate defense function of macrophages against mycobacterium tuberculosis. *Mucosal Immunology*. 4: 279-287.
- Belley, A., K. Keller, M. Goettke, and K. Chadee. 1999. Intestinal mucins in colonization and host defense against pathogens. *American Journal of Tropical Medicine and Hygiene*. 60: 1062-1062.
- Berg, R. D., and D. C. Savage. 1975. Immune-responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. *Infection and Immunity*. 11: 320-329.
- Bezuidenhout, A. J., and G. Vanaswegen. 1990. A light microscopic and immunocytochemical study of the gastrointestinal-tract of the ostrich (*struthio-camelus L*). *Onderstepoort Journal of Veterinary Research*. 57: 37-48.
- Bienenstock, J., and A. D. Befus. 1980. Mucosal immunology. *Immunology*. 41: 249-270.

- Biggs, P., C. M. Parsons, and G. C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. *Poultry Science*. 86: 2327-2336.
- Bjornvad, C. R., M. Schmidt, Y. M. Petersen, S. K. Jensen, H. Offenberg, J. Elnif et al. 2005. Preterm birth makes the immature intestine sensitive to feeding-induced intestinal atrophy. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*. 289: R1212-R1222.
- Blomberg, L., H. C. Krivan, P. S. Cohen, and P. L. Conway. 1993. Piglet ileal mucus contains protein and glycolipid (galactosylceramide) receptors specific for escherichia-coli K88 fimbriae. *Infection and Immunity*. 61: 2526-2531.
- Bogunovic, M., S. H. Dave, J. S. Tilstra, D. T. W. Chang, N. Harpaz, H. Xiong et al. 2007. Enteroendocrine cells express functional toll-like receptors. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 292: G1770-G1783.
- Boyd, F. M., and H. M. Edwards. 1967. Fat absorption by germ-free chicks. *Poultry Science*. 46: 1481-1483.
- Boyd, R., and D. Meredith. 2006. Structure-function studies on PepT1, a multimeric transport protein with broad substrate specificity. *77*: 3-5.
- Boyd, Y., M. Goodchild, S. Morroll, and N. Bumstead. 2001. Mapping of the chicken and mouse genes for toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. *Immunogenetics*. 52
- Bozkurt, M., K. Kucukyilmaz, A. U. Catli, and M. Cinar. 2009. Effect of dietary mannan oligosaccharide with or without oregano essential oil and hop extract supplementation on the performance and slaughter characteristics of male broilers. *South African Journal of Animal Science*. 39: 223-232.
- Bradford, M. M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*. 72:248-254.
- Brisbin, J. T., J. Gong, and S. Sharif. 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews*. 9: 101-110.
- Brockus, C. W., M. W. Jackwood, and B. G. Harmon. 1998. Characterization of beta-defensin prepropeptide mRNA from chicken and turkey bone marrow. *Animal Genetics*. 29: 283-289.
- Brown, K. M., and F. Moog. 1967. Invertase activity in intestine of developing chick. *Biochimica et Biophysica Acta*. 132: 185-187.

- Brownlie, R., and B. Allan. 2011. Avian toll-like receptors. *Cell and Tissue Research*. 343: 121-130.
- Burns, R. B., and M. H. Maxwell. 1986. Ultrastructure of peyer patches in the domestic-fowl and turkey. *Journal of Anatomy*. 147: 235-243.
- Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*. 25: 169-193.
- Cario, E. 2008. Barrier-protective function of intestinal epithelial toll-like receptor 2. *Mucosal Immunology*. 1: S62-S66.
- Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infection and Immunity*. 68: 7010-7017.
- Caviedes-Vidal, E., T. J. McWhorter, S. R. Lavin, J. G. Chediack, C. R. Tracy, and W. H. Karasov. 2007. The digestive adaptation of flying vertebrates: High intestinal paracellular absorption compensates for smaller guts. *Proceedings of the National Academy of Sciences of the United States of America*. 104: 19132-19137.
- Cervantes, H. 2006. Banning antibiotic growth promoters: Learning from the european experience. *Poultry International*. 45: 10-14.
- Cetin, N., B. Guclu, and E. Cetin. 2005. The effects of probiotic and mannanoligosaccharide on some haematological and immunological parameters in turkeys. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine*. 52: 263-267.
- Che, M. T., R. W. Johnson, K. W. Kelley, & J. E. Pettigrew. 2008. Effects of mannan oligosaccharide and mannan rich fraction of *saccharomyces cerevisiae* on production of cytokines by alveolar macrophages . *Journal of Animal Sciences*.86: 347. (Abstr.)
- Che, T. M., R. W. Johnson, K. W. Kelley, K. A. Dawson, C. A. Moran, and J. E. Pettigrew. 2012. Effects of mannan oligosaccharide on cytokine secretions by porcine alveolar macrophages and serum cytokine concentrations in nursery pigs. *Journal of Animal Science*. 90: 657-668.
- Che, T. M., R. W. Johnson, K. W. Kelley, W. G. Van Alstine, K. A. Dawson, C. A. Moran et al. 2011. Mannan oligosaccharide improves immune responses and growth efficiency of nursery pigs experimentally infected with porcine reproductive and respiratory syndrome virus. *Journal of Animal Science*. 89: 2592-2602.
- Chee, S. H., P. A. Iji, M. Choct, L. L. Mikkelsen, and A. Kocher. 2010. Characterisation and response of intestinal microflora and mucins to mannanoligosaccharide and antibiotic supplementation in broiler chickens. *British Poultry Science*. 51:368-380.

- Cheled-Shoval, S. L., E. Amit-Romach, M. Barbakov, and Z. Uni. 2011. The effect of in ovo administration of mannan oligosaccharide on small intestine development during the pre- and posthatch periods in chickens. *Poultry Science*. 90: 2301-2310.
- Chen, H., E. A. Wong, and K. E. Webb. 1999. Tissue distribution of a peptide transporter mRNA in sheep, dairy cows, pigs, and chickens. *Journal of Animal Science*. 77: 1277-1283.
- Chen, H., Y. X. Pan, E. A. Wong, and K. E. Webb. 2005. Dietary protein level and stage of development affect expression of an intestinal peptide transporter (cPepT1) in chickens. *Journal of Nutrition*. 135: 193-198.
- Chen, H., Y. X. Pan, E. A. Wong, J. R. Bloomquist, and K. E. Webb. 2002. Molecular cloning and functional expression of a chicken intestinal peptide transporter (cPepT1) in xenopus oocytes and Chinese hamster ovary cells. *Journal of Nutrition*. 132: 387-393.
- Cheng, H., and C. P. Leblond. 1974. Origin, differentiation and renewal of 4 main epithelial-cell types in mouse small intestine .1. columnar cell. *American Journal of Anatomy*. 141: 461-480.
- Chichlowski, M., W. J. Croom, F. W. Edens, B. W. McBride, R. Qiu, C. C. Chiang et al. 2007. Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poultry Science*. 86: 1121-1132.
- Choct, M. 1999. Soluble non-starch polysaccharides affect net utilisation of energy by chickens. *Recent Advances in Animal Nutrition in Australia*. 12: 31-35.
- Choct, M. 2009. Managing gut health through nutrition. *British Poultry Science*. 50: 9-15.
- Choct, M., and A. Kocher. 2000. Non-starch carbohydrates: Digestion and its secondary effects in monogastrics. *Proceedings of the Nutrition Society of Australia*. 24: 31-38.
- Choct, M., R. J. Hughes, J. Wang, M. R. Bedford, A. J. Morgan, and G. Annison. 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. *British Poultry Science*. 37: 609-621.
- Choct, M., Y. Dersjant-Li, J. McLeish, and M. Peisker. 2010. Soy oligosaccharides and soluble non-starch polysaccharides: A review of digestion, nutritive and anti-nutritive effects in pigs and poultry. *Asian-Australasian Journal of Animal Sciences*. 23: 1386-1398.
- Chotinsky, D., E. Toncheva, and Y. Profirov. 2001. Development of disaccharidase activity in the small intestine of broiler chickens. *British Poultry Science*. 42: 389-393.
- Coates, M. E. 1973. Gnotobiotic animals in nutrition research. *Proceedings of the Nutrition Society*. 32: 53-58.

- Coates, M. E. 1980. The Gut Microflora and Growth. Pages 175-227 in *Growth in Animals*. Lawrence, T. I. J., ed. Butterworths, Boston.
- Coates, M. E., G. F. Harrison, M. Lev, R. Fuller, and S. F. Suffolk. 1963. A comparison of growth of chicks in gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. *British Journal of Nutrition*. 17: 141-150.
- Coates, M. E., M. K. Davies, and S. K. Kon. 1955. The effect of antibiotics on the intestine of the chick. *British Journal of Nutrition*. 9: 110-119.
- Coates, M. E., M. K. Davies, and S. K. Kon. 1955. The effect of antibiotics on the intestine of the chick. *British Journal of Nutrition*. 9: 110-119.
- Cole, J. R., and F. M. Boyd. 1967. Fat absorption from the small intestine of gnotobiotic chicks. *Applied Microbiology*. 15: 1229-1234.
- Collett, S. R. 2004. Controlling Gastrointestinal Disease to Improve Absorption Membrane Integrity and Optimize Digestion Efficiency. Pages 77-91 in *Interfacing Immunity, Gut Health and Performance*. Waldron, L. A., and J. A. Taylor-Pickard, eds. Nottingham University Press, Nottingham, UK.
- Cook, R. H., and F. H. Bird. 1973. Duodenal villus area and epithelial cellular migration in conventional and germfree chicks. *Poultry Science*. 52: 2276-2280.
- Corredor, J., F. Yan, C. C. Shen, W. Tong, S. K. John, G. Wilson et al. 2003. Tumor necrosis factor regulates intestinal epithelial cell migration by receptor-dependent mechanisms. *American Journal of Physiology-Cell Physiology*. 284: C953-C961.
- Corring, T., C. Juste, and C. Simoesnunes. 1981. Digestive enzymes in the germfree animal. *Reproduction Nutrition Development*. 21: 355-370.
- Cotter, P. F., A. Malzone, B. Paluch, M. S. Lilburn, & A. E. Sefton. 2000. Modulation of humoral immunity in commercial laying hens by a dietary prebiotic. *Poultry Science*. 79 (Abstr.)
- Covert, M. W., T. H. Leung, J. E. Gaston, and D. Baltimore. 2005. Achieving stability of lipopolysaccharide-induced NF-kappa B activation. *Science*. 309: 1854-1857.
- Cummings, J. H., and G. T. Macfarlane. 2002. Gastrointestinal effects of prebiotics. *British Journal Nutrition*. 87: S145-S151.
- Dahlqvist, A. 1964. Method for assay of intestinal disaccharidases. *Analytical Biochemistry*. 7: 18-25.
- Dahlqvist, A., B. Bull, and Gustafss.be. 1965. Rat intestinal 6-bromo-2-naphthyl glycosidase and disaccharidase activities .I. enzymic properties and distribution in digestive tract of

- conventional and germ-free animals. *Archives of Biochemistry and Biophysics*. 109: 150-158.
- Damle, M., P. Harikumar, and S. Jamdar. 2010. Chicken intestine: A source of aminopeptidases. *Scienceasia*. 36: 137-141.
- Daniel, H. 2004. Molecular and integrative physiology of intestinal peptide transport. *Annual Review of Physiology*. 66: 361-384.
- Dautlick, J., and Strittma, C.F., 1970. Developmental and hormone-induced changes in chicken intestinal disaccharidases. *Biochimica et Biophysica Acta*. 222: 447-454.
- Davis, M. E., C. V. Maxwell, D. C. Brown, B. Z. de Rodas, Z. B. Johnson, E. B. Kegley et al. 2002. Effect of dietary mannan oligosaccharides and (or) pharmacological additions of copper sulfate on growth performance and immunocompetence of weanling and growing/finishing pigs. *Journal of Animal Science*. 80: 2887-2894.
- Davis, W. C., R. L. Konzek, K. Haas, D. M. Estes, M. J. Hamilton, D. R. Call et al. 2002. Use of the mannan receptor to selectively target vaccine antigens for processing and antigen presentation through the MHC class I and class II pathways. *Annals of the New York Academy of Sciences*. 969: 119-125.
- Dawson, K. A. 2001. Development of an appropriate microflora in the gut. *Proceedings of poultry beyond 2005—The 2nd international broiler nutritionists' conference, Rotorua, New Zealand*. 89-105.
- de Paiva, N. M., M. L. S. Ayrizono, M. Milanski, A. Coope, L. M. F. Oliveira, J. J. Fagundes et al. 2011. Differential expression of TLR2, TLR4 and JNK in mucosa of ileal pouches for ulcerative colitis. is there a role for bacterial antigen pathway in asymptomatic patients? *International Journal of Clinical and Experimental Medicine*. 4: 179-186.
- Denbow, D. M. 2000. *Gastrointestinal Anatomy and Physiology*. Pages 299-325 in *Sturkie's Avian Physiology*. Whittow, G. C., ed. 5th ed. Academic, San Diego; London.
- Denev, S. A., I. Dinev, I. Nikiforov, and V. Koinarski. 2005. Effects of mannanoligosaccharides on composition of the cecal microflora and performance of broiler chickens. *Proceedings of the 15th european symposium on poultry nutrition, Balatonfüred, Hungary*. 365-367.
- Deplancke, B., and H. R. Gaskins. 2001. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. *American Journal of Clinical Nutrition*. 73: 1131S-1141S.
- Diarra, M.S., and F. Malouin. 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2014.00282.

- Dibner, J. J., J. D. Richards, and C. D. Knight. 2008. Microbial imprinting in gut development and health. *Journal of Applied Poultry Research*. 17: 174-188.
- Dibner, J., and J. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*. 84: 634-643.
- Dicksved, J., H. Floistrup, A. Bergstrom, M. Rosenquist, G. Pershagen, A. Scheynius et al. 2007. Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Applied Environmental Microbiology*. 73: 2284-2289.
- Dignass, A. U. 2001. Mechanisms and modulation of intestinal epithelial repair. *Inflammatory Bowel Diseases*. 7: 68-77.
- Dilworth, L., M. Gardner, H. Asemota, and F. Omoruyi. 2012. Intestinal morphology assessments of rats fed phytic acid extract from sweet potato (*ipomoea batatas*) and IP6. *Basic and Applied Pathology*. 5: 84-90.
- Dimitroglou, A., D. L. Merrifield, P. Spring, J. Sweetman, R. Moate, and S. J. Davies. 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*sparus aurata*). *Aquaculture*. 300: 182-188.
- Drew, M., A. Van Kessel, and D. Maenz. 2003. Absorption of methionine and 2-hydroxy-4-methylthiobutoanic acid in conventional and germ-free chickens. *Poultry Science*. 82: 1149-1153.
- Drucker, D. J., P. Ehrlich, S. L. Asa, and P. L. Brubaker. 1996. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proceedings of the National Academy of Sciences of the United States of America*. 93: 7911-7916.
- Duguid, J. P., and D. C. Old. 1980. Adhesive Properties of Enterobacteriaceae. Pages 185-217 in *Bacterial Adherence, Receptors and Recognition*. Beachey, E. H., ed. Chapman and Hall, London.
- Duguid, J. P., M. R. Darekar, and D. W. F. Wheater. 1976. Fimbriae and infectivity in salmonella-typhimurium. *Journal of Medical Microbiology*. 9: 459-473.
- Dukes, H. H., M. J. Swenson, and W. O. Reece. 1993. *Dukes' physiology of domestic animals*. 11th ed. Ithaca, N.Y., Cornell University Press.
- Dumoncaux, T. J., J. E. Hill, S. A. Briggs, K. K. Amoako, S. M. Hemmingsen, and A. G. Van Kessel. 2006. Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. *Journal of Microbiological Methods*. 64: 46-62.

- Dumonceaux, T. J., J. E. Hill, S. M. Hemmingsen, and A. G. Van Kessel. 2006. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. *Applied Environmental Microbiology*. 72: 2815-2823.
- Duncan, M. J., E. L. Mann, M. S. Cohen, I. Ofek, N. Sharon, and S. N. Abraham. 2005. The distinct binding specificities exhibited by enterobacterial type 1 fimbriae are determined by their fimbrial shafts. *Journal of Biological Chemistry*. 280): 37707-37716.
- Edens, F. W., C. R. Parkhurst, I. A. Casas, and W. J. Dobrogosz. 1997. Principles of ex ovo competitive exclusion and in ovo administration of lactobacillus reuteri. *Poultry Science*. 76: 179-196.
- Ekino, S., K. Suginozono, T. Urano, H. Fujii, K. Matsuno, and M. Kotani. 1985. The bursa of fabricius - a trapping site for environmental antigens. *Immunology*. 55: 405-410.
- Engberg, R. M., M. S. Hedemann, T. D. Leser, and B. B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poultry Science*. 79: 1311-1319.
- Erickson, R. H., J. R. Gum, M. M. Lindstrom, D. Mckean, and Y. S. Kim. 1995. Regional expression and dietary-regulation of rat small-intestinal peptide and amino-acid transporter messenger-rnas. *Biochemical Biophysical Research Communications*. 216: 249-257.
- Eshdat, Y., I. Ofek, Y. Yashouvgan, N. Sharon, and D. Mirelman. 1978. Isolation of a mannose-specific lectin from escherichia-coli and its role in the adherence of the bacteria to epithelial-cells. *Biochemical Biophysical Research Communications*. 85: 1551-1559.
- Evans, E. W., and B. G. Harmon. 1995. A review of antimicrobial peptides: Defensins and related cationic peptides. *Veterinary Clinical Pathology*. 24: 109-116.
- Eyssen, H. 1973. Role of gut microflora in metabolism of lipids and sterols. *Proceedings of the Nutrition Society*. 32: 59-63.
- Falk, P. G., L. V. Hooper, T. Midtvedt, and J. I. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. *Microbiology and Molecular Biology Reviews*. 62: 1157-1170.
- Fan, M. Z., B. Stoll, R. Jiang, and D. G. Burrin. 2001. Enterocyte digestive enzyme activity along the crypt-villus and longitudinal axes in the neonatal pig small intestine. *Journal of Animal Science*. 79: 371-381.
- Fasano, A., and T. Shea-Donohue. 2005. Mechanisms of disease: The role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nature Clinical Practice Gastroenterology & Hepatology*. 2: 416-422.

- Fasina, Y. O., P. S. Holt, E. T. Moran, R. W. Moore, D. E. Conner, and S. R. McKee. 2008. Intestinal cytokine response of commercial source broiler chicks to salmonella typhimurium infection. *Poultry Science*. 87: 1335-1346.
- Fawcett, D. W., and R. P. Jensch. 2002. Bloom and Fawcett's concise histology. 2nd edn. ed. London, Hodder Arnold.
- Fay, J. P., and R. N. Farias. 1975. Inhibitory action of fatty-acids on growth of escherichia-coli. *Journal of general microbiology*. 91: 233-240.
- Feduccia, A. 2011. Cretaceous avian crops reveal dietary secrets and pose evolutionary questions. *Proceedings of the National Academy of Sciences of the United States of America*. 108: 16487-16488.
- Fei, Y. J., Y. Kanai, S. Nussberger, V. Ganapathy, F. H. Leibach, M. F. Romero et al. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature*. 368: 563-566.
- Fellermann, K., and E. F. Stange. 2001. Defensins - innate immunity at the epithelial frontier. *European Journal of Gastroenterology and Hepatology*. 13: 771-776.
- Feracci, H., and S. Maroux. 1980. Rabbit intestinal aminopeptidase-N purification and molecular-properties. *Biochimica and Biophysica Acta*. 599: 448-463.
- Feracci, H., S. Maroux, J. Bonicel, and P. Desnuelle. 1982. The amino-acid-sequence of the hydrophobic anchor of rabbit intestinal brush-border aminopeptidase-N. *Biochimica and Biophysica Acta*. 684: 133-136.
- Ferket, P. R. 1991. Effect of diet on gut microflora of poultry. *Zootecnica International*. 7/8: 44-49.
- Ferket, P. R. 2004. Alternatives to antibiotics in poultry production: Responses, practical experience and recommendations. *nutritional biotechnology in the feed and food industries. Proceedings of alltech's 20th annual symposium, Kentucky, USA*. 57-67.
- Ferket, P. R., C. W. Parks, and J. L. Grimes. 2002. Benefits of dietary antibiotic and mannan oligosaccharide supplementation for poultry. *Proceedings of the multi-state poultry feeding and nutrition conference. Indianapolis, USA*. 14-16.
- Fernandez, F., M. Hinton, and B. Van Gils. 2000. Evaluation of the effect of mannan-oligosaccharides on the competitive exclusion of salmonella enteritidis colonization in broiler chicks. *Avian Pathology*. 29: 575-581.
- Fernandez, F., M. Hinton, and B. Van Gils. 2002. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to salmonella enteritidis colonization. *Avian Pathology*. 31: 49-58.

- Ferraris, R. P. 2001. Dietary and developmental regulation of intestinal sugar transport. *Biochemical Journal*. 360: 265-276.
- Ferrer, R., J. M. Planas, and M. Moreto. 1995. Cell apical surface area in enterocytes from chicken small and large intestine during development. *Poultry Science*. 74: 1995-2002.
- Ford, D. J., and M. E. Coates. 1971. Absorption of glucose and vitamins of B complex by germ-free and conventional chicks. *Proceedings of the Nutrition Society*. 30: A10-A11.
- Forder, R. E. A., G. S. Howarth, D. R. Tivey, and R. J. Hughes. 2007. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. *Poultry Science*. 86: 2396-2403.
- Frankel, W. L., W. Zhang, A. Singh, D. M. Klurfeld, S. Don, T. Sakata et al. 1994. Mediation of the trophic effects of short-chain fatty-acids on the rat jejunum and colon. *Gastroenterology*. 106: 375-380.
- Franklin, S. T., K. E. Newman, and M. C. Newman. 2002. Evaluation of mannan oligosaccharide on the immune status of dairy cows and their calves. *Journal of Dairy Science*. 85: 192-192.
- Franklin, S. T., M. C. Newman, K. E. Newman, and K. I. Meek. 2005. Immune parameters of dry cows fed mannan oligosaccharide and subsequent transfer of immunity to calves. *Journal of Dairy Science*. 88: 766-775.
- Franti, C. E., A. D. Wiggins, H. E. Adler, and L. M. Julian. 1972. Antibiotic growth promotion - effects of zinc bacitracin and oxytetracycline on digestive, circulatory, and excretory systems of new-hampshire cockerels. *Poultry Science*. 51: 1137-1145.
- Friedman, A., E. Bar-Shira, and D. Sklan. 2003. Ontogeny of gut associated immune competence in the chick. *Worlds Poultry Science Journal*. 59: 209-219.
- Fuku, A., N. Inoue, M. Matsumoto, M. Nomura, K. Yamada, Y. Matsuda et al. 2001. Molecular cloning and functional characterization of chicken toll-like receptors - A single chicken toll covers multiple molecular patterns. *Journal of Biological Chemistry*. 276: 47143-47149.
- Fuller, R. 1984. Microbial activity in the alimentary-tract of birds. *Proceedings of the Nutrition Society*. 43: 55-61.
- Fuller, R. 1989. Probiotics in man and animals. *Journal of the Applied Bacteriology*. 66: 365-378.
- Furuse, M., and H. Yokota. 1984a. Effect of the gut microflora on the size and weight of organs of chicks fed diets of different protein-content. *British Poultry Science*. 25: 429-439.

- Furuse, M., and H. Yokota. 1984b. Protein and energy-utilization in germ-free and conventional chicks given diets containing different levels of dietary-protein. *British Journal of Nutrition*. 51: 255-264.
- Furuse, M., and J. Okumura. 1994. Nutritional and physiological-characteristics in germ-free chickens. *Comparative Biochemisry and Physiology*. 109: 547-556.
- Gabella, G. 1985. Structure of the musculature of the chicken small-intestine. *Anatomy and Embryology*. 171: 139-149.
- Gabriel, I., M. Lessire, S. Mallet, and J. F. Guillot. 2006. Microflora of the digestive tract: Critical factors and consequences for poultry. *Worlds Poultry Science Journal*. 62: 499-511.
- Gabriel, I., S. Mallet, M. Leconte, G. Fort, and M. Naciri. 2003. Effects of whole wheat feeding on the development of coccidial infection in broiler chickens. *Poultry Science*. 82: 1668-1676.
- Galfi, P., and J. Bokori. 1990. Feeding trial in pigs with a diet containing sodium normal-butyrate. *Acta Veterinaria Hungarica*. 38: 3-17.
- Gal-Garber, O., and Z. Uni. 2000. Chicken intestinal aminopeptidase: Partial sequence of the gene, expression and activity. *Poultry Science*. 79: 41-45.
- Gal-Garber, O., S. J. Mabweesh, D. Sklan, and Z. Uni. 2000. Partial sequence and expression of the gene for and activity of the sodium glucose transporter in the small intestine of fed, starved and re-fed chickens. *Journal of Nutrition*. 130: 2174-2179.
- Ganz, T. 2003. Defensins: Antimicrobial peptides of innate immunity. *Nature Reviews Immunology*. 3: 710-720.
- Garlanda, C., F. Riva, N. Polentarutti, C. Buracchi, M. Sironi, M. De Bortoli et al. 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proceedings of the National Academy of Sciences of the United States of America*. 101: 3522-3526.
- Gasbarrini, G., and M. Montalto. 1999. Structure and function of tight junctions. role in intestinal barrier. *Italian Journal of Gastroenterology and Hepatology*. 31: 481-488.
- Gaskins, H. 2003.
The commensal microbiota and development of mucosal defense in the mammalian intestine. 9th international symposium of digestive physiology in pigs, Department of Agricultural, Food and Nutritional Science, University of Alberta, Banff, Alberta.
- Gaskins, H. R. 1997. Immunological Aspects of host/microbiota Interactions at the Intestinal Epithelium. Pages 537-587 in *Gastrointestinal Microbiology and Host Interactions*. Mackie, R. I., B. A. White, and R. E. Isaacson, eds. Chapman & Hall, New York.

- Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants: Mode of action. *Animal Biotechnology*. 13: 29-42.
- George, S., K. Circle, S. Lindblom, S. Vilain, A. J. M. Rosa, D. Francis et al. 2012. Assessment of toll-like receptors in the ileum of weanling pigs- responses to feed antibiotic chlortetracycline and gnotobiotic conditions. *Journal of Clinical Cell Immunology*. 3: 125.
- Geyra, A., Z. Uni, and D. Sklan. 2001. Enterocyte dynamics and mucosal development in the posthatch chick. *Poultry Science*. 80: 776-782.
- Geyra, A., Z. Uni, and D. Sklan. 2001. The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. *British Journal of Nutrition*. 86: 53-61.
- Gibbons, R. J., and J. Vanhoute. 1975. Bacterial adherence in oral microbial ecology. *Annual Review of Microbiology*. 29: 19-44.
- Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *Journal of Nutrition*. 125: 1401-1412.
- Gibson, G. R., and X. Wang. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *Journal of Applied Bacteriology*. 77: 412-420.
- Gilbert, E. R., H. Li, D. A. E., K. E. Webb Jr., and E. A. Wong. 2008. Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. *Journal of Nutrition*. 138: 262-271.
- Gilbert, E. R., H. Li, D. A. Ernmersonj, K. E. Webb Jr., and E. A. Wong. 2007. Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. *Poultry Science*. 86: 1739-1753.
- Goebel, T. W. F., B. Kaspers, and M. Stangassinger. 2001. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *Intestinal Immunology*. 13: 757-762.
- Goitsuka, R., C. H. Chen, L. Benyon, Y. Asano, D. Kitamura, and M. D. Cooper. 2007. Chicken cathelicidin-B1, an antimicrobial guardian at the mucosal M cell gateway. *Proceedings of the National Academy of Sciences of the United States of America*. 104: 15063-15068.
- Gong, J., H. Yu, T. Liu, J. J. Gill, J. R. Chambers, R. Wheatcroft et al. 2008. Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *Journal of Applied Microbiology*. 104: 1372-1382.
- Gordon, A. H., and A. Koy. 1985. The acute phase response to injury and infection. The roles of interleukin 1 and other mediators. Amsterdam, Elsevier.

- Gordon, H. A., and E. Brucknerkardoss. 1961. Effects of normal microbial flora on various tissue elements of small intestine. *Acta Anatomica*. 44: 210-225.
- Gordon, H. A., and L. Pesti. 1971. Gnotobiotic animal as a tool in study of host microbial relationships. *Bacteriological Reviews*. 35: 390-429.
- Gordon, H. A., Bruckner, E. T. E. Staley, M. Wagner, and B. S. Wostmann. 1966. Characteristics of germfree rat. *Acta Anatomica*. 64: 367-389.
- Gordon, J. I. 1989. Intestinal epithelial differentiation - new insights from chimeric and transgenic mice. *Journal of Cellular Biology*. 108: 1187-1194.
- Greko, C. 2001. Safety Aspects on Non-use of Antimicrobials as Growth Promoters. Pages 219-230 in *Gut Environment of Pigs*. Piva, A., K. E. Bach Knudsen, and J. E. Lindberg, eds. Nottingham University Press, Nottingham, U.K.
- Grootjans, J., G. Thuijls, F. Verdam, J. Derikx, K. Lenaerts, and W. A. Buurman. 2010. Non-invasive assessment of barrier integrity and function of the human gut. *World Journal of Gastrointestinal Surgery*. 2: 61-69.
- Gunther, N. W., J. A. Snyder, V. Lockett, I. Blomfield, D. E. Johnson, and H. L. T. Mobley. 2002. Assessment of virulence of uropathogenic *Escherichia coli* type 1 fimbrial mutants in which the invertible element is phase-locked on or off. *Infection and Immunity*. 70: 3344-3354.
- Gusseklou, S. W. S. 2006. Feeding Structures in Birds . Pages 14-32 in *Feeding in Domestic Vertebrates : From Structure to Behaviour*. Bels, V. L., ed. CABI Publication, Wallingford, UK; Cambridge, MA.
- Hardin, J., K. Kroeker, B. Chung, and D. Gall. 2000. Effect of proinflammatory interleukins on jejunal nutrient transport. *Gut*. 47: 184-191.
- Harmon, B. G. 1998. Avian heterophils in inflammation and disease resistance. *Poultry Science*. 77: 972-977.
- Hasenstein, J. R., G. L. Zhang, and S. J. Lamont. 2006. Analyses of five gallinacin genes and the salmonella enterica serovar enteritidis response in poultry. *Infection and Immunity*. 74: 3375-3380.
- Hausmann, M. 2010. How bacteria-induced apoptosis of intestinal epithelial cells contributes to mucosal inflammation. *International Journal of Inflammation*. 2010: 574568-574568.
- He, H., K. M. MacKinnon, K. J. Genovese, and M. H. Kogut. 2011. CpG oligodeoxynucleotide and double-stranded RNA synergize to enhance nitric oxide production and mRNA expression of inducible nitric oxide synthase, pro-inflammatory cytokines and chemokines in chicken monocytes. *Innate Immunity*. 17: 137-144.

- Hediger, M. A., and D. B. Rhoads. 1994. Molecular physiology of sodium-glucose cotransporters. *Physiological Reviews*. 74: 993-1026.
- Hegde, S. N., B. A. Rolls, A. Turvey, and M. E. Coates. 1982. Influence of gut microflora on the lymphoid-tissue of the chicken (*Gallus domesticus*) and Japanese quail (*Coturnix coturnix-japonica*). *Comparative Biochemistry and Physiology A-Physiology*. 72: 205-209.
- Heneghan, J. B. 1963. Influence of microbial flora on xylose absorption in rats and mice. *American Journal of Physiology*. 205: 417-420.
- Herskovi, T., J. Katz, M. H. Floch, R. P. Spencer, and H. M. Spiro. 1967. Small intestinal absorption and morphology in germfree monocontaminated and conventionalized mice. *Gastroenterology*. 52: 1136.(Abstr).
- Hill, J. E., R. P. Seipp, M. Betts, L. Hawkins, A. G. Van Kessel, W. L. Crosby et al. 2002. Extensive profiling of a complex microbial community by high-throughput sequencing. *Applied and Environmental Microbiology*. 68: 3055-3066.
- Hill, J. E., S. L. Penny, K. G. Crowell, S. H. Goh, and S. M. Hemmingsen. 2004. cpnDB: A chaperonin sequence database. *Genome Research*. 14: 1669-1675.
- Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: Repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *Journal of Immunology*. 165: 618-622.
- Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*. 424(6950): 743-748.
- Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. *Journal of Applied Poultry Research*. 12: 60-64.
- Holmes, R., and R. W. Lobley. 1989. Intestinal brush-border revisited. *Gut*. 30: 1667-1678.
- Honjo, K., T. Hagiwara, K. Itoh, E. Takahashi, and Y. Hirota. 1993. Immunohistochemical analysis of tissue distribution of B and T-cells in germ-free and conventional chickens. *Journal of Veterinary Medical Science*. 55: 1031-1034.
- Hooge, D. M. 2003. Dietary mannan oligosaccharides improve broiler and turkey performance: Meta-analysis of pen trials around the world. *Nutritional biotechnology in the feed and food industries. Proceedings of alltech's 19th annual symposium.* , Lexington, Kentucky, USA.
- Hooge, D. M. 2004. Meta-analysis of broiler chicken pen trials evaluating dietary mannan oligosaccharide, 1993-2003. *International Journal of Poultry Science*. 3: 163-174.

- Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. C. Falk, and J. I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science*. 291: 881-884.
- Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda et al. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the *Ips* gene product. *Journal of Immunology*. 162: 3749-3752.
- Huang, F. S., C. J. Kemp, J. L. Williams, C. R. Erwin, and B. W. Warner. 2002. Role of epidermal growth factor and its receptor in chemotherapy-induced intestinal injury. *American Journal of Physiology*. 282: G432-G442.
- Hughes, R. J. 2003. Energy Metabolism of Chickens: Physiological Limitations : A Report for the Rural Industries Research and Development Corporation. Pages 172-176 RIRDC Publication, Kingston.
- Humphrey, B. D., E. A. Koutsos, and K. C. Klasing. 2002. Requirements and Priorities of the Immune System for Nutrients. Pages 69-77 in *Nutrition Biotechnology in Feed and Food Industries: Proceedings of Alltech's 18th Annual Symposium*. Lyons, T. P. J., K.A., ed. Nottingham University press, Nottingham, UK.
- Hung, I. F., M. D. Lindemann, G. L. Cromwell, B. G. Kim, & M. G. Holt. 2008. Effect of supplying mannan oligosaccharide (MOS) to pig diets on response to an immune challenge. *Journal of Animal Science*. 86: 57. (Abstr.)
- Iji, P. A., A. Saki, and D. R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. *British Poultry Science*. 42: 505-513.
- Iji, P. A., A. Saki, and D. R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 2. Development and characteristics of intestinal enzymes. *British Poultry Science*. 42: 514-522.
- Iji, P., A. Saki, and D. Tivey. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. *Journal of the Science of Food and Agriculture*. 81: 1186-1192.
- Ingenbleek, Y., and V. Young. 1994. Transthyretin (prealbumin) in health and disease: Nutritional implications. *Annual Review of Nutrition*. 14: 495-533.
- Ivanov, I., B. R. Chapados, J. A. McCammon, and J. A. Tainer. 2006. Proliferating cell nuclear antigen loaded onto double-stranded DNA: Dynamics, minor groove interactions and functional implications. *Nucleic Acids Research*. 34: 6023-6033.

- Jamroz, D., A. Wiliczekiewicz, J. Orda, T. Wertelecki, and J. Skorupinska. 2003. Effect of a feed antibiotic or mannan oligosaccharides in broiler chickens. Alltech's 20th annual symposium on nutritional biotechnology in feed and food industries, poster, Lexington, Kentucky, USA.
- Jamroz, D., A. Wiliczekiewicz, J. Skorupinska, and J. Orda. 1998. Fermentation and apparent digestion of the structural carbohydrates in chicks, ducks and geese fed triticale mixtures supplemented with enzyme. *Journal of Animal Physiology and Animal Nutrition*. 79: 1-17.
- Jamroz, D., K. Jakobsen, K. E. B. Knudsen, A. Wiliczekiewicz, and J. Orda. 2002. Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley. *Comparative Biochemistry and Physiology a- Molecular and Integrative Physiology*. 131: 657-668.
- Jensen, B. 1993. The possibility of manipulation of microbial activity in the digestive tract of monogastric animals. in: *Proceedings of the 44th annual meeting for Animal Production*. 44th annual meeting. European association for animal production, Arhus, Denmark. pp.313.
- Jensen, B. B. 1998. The impact of feed additives on the microbial ecology of the gut in young pigs. *Journal of Animal and Feed Sciences*. 7: 45-64.
- Jeurissen, S. H. M., F. Lewis, J. D. van der Klis, Z. Mroz, J. M. J. Rebel, and A. A. H. M. ter Huurne. 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Current Issues of Intestinal Microbiology*. 3: 1-14.
- Johansen, C. H., L. Bjerrum, K. Finster, and K. Pedersen. 2006. Effects of a campylobacter jejuni infection on the development of the intestinal microflora of broiler chickens. *Poultry Science*. 85: 579-587.
- Johnson, L. R. 2006. *Physiology of the gastrointestinal tract*. 4th ed. Amsterdam; Boston, Elsevier Academic Press.
- Johnson, R. W. 1997. Inhibition of growth by pro-inflammatory cytokines: An integrated view. *Journal of Animal Science*. 75: 1244-1255.
- Jones, C. H., J. S. Pinkner, R. Roth, J. Heuser, A. V. Nicholes, S. N. Abraham et al. 1995. Fimh adhesin of type-1 pili is assembled into a fibrillar tip structure in the enterobacteriaceae. *Proceedings of the National Academy of Sciences of the United States of America*. 92: 2081-2085.
- Jorgensen, H., X. Q. Zhao, K. E. B. Knudsen, and B. O. Eggum. 1996. The influence of dietary fibre source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. *British Journal of Nutrition*. 75: 379-395.
- Jozefiak, D., A. Rutkowski, and S. A. Martin. 2004. Carbohydrate fermentation in the avian ceca: A review. *Animal Feed Science and Technology*. 113: 1-15.

- Jozefiak, D., A. Rutkowski, B. B. Jensen, and R. M. Engberg. 2006. The effect of beta-glucanase supplementation of barley- and oat-based diets on growth performance and fermentation in broiler chicken gastrointestinal tract. *British Poultry Science*. 47: 57-64.
- Jukes, H. G., D. C. Hill, and H. D. Branion. 1956. Effect of feeding antibiotics on the intestinal tract of the chick. *Poultry Science*. 35: 716-723.
- Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzyckawroblewska et al. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial-cells in response to bacterial invasion. *Journal of Clinical Investigation*. 95: 55-65.
- Kadowaki, N., S. Ho, S. Antonenko, R. D. Malefyt, R. A. Kastelein, F. Bazan et al. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine*. 194: 863-869.
- Kalita, P. C., and G. K. Singh. 2014. Gross and microscopic anatomy on small intestine of the kadaknath fowl. LAP LAMBERT Academic Publishing.
- Kalliomaki, M., R. Satokari, H. Lahteenoja, S. Vahamiko, J. Gronlund, T. Routi et al. 2012. Expression of microbiota, toll-like receptors, and their regulators in the small intestinal mucosa in celiac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 54: 727-732.
- Kanai, Y., and M. A. Hediger. 2004. The glutamate/neutral amino acid transporter family SLC1: Molecular, physiological and pharmacological aspects. *Pflugers Archiv-European Journal of Physiology*. 447: 469-479.
- Kandori, H., K. Hirayama, M. Takeda, and K. Doi. 1996. Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. *Experimental Animals*. 45: 155-160.
- Kania, R. K., N. A. Santiago, and G. M. Gray. 1977. Intestinal surface amino-oligopeptidases .2. substrate kinetics and topography of active-site. *Journal of Biological Chemistry*. 252: 4929-4934.
- Kannaki, T. R., M. R. Reddy, M. Shanmugam, P. C. Verma, and R. P. Sharma. 2010. Chicken toll-like receptors and their role in immunity. *Worlds Poultry Science Journal*. 66: 727-738.
- Karrer, O., and D. S. Parsons. 1968. Disaccharidase activity in small intestine of developing chick. *Life Sciences Part 1 Physiology and Pharmacology and Part 2 Biochemistry General and Molecular Biology*. 7: 85-89.
- Katanbaf, M. N., E. A. Dunnington, and P. B. Siegel. 1988. Allomorphic relationships from hatching to 56 days in parental lines and F1 crosses of chickens selected 27 generations for high or low body-weight. *Growth Development and Aging*. 52: 11-21.

- Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*. 11: 115-122.
- Kawai, Y., and M. Morotomi. 1978. Intestinal enzyme-activities in germfree, conventional, and gnotobiotic rats associated with indigenous microorganisms. *Infection and Immunity*. 19: 771-778.
- Kawai, Y., Y. Ishii, K. Arakawa, K. Uemura, B. Saitoh, J. Nishimura et al. 2004. Structural and functional differences in two cyclic bacteriocins with the same sequences produced by lactobacilli. *Applied and Environmental Microbiology*. 70: 2906-2911.
- Kellogg, T. F., and B. S. Wostmann. 1969. Fecal neutral steroids and bile acids from germfree rats. *Journal of Lipid Research*. 10: 495-503.
- Kenny, J. 1986. Cell-surface peptidases are neither peptide-specific nor organ-specific. *Trends in Biochemical Sciences*. 11: 40-42.
- Kenworth, R. 1967. Influence of bacteria on absorption from small intestine. *Proceedings of the Nutrition Society*. 26: 18-23.
- Khoury, K. A., M. H. Floch, and T. Hersh. 1969. Small intestinal mucosal cell proliferation and bacterial flora in conventionalization of germfree mouse. *Journal of Experimental Medicine*. 130: 659-670.
- Kim, Y. S., and E. J. Brophy. 1976. Rat intestinal brush-border membrane peptidases .1. solubilization, purification, and physicochemical properties of 2 different forms of enzyme. *Journal of Biological Chemistry*. 251: 3199-3205.
- Kimura, N., F. Mimura, S. Nishida, A. Kobayashi, and T. Mitsuoka. 1976. Studies on relationship between intestinal flora and cecal coccidiosis in chicken. *Poultry Science* 55: 1375-1383.
- Klasing, K. C. 2007. Nutrition and the immune system. *British Poultry Science*. 48: 525-537.
- Klasing, K. C., B. K. Johnstone, and B. N. Benson. 1999. Implications of an Immune Response on Growth and Nutrient Requirements of Chicks. Pages 35-48 in *Recent Developments in Poultry Nutrition 2*. Garnsworthy, P. C., and J. Wiseman, eds. Nottingham University Press, Nottingham, UK.
- Kleessen, B., L. Hartmann, and M. Blaut. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. *British Journal of Nutrition*. 89: 597-606.
- Klis, F. M., P. Mol, K. Hellingwerf, and S. Brul. 2002. Dynamics of cell wall structure in *saccharomyces cerevisiae*. *FEMS Microbiology Reviews*. 26: 239-256.

- Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and Environmental Microbiology*. 68: 5918-5924.
- Kocher, A., N. J. Rodgers, and M. Choct. 2004. Efficacy of alternatives to AGPS in broilers challenged with clostridium perfringens. *Proceedings of the 16th Australian poultry science symposium, Sydney, New South Wales, Australia*. pp. 130-133.
- Korver, D. R. 2006. Overview of the immune dynamics of the digestive system. *Journal of Applied Poultry Research*. 15: 123-135.
- Korver, D., and K. Klasing. 2004. Influence of nutrition on immune status of the bird. *Proceedings of the 24th technical turkey conference*. pp. 43.
- Koutsos, E. A., and V. J. Arias. 2006. Intestinal ecology: Interactions among the gastrointestinal tract, nutrition, and the microflora. *Journal of Applied Poultry Research*. 15: 161-173.
- Kraft, R., C. Ruchti, A. Burkhardt, and H. Cottier. 1992. Pathogenic principles in the development of gut-derived infectious-toxic shock gits and multiple organ failure. *Current Studies in Hematology and Blood Transfusion*. 59: 204-240.
- Krajmalnik-Brown, R., Z. Ilhan, D. Kang, and J. K. DiBaise. 2012. Effects of gut microbes on nutrient absorption and energy regulation. *Nutrition in Clinical Practice*. 27: 201-214.
- Kumprecht, I., and P. Zobac. 1997. The effect of mannan-oligosaccharides in feed mixtures on the performance of chicken broilers. *Zivocisna Vyroba*. 42: 117-124.
- Kussaibati, R., J. Guillaume, and B. Leclercq. 1982. The effects of the gut microflora on the digestibility of starch and proteins in young chicks. *Annales De Zootechnie*. 31: 483-488.
- Lacy, E. R. 1988. Epithelial restitution in the gastrointestinal-tract. *Journal of Clinical Gastroenterology*. 10: S72-S77.
- Lamkanfi, M., N. Festjens, W. Declercq, T. Vanden Berghe, and P. Vandenabeele. 2007. Caspases in cell survival, proliferation and differentiation. *Cell Death and Differentiation*. 14: 44-55.
- Lan, Y., M. Verstegen, S. Tamminga, and B. Williams. 2005. The role of the commensal gut microbial community in broiler chickens. *Worlds Poultry Science Journal*. 61: 95-104.
- Langhans, W. 1996. Bacterial products and the control of ingestive behavior: Clinical implications. *Nutrition*. 12: 303-315.

- Laurent, F., R. Mancassola, S. Lacroix, R. Menezes, and M. Naciri. 2001. Analysis of chicken mucosal immune response to eimeria tenella and eimeria maxima infection by quantitative reverse transcription-PCR. *Infection and Immunity*. 69: 2527-2534.
- Lavoie, J. N., M. Nguyen, R. C. Marcellus, P. E. Branton, and G. C. Shore. 1998. E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. *Journal of Cell Biology*. 140: 637-645.
- Lei, F., Y. Yin, Y. Wang, B. Deng, H. D. Yu, L. Li et al. 2012. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Applied and Environmental Microbiology*. 78: 5763-5772.
- Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow et al. 2003. Allelic variation in TLR4 is linked to susceptibility to salmonella enterica serovar typhimurium infection in chickens. *Infection and Immunity*. 71: 1116-1124.
- Li, H., E. R. Gilbert, Y. Zhang, O. Crasta, D. Emmerson, K. E. Webb Jr. et al. 2008. Expression profiling of the solute carrier gene family in chicken intestine from the late embryonic to early post-hatch stages. *Animal Genetics*. 39: 407-424.
- Lieberman, M., A. D. Marks, and A. Peet. 2013. Marks' basic medical biochemistry :A clinical approach. 4th ed. Philadelphia, Wolter Kluwer Health/Lippincott Williams & Wilkins.
- Lilja, C. 1983. A comparative-study of postnatal-growth and organ development in some species of birds. *Growth*. 47: 317-339.
- Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to eimeria parasites. *Clinical Microbiology Reviews*. 9: 349-360.
- Lillehoj, H. S., and K. S. Chung. 1992. Postnatal-development of lymphocyte-T subpopulations in the intestinal intraepithelium and lamina propria in chickens. *Veterinary Immunology and Immunopathology*. 31: 347-360.
- Liu, N., Y. J. Ru, F. D. Li, and A. J. Cowieson. 2008. Effect of diet containing phytate and phytase on the activity and messenger ribonucleic acid expression of carbohydrase and transporter in chickens. *Journal of Animal Science*. 86: 3432-3439.
- Loddi, M. M., L. S. O. Nakaghi, F. Edens, F. M. Tucci, M. I. Hannas, V. M. B. Moraes, J. Ariki and L.D.G. Bruno. 2002. Mannan oligosaccharide and organic acids on intestinal morphology integrity of broilers evaluated by scanning electron microscopy. *Proceeding 11th European Poultry Science Conference, Bremen, Germany, 2002*; pp.121.
- Lodoen, M. B., and L. L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Current Opinion in Immunology*. 18: 391-398.

- Lovshin, J., B. Yusta, I. Iliopoulos, A. Migirdicyan, L. Dableh, P. L. Brubaker et al. 2000. Ontogeny of the glucagon-like peptide-2 receptor axis in the developing rat intestine. *Endocrinology*. 141: 4194-4201.
- Low, A. G., and T. Zebrowska. 1986. Digestion in Poultry. Pages 122-142 in *Avian Physiology* Sturkie, P. D., and C. A. Benzo , eds. 4th ed. Springer-Verlag, New York.
- Low, M. G. 1987. Biochemistry of the glycosyl-phosphatidylinositol membrane-protein anchors. *Biochemical Journal*. 244: 1-13.
- Low, M. G., M. A. J. Ferguson, A. H. Futerman, and I. Silman. 1986. Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane-proteins. *Trends in Biochemical Sciences*. 11: 212-215.
- Lu, J. R., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology*. 69: 6816-6824.
- Luo, C. H., and L. B. Zheng. 2000. Independent evolution of toll and related genes in insects and mammals. *Immunogenetics*. 51: 92-98.
- Lynch, M. P., S. Nawaz, and L. E. Gerschenson. 1986. Evidence for soluble factors regulating cell-death and cell-proliferation in primary cultures of rabbit endometrial cells grown on collagen. *Proceedings of the National Academy of Sciences of the United States of America*. 83: 4784-4788.
- Lynn, D. J., R. Higgs, A. T. Lloyd, C. O'Farrelly, V. Herve-Grepinet, Y. Nys et al. 2007. Avian beta-defensin nomenclature: A community proposed update. *Immunology Letters*. 110: 86-89.
- Lynn, D., R. Higgs, S. Gaines, J. Tierney, T. James, A. Lloyd et al. 2004. Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics*. 56: 170-177.
- Lyons, T. P. 1994. Biotechnology in the feed industry - 1994 and beyond - a panorama of techniques, processes and products to address animal production problems today and tomorrow. *Alltechs 10th annual symposium on biotechnology in the feed industry*, Nottingham University Press :pp 1-47.
- MacDonald, T. T., and G. Monteleone. 2005. Immunity, inflammation, and allergy in the gut. *Science*. 307: 1920-1925.
- Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth. 1999. Probiotics inhibit enteropathogenic E-coli adherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 276: G941-G950.

- Macpherson, A. J., and N. L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology*. 4: 478-485.
- Madara, J. L., S. Nash, R. Moore, and K. Atisook. 1990. Structure and function of the intestinal epithelial barrier in health and disease. *Monographs in Pathology*. 31: 306-24.
- Mahagna, M., and I. Nir. 1996. Comparative development of digestive organs, intestinal disaccharidases and some blood metabolites in broiler and layer-type chicks after hatching. *British Poultry Science*. 37: 359-371.
- Maisonnier, S., J. Gomez, A. Bree, C. Berri, E. Baeza, and B. Carre. 2003. Effects of microflora status, dietary bile salts and guar gum on lipid digestibility, intestinal bile salts, and histomorphology in broiler chickens. *Poultry Science*. 82: 805-814.
- Mammen, J. M. V., and J. B. Matthews. 2003. Mucosal repair in the gastrointestinal tract. *Critical Care Medicine*. 31: S532-S537.
- Mane, S., M. Damle, P. Harikumar, S. Jamdar, and W. Gade. 2010. Purification and characterization of aminopeptidase N from chicken intestine with potential application in debittering. *Process Biochemistry*. 45: 1011-1016.
- Margalef, R. A. M. O. N. 1957. Information theory in ecology. *Mem R Acad Cienc Y Artes Barcelona*. 32: 373-449.
- Maroux, S., and H. Feracci. 1983. Structure and biosynthesis of aminopeptidases. *Methods in Enzymology*. 96: 406-423.
- Maroux, S., D. Louvard, and J. Baratti. 1973. Aminopeptidase from hog intestinal brush border. *Biochimica et Biophysica Acta*. 321
- Mast, J., and B. M. Goddeeris. 1999. Development of immunocompetence of broiler chickens. *Veterinary Immunology and Immunopathology*. 70: 245-256.
- Mayhew, T. M., R. Myklebust, A. Whybrow, and R. Jenkins. 1999. Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histology and Histopathology*. 14: 257-267.
- Mazmanian, S. K., C. H. Liu, A. O. Tzianabos, and D. L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*. 122: 107-118.
- Mcbride, B. W., and J. M. Kelly. 1990. Energy-cost of absorption and metabolism in the ruminant gastrointestinal-tract and liver - a review. *Journal of Animal Science*. 68: 2997-3010.

- McCarthy, N. J., M. K. B. Whyte, C. S. Gilbert, and G. I. Evan. 1997. Inhibition of ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the bcl-2 homologue bak. *Journal of Cell Biology*.136: 215-227.
- McKay, D. M., and A. W. Baird. 1999. Cytokine regulation of epithelial permeability and ion transport. *Gut*. 44: 283-289.
- Mead, G. C., and B. W. Adams. 1975. Some observations on cecal microflora of chick during first 2 weeks of life. *British Poultry Science*. 16: 169-176.
- Mead, G. C., and P. A. Barrow. 1990. Salmonella control in poultry by competitive-exclusion or immunization. *Letters in Applied Microbiology*. 10: 221-227.
- Meade, K. G., R. Higgs, A. T. Lloyd, S. Giles, and C. O'Farrelly. 2009. Differential antimicrobial peptide gene expression patterns during early chicken embryological development. *Developmental & Comparative Immunology*. 33: 516-524.
- Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nature Reviews Immunology*. 1: 135-145.
- Meslin, J. C., and J. L. Guenet. 1973. Action of bacterial-flora on morphology and mucous surface of small-intestine of rat. *Annales De Biologie Animale Biochimie Biophysique*. 13: 203-214.
- Meslin, J. C., N. Fontaine, and C. Andrieux. 1999. Variation of mucin distribution in the rat intestine, caecum and colon: Effect of the bacterial flora. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology*. 123: 235-239.
- Metges, C. C. 2000. Contribution of microbial amino acids to amino acid homeostasis of the host. *Journal of Nutrition*. 130 : 1857S-1864S.
- Michard, J. 2008. Seeking new broiler growth promoters. *Poultry International*. 47: 28-30.
- Midtvedt, T. 1974. Microbial bile-acid transformation. *American Journal of Clinical Nutrition*. 27: 1341-1347.
- Miller, L. L., P. B. Siegel, and E. A. Dunnington. 1992. Inheritance of antibody-response to sheep erythrocytes in lines of chickens divergently selected for 56-day body-weight and their crosses. *Poultry Science*. 71: 47-52.
- Miller, T. M., K. L. Moulder, C. M. Knudson, D. J. Creedon, M. Deshmukh, S. J. Korsmeyer et al. 1997. Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. *Journal of Cell Biology*. 139: 205-217.

- Milona, P., C. L. Townes, R. M. Bevan, and J. Hall. 2007. The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against salmonella serovars. *Biochemical and Biophysical Research Communications*. 356: 169-174.
- Miniats, O. P., and V. E. Valli. 1973. The Gastrointestinal Tract of Gnotobiotic Pigs. Pages 575-588 in *Germfree Research. Biological Effects of Gnotobiotic Environments*. Henegan, J. B., ed. Academic Press, the University of Michigan.
- Miyamoto, K., K. Hase, T. Takagi, T. Fujii, Y. Taketani, H. Minami et al. 1993. Differential responses of intestinal glucose-transporter messenger-rna transcripts to levels of dietary sugars. *Biochemical Journal*. 295: 211-215.
- Moal, V. L. L., and A. L. Servin. 2006. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: Mucins, antimicrobial peptides, and microbiota. *Clinical Microbiology Reviews*. 19: 315-337.
- Mohamed, M. A., H. M. A. Hassan, and E. M. A. El-Barkouky. 2008. Effect of mannan oligosaccharide on performance and carcass characteristics of broiler chicks. *Journal of Agriculture and Social Sciences*. 4: 13-17.
- Monteleone, G., F. Pallone, and T. T. MacDonald. 2004. Smad7 in TGF-beta-mediated negative regulation of gut inflammation. *Trends in Immunology*. 25: 513-517.
- Moran, E. T. 1985. Digestion and absorption of carbohydrates in fowl and events through perinatal-development. *Journal of Nutrition*. 115: 665-674.
- Moreno, M. J., S. Pellicer, and M. P. Fernandezotero. 1995. Lindane treatment alters both intestinal-mucosa composition and brush-border enzymatic-activity in chickens. *Pesticide Biochemistry and Physiology*. 52: 212-221.
- Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews Immunology*. 3: 331-341.
- Mozdziak, P. E., J. J. Dibner, and D. W. McCoy. 2003. Glyceraldehyde-3-phosphate dehydrogenase expression varies with age and nutrition status. *Nutrition*. 19: 438-440.
- Muir, W. I., W. L. Bryden, and A. J. Husband. 2000. Immunity, vaccination and the avian intestinal tract. *Developmental & Comparative Immunology*. 24 :325-342.
- Mulder, I. E., B. Schmidt, C. R. Stokes, M. Lewis, M. Bailey, R. I. Aminov et al. 2009. Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biology*. 7: 79-98.
- Mulder, R. W. A. W., R. Havenaar, and J. H. J. Huis. 1997. Intervention Strategies: The use of Probiotics and Competitive Exclusion Microfloras Against Contamination with Pathogens

- in Pigs and Poultry. Pages 186-207 in *Probiotics 2: Applications and Practical Aspects*. Fuller, R., ed. Chapman & Hall, London.
- Murakami, H., Y. Akiba, and M. Horiguchi. 1988. Energy and protein utilization in newly-hatched broiler chicks studies on the early nutrition of poultry. *Japanese Journal of Zootechnical Science*. 59: 890-895.
- Muramatsu, T., M. E. Coates, D. Hewitt, D. N. Salter, and P. J. Garlick. 1983. The influence of the gut microflora on protein-synthesis in liver and jejunal mucosa in chicks. *British Journal of Nutrition*. 49: 453-462.
- Muramatsu, T., O. Takasu, M. Furuse, I. Tasaki, and J. Okumura. 1987. Influence of the gut microflora on protein-synthesis in tissues and in the whole-body of chicks. *Biochemical Journal*. 246: 475-479.
- Musso, G., R. Gambino, and M. Cassader. 2011. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annual Review of Medicine*, Vol 62: 361-380.
- Nabuurs, M. J. A., A. Hoogendoorn, E. J. Vandermolen, and A. L. M. Vanosta. 1993. Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. *Research in Veterinary Science*. 55: 78-84.
- Naik, S., E. J. Kelly, L. Meijer, T. Pettersson, and I. R. Sanderson. 2001. Absence of toll-like receptor 4 explains endotoxin hyporesponsiveness in human intestinal epithelium. *Journal of Pediatric Gastroenterology Nutrition*. 32: 449-453.
- Nakabayashi, T., and H. Ikezawa. 1986. Alkaline phosphodiesterase-i release from eukaryotic plasma-membranes by phosphatidylinositol-specific phospholipase-C .2. the release from brush-border membranes of porcine intestine. *Toxicon*. 24: 975-984.
- Newman, K. 1994. Mannan-Oligosaccharides - Natural Polymers with Significant Impact on the Gastrointestinal Microflora and the Immune System. Pages 167-174 in *Biotechnology in the Feed Industry*. Proc. Alltech's 10th Annual Symposium. Lyons, T. J.,KA, ed. Nottingham University Press, Nottingham, UK.
- Newman, K. E., and M. C. Newman. 2001. Evaluation of mannan oligosaccharide on the microflora and immunoglobulin status of sows and piglet performance. *Journal of Dairy Science*. 84: 189 (Abstr.).
- Newton, K., and V. M. Dixit. 2012. Signaling in innate immunity and inflammation. *Cold Spring Harbor Perspectives in Biology*. 4: a006049. doi: 10.1101/cshperspect.a006049.
- Nguyen, T. N., J. S. Lee, B. M. Song, Y. M. Kang, H. S. Kim, and S. H. Seo. 2011. Induction of inflammatory cytokines and toll-like receptors in chickens infected with avian H9N2 influenza virus. *Veterinary Research*. 42: 64.

- Nicholson, D. W., and N. A. Thornberry. 1997. Caspases: Killer proteases. *Trends in Biochemical Science*.22: 299-306.
- Nitsan, Z., G. Benavraham, Z. Zoref, and I. Nir. 1991. Growth and development of the digestive organs and some enzymes in broiler chicks after hatching. *British Poultry Science*. 32: 515-523.
- Nollet, L. 2005. EU close to a future without antibiotic growth promoters. AGP alternatives - part 1. *World Poultry*. 21: 14-15.
- Noy, Y., A. Geyra, and D. Sklan. 2001. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poultry Science*. 80: 912-919.
- Nurmi, E., and M. Rantala. 1973. New aspects of salmonella infection in broiler production. *Nature*. 241: 210-211.
- Nusrat, A., C. Delp, and J. L. Madara. 1992. Intestinal epithelial restitution - characterization of a cell-culture model and mapping of cytoskeletal elements in migrating cells. *Journal of Clinical Investigation*. 89: 1501-1511.
- Ogihara, H., T. Suzuki, Y. Nagamachi, K. Inui, and K. Takata. 1999. Peptide transporter in the rat small intestine: Ultrastructural localization and the effect of starvation and administration of amino acids. *Histochemical Journal*. 31: 169-174.
- Ohimain, E. I., and R. T. S. Ofongo. 2012. The effect of probiotic and prebiotic feed supplementation on chicken health and gut microflora: A review. *International Journal of Animal and Veterinary Advances*. 4: 135-143.
- Ohland, C. L., and W. K. MacNaughton. 2010. Probiotic bacteria and intestinal epithelial barrier function. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 298: G807-G819.
- Olson, G. B., and B. S. Wostmann. 1966. Lymphocytopoiesis plasmacytopoiesis and cellular proliferation in nonantigenically stimulated germfree mice. *Journal of Immunology*. 97: 267-&.
- O'Quinn, P. R., D. W. Funderburke, and G. W. Tibbetts. 2001. Effects of dietary supplementation with mannan oligosaccharides on sow and litter performance in a commercial production system. *Journal of Dairy Science*. 84: 212 (Abstr.).
- Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in toll-like receptor 3-mediated interferon-beta induction. *Nature Immunology*. 4: 161-167.
- Osman, A. M. 1982. Amylase in chicken intestine and pancreas. *Comparative Biochemistry and Physiology B*. 73: 571-574.

- Oswald, I. P. 2006. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. *Veterinary Research* .37: 359-368.
- Otte, J. M., E. Cario, and D. K. Podolsky. 2004. Mechanisms of cross hyporesponsiveness to toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology*. 126: 1054-1070.
- Overton, J., and J. Shoup. 1964. Fine structure of cell surface specializations in maturing duodenal mucosa of chick. *Journal of Cell Biology*. 21: 75-85.
- Oyofe, B. A., J. R. Deloach, D. E. Corrier, J. O. Norman, R. L. Ziprin, and H. H. Mollenhauer. 1989. Effect of carbohydrates on salmonella-typhimurium colonization in broiler-chickens. *Avian Diseases*. 33: 531-534.
- Oyofe, B. A., J. R. Deloach, D. E. Corrier, J. O. Norman, R. L. Ziprin, and H. H. Mollenhauer. 1989. Prevention of salmonella-typhimurium colonization of broilers with D-mannose. *Poultry Science*. 68: 1357-1360.
- Oyofe, B. A., R. E. Droleskey, J. O. Norman, H. H. Mollenhauer, R. L. Ziprin, D. E. Corrier et al. 1989. Inhibition by mannose of invitro colonization of chicken small-intestine by salmonella-typhimurium. *Poultry Science*. 68: 1351-1356.
- Page, S. W. 2006. Current use of Antimicrobial Growth Promoters in Food Animals: The Benefits. Pages 19-51 in *Antimicrobial Growth Promoters: Where do we Go from here?* Barug, D., J. de Jong, A. K. Kies, and M. Verstegen, eds. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Palacin, M., and Y. Kanai. 2004. The ancillary proteins of HATs: SLC3 family of amino acid transporters. *Pflügers Archiv-European Journal of Physiology*. 447: 490-494.
- Palacin, M., R. Estevez, J. Bertran, and A. Zorzano. 1998. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiological Reviews*. 78: 969-1054.
- Parsons, C. M., L. M. Potter, and R. D. Brown. 1983. Effects of dietary carbohydrate and of intestinal microflora on excretion of endogenous amino-acids by poultry. *Poultry Science*. 62: 483-489.
- Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. *Poultry Science*. 82: 627-631.
- Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences of the United States of America*. 85: 2444-2448.

- Pelicano, E. R. L., P. A. d. Souza, H. B. A. d. Souza, F. R. Leonel, N. M. B. L. Zeola, and M. M. Boiago. 2004. Productive traits of broiler chickens fed diets containing different growth promoters. *Revista Brasileira De Ciencia Avicola*. 6: 177-182.
- Perdue, M. H. 1999. Mucosal immunity and inflammation III. the mucosal antigen barrier: Cross talk with mucosal cytokines. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 277: G1-G5.
- Perez-Vilar, J., and R. L. Hill. 1999. The structure and assembly of secreted mucins. *Journal of Biological Chemistry*. 274: 31751-31754.
- Philpott, D. J., D. M. McKay, W. Mak, M. H. Perdue, and P. M. Sherman. 1998. Signal transduction pathways involved in enterohemorrhagic escherichia coli-induced alterations in T84 epithelial permeability. *Infection and Immunity*. 66: 1680-1687.
- Piard, J. C., and M. Desmazeaud. 1991. Inhibiting factors produced by lactic-acid bacteria .1. oxygen metabolites and catabolism end-products. *Lait*. 71: 525-541.
- Piguet, P. F., C. Vesin, Y. Donati, and C. Barazzone. 1999. TNF-induced enterocyte apoptosis and detachment in mice: Induction of caspases and prevention by a caspase inhibitor, ZVAD-fmk. *Laboratory Investigation*. 79: 495-500.
- Pinchasov, Y., and S. Elmaliah. 1994. Broiler chick responses to anorectic agents .1. dietary acetic and propionic acids and the digestive-system. *Pharmacology Biochemistry and Behavior*. 48: 371-376.
- Pinchasov, Y., and Y. Noy. 1994. Early postnatal amylolysis in the gastrointestinal-tract of turkey poults meleagris-gallopavo. *Comparative Biochemistry and Physiology A-Physiology*. 107: 221-226.
- Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: A review. *Livestock Production Science*. 51: 215-236.
- Pluske, J. R., M. J. Thompson, C. S. Atwood, P. H. Bird, I. H. Williams, and P. E. Hartmann. 1996. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *British Journal of Nutrition*. 76: 409-422.
- Pluske, J. R., Z. Durmic, D. W. Pethick, B. P. Mullan, and D. J. Hampson. 1998. Confirmation of the role of rapidly fermentable carbohydrates in the expression of swine dysentery in pigs after experimental infection. *Journal of Nutrition*. 128: 1737-1744.
- Podmaniczky, B., A. Kocher, Z. S. Szabo, B. Vegi, K. Horel, L. Korosi et al. 2006. The effect of mannan oligosaccharide on growth performance of challenged broilers. *World Poultry Science Journal*. 62: 319.

- Podzorski, R. P., G. R. Gray, and R. D. Nelson. 1990. Different effects of native candida-albicans mannan and mannan-derived oligosaccharides on antigen-stimulated lymphoproliferation invitro. *Journal of Immunology*. 144: 707-716.
- Pond, W. G., and J. T. Yen. 1987. Effect of supplemental carbadox, and antibiotic combination, or clinoptilolite on weight-gain and organ weights of growing swine fed maize or rye as the grain sources. *Nutrition Reports International*. 35: 801-809.
- Quaroni, A. 1985. Crypt cell-development in newborn rat small-intestine. *Journal of Cell Biology*. 100: 1601-1610.
- Quaroni, A. 1985. Prenatal and postnatal-development of differentiated functions in rat intestinal epithelial-cells. *Developmental Biology*. 111: 280-292.
- Qureshi, M. A., and G. B. Havenstein. 1994. A comparison of the immune performance of a 1991 commercial broiler with a 1957 random-bred strain when fed typical 1957 and 1991 broiler diets. *Poultry Science*. 73: 1805-1812.
- Raetz, C. R. H., and C. Whitfield. 2002. Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*. 71: 635-700.
- Rakoff-Nahoum, S., and A. Bousvaros. 2010. Innate and adaptive immune connections in inflammatory bowel diseases. *Current Opinion in Gastroenterology*. 26: 572-577.
- Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. 118: 229-241.
- Ramachandran, A., M. Madesh, and K. A. Balasubramanian. 2000. Apoptosis in the intestinal epithelium: Its relevance in normal and pathophysiological conditions. *Journal of Gastroenterology and Hepatology*. 15: 109-120.
- Ratcliffe, M. J. H. 2006. Antibodies, immunoglobulin genes and the bursa of fabricius in chicken B cell development. *Developmental & Comparative Immunology*. 30: 101-118.
- Reddy, B. S., and B. S. Wostmann. 1966. Intestinal disaccharidase activities in growing germfree and conventional rats. *Archives of Biochemistry and Biophysics*. 113: 609-616.
- Reddy, B. S., Pleasant, Jr, and B. S. Wostmann. 1968. Effect of dietary carbohydrates on intestinal disaccharidases in germfree and conventional rats. *Journal of Nutrition*. 95: 413-419.
- Robayo-Torres, C. C., R. Quezada-Calvillo, and B. L. Nichols. 2006. Disaccharide digestion: Clinical and molecular aspects. *Clinical Gastroenterology and Hepatology*. 4: 276-287.

- Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan. 1998. A family of human receptors structurally related to drosophila toll. *Proceedings of the National Academy of Sciences of the United States of America*. 95: 588-593.
- Roe, M. T., and S. D. Pillai. 2003. Monitoring and identifying antibiotic resistance mechanisms in bacteria. *Poultry Science*. 82: 622-626.
- Rolfe, R. D. 1991. Population Dynamics of the Intestinal Tract. Pages 59-76 in *Colonization Control of Human Bacterial Enteropathogens in Poultry*. Blankenship, L. C., ed. Academic Press Inc, San Diego, California.
- Rolli, J., N. Loukili, S. Levrant, N. Rosenblatt-Velin, S. Rignault-Clerc, B. Waeber, Feihl F, Pacher P, Liaudet L. 2010. Bacterial flagellin elicits widespread innate immune defense mechanisms, apoptotic signaling, and a sepsis-like systemic inflammatory response in mice. *Critical Care*. 14: R160. doi: 10.1186/cc9235.
- Rolls, B. A., A. Turvey, and M. E. Coates. 1978. Influence of gut microflora and of dietary fiber on epithelial-cell migration in chick intestine. *British Journal of Nutrition*. 39: 91-98.
- Romanoff, A. L. 1960. *The avian embryo. structural and functional development*. New York, Macmillan.
- Rose, F. R. A. J., K. Bailey, J. W. Keyte, W. C. Chan, D. Greenwood, and Y. R. Mahida. 1998. Potential role of epithelial cell-derived histone H1 proteins in innate antimicrobial defense in the human gastrointestinal tract. *Infection and Immunity*. 66: 3255-3263.
- Rosen, G. D. 2007. Holo-analysis of the efficacy of bio-mos (R) in broiler nutrition. *British Poultry Science*. 48: 21-26.
- Rothkotter, H. J., T. Kirchhoff, and R. Pabst. 1994. Lymphoid and nonlymphoid cells in the epithelium and lamina propria of intestinal-mucosa of pigs. *Gut*. 35: 1582-1589.
- Rothwell, L., R. A. Gramzinski, M. E. Rose, and P. Kaiser. 1995. Avian coccidiosis - changes in intestinal lymphocyte populations associated with the development of immunity to eimeria-maxima. *Parasite Immunology*. 17: 525-533.
- Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunological stress contributes to the growth-permitting ability of dietary antibiotics in chicks. *Journal of Nutrition*. 122: 2383-2390.
- Sahl, H. G., U. Pag, S. Bonness, S. Wagner, N. Antcheva, and A. Tossi. 2005. Mammalian defensins: Structures and mechanism of antibiotic activity. *Journal of Leukocyte Biology*. 77: 466-475.
- Sajjan, S. U., and J. F. Forstner. 1990. Characteristics of binding of escherichia-coli serotype O157-H7 strain cl-49 to purified intestinal mucin. *Infection and Immunity*. 58: 860-867.

- Sakata, T. 1987. Stimulatory effect of short-chain fatty-acids on epithelial-cell proliferation in the rat intestine - a possible explanation for trophic effects of fermentable fiber, gut microbes and luminal trophic factors. *British Journal of Nutrition*. 58: 95-103.
- Sakata, T., and A. Inagaki. 2001. Organic Acid Production in the Large Intestine: Implication for Epithelial Cell Proliferation and Cell Death. Pages 85-94 in *Gut Environment of Pigs*. Piva, A., K. E. Bach Knudsen, and J. E. Lindberg, eds. Nottingham University Press, Nottingham, U.K.
- Salminen, S., C. Bouley, M. C. Boutron-Ruault, J. H. Cummings, A. Franck, G. R. Gibson et al. 1998. Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition*. 80: S147-S171.
- Salter, D. N., and M. E. Coates. 1971. Influence of microflora of alimentary tract on protein digestion in chick. *British Journal of Nutrition*. 26: 55-69.
- Salter, D. N., M. E. Coates, and D. Hewitt. 1974. Utilization of protein and excretion of uric-acid in germfree and conventional chicks. *British Journal of Nutrition*. 31: 307-318.
- Samanya, M., and K. Yamauchi. 2002. Histological alterations of intestinal villi in chickens fed dried bacillus subtilis var. natto. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology*. 133: 95-104.
- Samli, H. E., N. Senkoğlu, F. Koc, M. Kanter, and A. Ağma. 2007. Effects of enterococcus faecium and dried whey on broiler performance, gut histomorphology and intestinal microbiota. *Archives of Animal Nutrition*. 61: 42-49.
- Sanderink, G. J., Y. Artur, and G. Siest. 1988. Human aminopeptidases - a review of the literature. *Journal of Clinical Chemistry and Clinical Biochemistry*. 26: 795-807.
- Sanllorenti, P. M., D. B. Tardivo, and R. D. Conde. 1992. Dietary level of protein regulates glyceraldehyde-3-phosphate dehydrogenase content and synthesis rate in mouse-liver cytosol. *Molecular and Cellular Biochemistry*. 115: 117-128.
- Santaolalla, R., and M. T. Abreu. 2012. Innate immunity in the small intestine. *Current Opinion in Gastroenterology*. 28: 124-129.
- Santin, E., A. Maiorka, M. Macari, M. Grecco, J. C. Sanchez, T. M. Okada et al. 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing saccharomyces cerevisiae cell wall. *Journal of Applied Poultry Research*. 10: 236-244.
- Sarica, S., S. Erdogan, A. Koc, and Z. Erdogan. 2005. Addition of avilamycin, mannanoligosaccharide and organic acids mixture to corn-soybean meal based broiler diets. *Indian Journal of Animal Science*. 75: 961-964.

- Sarin, A., M. S. Williams, M. A. Alexander-Miller, J. A. Berzofsky, C. M. Zacharchuk, and P. A. Henkart. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity*. 6: 209-215.
- Sartor, R. B. 2002. Mucosal Immunology and Mechanisms of Gastrointestinal Inflammation. Pages 21-51 in Sleisenger & Fordtran's Gastrointestinal and Liver Disease. Feldman, M., L. S. Friedman, and M. H. Sleisenger, eds. 7th ed. Saunders, Philadelphia.
- Savage, T. F., E. I. Zakrzewska, and J. R. Andreasen . 1997. The effects of feeding mannan oligosaccharide supplemented diets to poult on performance and the morphology of the small intestine. *Poultry Science*. 76(Suppl. 1): 139.
- Savage, T. F., P. F. Cotter, and E. I. Zakrzewska. 1996. The effect of feeding of mannan oligosaccharide on immunoglobulins, plasma IgG and bile IgA, of wrolstad MW male turkeys. *Poultry Science*. 75: 143-149.
- Schmidt, B., I. E. Mulder, C. C. Musk, R. I. Aminov, M. Lewis, C. R. Stokes et al. 2011. Establishment of normal gut microbiota is compromised under excessive hygiene conditions. *Plos One*. 6: e28284. doi: 10.1371/journal.pone.0028284.
- Sell, J. L., C. R. Angel, F. J. Piquer, E. G. Mallarino, and H. A. Albatshan. 1991. Developmental patterns of selected characteristics of the gastrointestinal-tract of young turkeys. *Poultry Science*. 70: 1200-1205.
- Semenza, G. 1986. Anchoring and biosynthesis of stalked brush-border membrane-proteins - glycosidases and peptidases of enterocytes and renal tubuli. *Annual Review of Cell Biology*. 2: 255-313.
- Shanahan, F. 2002. The host-microbe interface within the gut. *Best Practice & Research in Clinical Gastroenterology*. 16: 915-931.
- Shane, S. M. 2001. Mannan Oligosaccharides in Poultry Nutrition: Mechanisms and Benefits. Pages 65-77 in *Biotechnology in the Feed Industry: Proceedings of Alltech's 17th Annual Symposium*. Lyons, T. P. J., K.A., ed. Nottingham University press, Nottingham, UK.
- Shannon, C. E. 1948. A mathematical theory of communication. *Bell System Technical Journal*. 27: 379-423.
- Sharma, J. M. 2003. The Avian Immune System. Pages 5-16 in *Diseases of Poultry*. Saif, Y. M., ed. Iowa State University Press, Ames.
- Sharma, J. M., and B. D. Coulson. 1979. Presence of natural killer cells in specific-pathogen-free chickens. *Journal of the National Cancer Institute*. 63: 527-531.
- Sharma, R. P., and U. Schumacher. 2001. Carbohydrate expression in the intestinal mucosa. . Berlin; New York, Springer.

- Sharma, R., C. Young, and J. Neu. 2010. Molecular modulation of intestinal epithelial barrier: Contribution of microbiota. *Journal of Biomedicine and Biotechnology*. : 305879. doi:10.1155/2010/305879.
- Shashidhara, R. G., and G. Devegowda. 2003. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity. *Poultry Science*. 82: 1319-1325.
- Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, J. J. Callanan et al. 2009. Innate immune gene expression differentiates the early avian intestinal response between salmonella and campylobacter. *Veterinary Immunology and Immunopathology*. 132: 191-198.
- Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, and C. O'Farrelly. 2008. Comparative innate immune gene expression in chicken intestine after in vivo challenge with commensal (campylobacter) and pathogenic (salmonella) bacteria. *FASEB Journal*. 22: 864.2 (Abstr.).
- Sheng, K. C., D. S. Pouniotis, M. D. Wright, C. K. Tang, E. Lazoura, G. A. Pietersz et al. 2006. Mannan derivatives induce phenotypic and functional maturation of mouse dendritic cells. *Immunology*. 118: 372-383.
- Shibahara, T., N. Sato, S. Waguri, T. Iwanaga, A. Nakahara, H. Fukutomi et al. 1995. The fate of effete epithelial-cells at the villus tips of the human small-intestine. *Archives of Histology and Cytology*. 58: 205-219.
- Shiraga, T., K. I. Miyamoto, H. Tanaka, H. Yamamoto, Y. Taketani, K. Morita et al. 1999. Cellular and molecular mechanisms of dietary regulation on rat intestinal H⁺/peptide transporter PepT1. *Gastroenterology*. 116: 354-362.
- Shirkey, T. W., R. H. Siggers, B. G. Goldade, J. K. Marshall, M. D. Drew, B. Laarveld et al. 2006. Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. *Experimental Biology and Medicine*. 231: 1333-1345.
- Shurson, G. C., P. K. Ku, G. L. Waxler, M. T. Yokoyama, and E. R. Miller. 1990. Physiological relationships between microbiological status and dietary copper levels in the pig. *Journal of Animal Science*. 68: 1061-1071.
- Siddons, R. C. 1969. Intestinal disaccharidase activities in chick. *Biochemical Journal*. 112: 51-59.
- Siddons, R. C., and M. E. Coates. 1972. Influence of intestinal microflora on disaccharidase activities in chick. *British Journal of Nutrition*. 27: 101-112.

- Siegel, P. B., E. A. Dunnington, D. E. Jones, C. O. Ubosi, W. B. Gross, and J. A. Cherry. 1984. Phenotypic profiles of broiler stocks fed 2 levels of methionine and lysine. *Poultry Science*. 63: 855-862.
- Siegel, P. B., W. B. Gross, and E. A. Dunnington. 1989. Effects of dietary corticosterone in young leghorn and meat-type cockerels. *British Poultry Science*. 30: 185-192.
- Siggers, R. H., T. W. Shirkey, M. D. Drew, B. Laarveld, and A. G. Van Kessel. 2008. Cloning of porcine proglucagon and effect of commensal bacteria on relative gene expression in the intestine of gnotobiotic pigs. *Canadian Journal of Animal Science*. 88: 429-438.
- Sihn, G., K. Savary, A. Michaud, M. C. Fournie-Zaluski, B. P. Roques, P. Corvol et al. 2006. Aminopeptidase N during the ontogeny of the chick. *Differentiation*. 74: 119-128.
- Simpson, E. H. 1949. Measurement of diversity. *Nature*. 163: 688-688.
- Singboottra, P., M. A. Qureshi, and F. W. Edens. 2003. Effect of bio-mos on a chicken macrophage cell line. Alltech's 19th annual nutritional biotechnology in the feed and food industries symposium, Lexington, Ky.
- Sklan, D. 2001. Development of the digestive tract of poultry. *Worlds Poultry Science Journal*. 57: 415-428.
- Sklan, D., A. Geyra, E. Tako, O. Gal-Gerber, and Z. Uni. 2003. Ontogeny of brush border carbohydrate digestion and uptake in the chick. *British Journal of Nutrition*. 89: 747-753.
- Smirnov, A., R. Perez, E. Amit-Romach, D. Sklan, and Z. Uni. 2005. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. *Journal of Nutrition*. 135: 187-192.
- Smith, D. L., J. A. Johnson, A. D. Harris, J. P. Furuno, E. N. Perencevich, and J. G. Morris. 2003. Assessing risks for a pre-emergent pathogen: Virginiamycin use and the emergence of streptogramin resistance in enterococcus faecium. *Lancet Infectious Diseases*. 3: 241-249.
- Smith, H. W. 1965. Observations on flora of alimentary tract of animals and factors affecting its composition. *Journal of Pathology and Bacteriology*. 89: 95-122.
- Smith, K., K. D. McCoy, and A. J. Macpherson. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology*. 19: 59-69.
- Smith, M. W., and M. A. Peacock. 1989. Comparative aspects of microvillus development in avian and mammalian enterocytes. *Comparative Biochemistry and Physiology*. 93: 617-622.
- Snel, J., H. J. M. Harmsen, P. W. J. J. v. d. Wielen, B. A. Williams, P. W. J. J. v. der Wielen, and P. W. J. J. van der Wielen. 2002. Dietary Strategies to Influence the Gastro-Intestinal

- Microflora of Young Animals, and its Potential to Improve Intestinal Health. Pages 37-69 in Nutrition and Health of the Gastrointestinal Tract. Blok, M. C. Vahl, H. A. Lange, L. de. Braak, A. E. van de. Hemke, G. Hessing, M., ed. Wageningen Academic Publishers, The Netherlands.
- Solis de los Santos, F., A. M. Donoghue, M. B. Farnell, G. R. Huff, W. E. Huff, and D. J. Donoghue. 2007. Gastrointestinal maturation is accelerated in turkey poultlets supplemented with a mannan-oligosaccharide yeast extract (alphanune). *Poultry Science*. 86: 921-930.
- Sommer, F., and F. Baeckhed. 2013. The gut microbiota - masters of host development and physiology. *Nature Reviews Microbiology*. 11: 227-238.
- Sonmez, G., and M. Eren. 1999. Effects of supplementation of zinc bacitracin, mannanoligosaccharide and probiotic into the broiler feeds on morphology of the small intestine. *Veteriner Fakultesi Dergisi, Uludag Universitesi*. 18: 125-138.
- Spring, P. 1999. Mannan oligosaccharide as an alternative to antibiotic use in europe. *Zootecnica International*. 22: 38-41.
- Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 2000. The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. *Poultry Science*. 79: 205-211.
- Spurlock, M. E. 1997. Regulation of metabolism and growth during immune challenge: An overview of cytokine function. *Journal of Animal Science*. 75: 1773-1783.
- Stadnyk, A. W. 2002. Intestinal epithelial cells as a source of inflammatory cytokines and chemokines. *Canadian Journal of Gastroenterology*. 16: 241-246.
- Stahl, P. D., and R. A. B. Ezekowitz. 1998. The mannose receptor is a pattern recognition receptor involved in host defense. *Current Opinion in Immunology*. 10: 50-55.
- Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via paneth cells. *Proceedings of the National Academy of Sciences of the United States of America*. U. S. A. 99: 15451-15455.
- Steel, A., S. Nussberger, M. F. Romero, W. F. Boron, C. A. R. Boyd, and M. A. Hediger. 1997. Stoichiometry and pH dependence of the rabbit proton-dependent oligopeptide transporter PepT1. *Journal of Physiology-London*. 498: 563-569.
- Steidler, L., W. Hans, L. Schotte, S. Neiryneck, F. Obermeier, W. Falk et al. 2001. Treatment of murine colitis by lactococcus lactis secreting IL-10. *International Journal of Antimicrobial Agents*. 17: S4-S5.
- Stevens, C. E., and I. D. Hume. 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews*. 78: 393-427.

- Stevens, C. E., and I. D. Hume. 2004. *Comparative Physiology of the Vertebrate Digestive System*. Pages 11-45 2nd ed. Cambridge University Press, Cambridge; New York.
- Strzalka, W., and A. Ziemienowicz. 2011. Proliferating cell nuclear antigen (PCNA): A key factor in DNA replication and cell cycle regulation. *Annals of Botany*. 107: 1127-1140.
- Subedi, K., N. Isobe, M. Nishibori, and Y. Yoshimura. 2007. Changes in the expression of gallinacins, antimicrobial peptides, in ovarian follicles during follicular growth and in response to lipopolysaccharide in laying hens (*Gallus domesticus*). *Reproduction*. 133: 127-133.
- Sugiarto, H., and P. L. Yu. 2004. Avian antimicrobial peptides: The defense role of beta-defensins. *Biochemical and Biophysical Research Communications*. 323: 721-727.
- Suzuki, H., A. Yanaka, T. Shibahara, H. Matsui, A. Nakahara, N. Tanaka et al. 2002. Ammonia-induced apoptosis is accelerated at higher pH in gastric surface mucous cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 283: G986-G995.
- Suzuki, T., P. Higgins, and D. Crawford. 2000. Control selection for RNA quantitation. *Biotechniques*. 29: 332-337.
- Swann, M. M. 1969. Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. HMSO, London.
- Swartz, T. D., F. A. Duca, T. de Wouters, Y. Sakar, and M. Covasa. 2012. Up-regulation of intestinal type 1 taste receptor 3 and sodium glucose luminal transporter-1 expression and increased sucrose intake in mice lacking gut microbiota. *British Journal of Nutrition*. 107: 621-630.
- Tada, H., E. Nemoto, H. Shimauchi, T. Watanabe, T. Mikami, T. Matsumoto et al. 2002. *Saccharomyces cerevisiae*- and *Candida albicans*-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and toll-like receptor 4-dependent manner. *Microbiology and Immunology*. 46: 503-512.
- Takeda, K., and S. Akira. 2004. TLR signaling pathways. *Seminars in Immunology*. 16: 3-9.
- Takesue, Y., K. Yokota, Y. Nishi, R. Taguchi, and H. Ikezawa. 1986. Solubilization of trehalase from rabbit renal and intestinal brush-border membranes by a phosphatidylinositol-specific phospholipase-C. *FEBS Letters*. 201: 5-8.
- Takeuchi, T., H. Kitagawa, T. Imagawa, and M. Uehara. 1999. Apoptosis of villous epithelial cells and follicle-associated epithelial cells in chicken cecum. *Journal of Veterinary Medical Science*. 61: 149-154.

- Tang, Y. Q., J. Yuan, G. Osapay, K. Osapay, D. Tran, C. J. Miller et al. 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science*. 286: 498-502.
- Tappenden, K. A., D. M. Albin, A. L. Bartholome, and H. F. Mangian. 2003. Glucagon-like peptide-2 and short-chain fatty acids: A new twist to an old story. *Journal of Nutrition*. 133: 3717-3720.
- Tellez, G., S. Higgins, A. Donoghue, and B. Hargis. 2006. Digestive physiology and the role of microorganisms. *Journal of Applied Poultry Research*. 15: 136-144.
- Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt. 2008. Evolution of the chicken toll-like receptor gene family: A story of gene gain and gene loss. *BMC Genomics*. 9: 62. doi:10.1186/1471-2164-9-62.
- Tennant, B., Reinague.M, and D. Harrold. 1971. Influence of microorganisms on intestinal absorption. *Annals of the New York Academy of Sciences*. 176: 262-272.
- Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen et al. 1999. Housekeeping genes as internal standards: Use and limits. *Journal of Biotechnology*. 75: 291-295.
- Thomas, W. E., L. M. Nilsson, M. Forero, E. V. Sokurenko, and V. Vogel. 2004. Shear-dependent 'stick-and-roll' adhesion of type 1 fimbriated escherichia coli. *Molecular Microbiology*. 53: 1545-1557.
- Thorbecke, G. J., H. A. Gordon, B. Wostman, M. Wagner, and J. A. Reyniers. 1957. Lymphoid tissue and serum gamma globulin in young germfree chickens. *Journal of Infectious Diseases*. 10: 237-251.
- Tizard, I. R., R. H. Carpenter, B. H. Mcanalley, and M. C. Kemp. 1989. The biological activities of mannans and related complex carbohydrates. *Molecular Biotherapy*. 1: 290-296.
- Traber, P. G., D. L. Gumucio, and W. Wang. 1991. Isolation of intestinal epithelial-cells for the study of differential gene-expression along the crypt-villus axis. *American Journal of Physiology*. 260: G895-G903.
- Trinchieri, G. 1989. Biology of natural-killer cells. *Advances in Immunology*. 47: 187-376.
- Truscott, R. B., and F. Alsheikhly. 1977. Reproduction and treatment of necrotic enteritis in broilers. *American Journal of Veterinary Research*. 38: 857-861.
- Turin, L., and F. Riva. 2008. Toll-like receptor family in domestic animal species. *Critical Reviews in Immunology*. 28: 513-538.

- Umesaki, Y., H. Setoyama, S. Matsumoto, A. Imaoka, and K. Itoh. 1999. Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. *Infection and Immunity*. 67: 3504-3511.
- Umesaki, Y., H. Setoyama, S. Matsumoto, and Y. Okada. 1993. Expansion of alpha-beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. *Immunology*. 79: 32-37.
- Uni, Z. 2006. Early Development of Small Intestinal Function. Pages 29-42 in *Avian Gut Function in Health and Disease*. Perry, G. C., ed. CABI, Wallingford, UK :Cambridge, MA.
- Uni, Z. and Smirnov, A. (2006) Modulating mucin dynamics using functional carbohydrates. *Gut microbiology: research to improve health, immune response and nutrition*. Fifth RRI-INRA Joint Symposium 21-23 June, Aberdeen, UK.
- Uni, Z., A. Geyra, H. Ben-Hur, and D. Sklan. 2000. Small intestinal development in the young chick: Crypt formation and enterocyte proliferation and migration. *British Poultry Science*. 41: 544-551.
- Uni, Z., E. Tako, O. Gal-Garber, and D. Sklan. 2003. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. *Poultry Science*. 82: 1747-1754.
- Uni, Z., R. Platin, and D. Sklan. 1998b. Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*. 168: 241-247.
- Uni, Z., S. Ganot, and D. Sklan. 1998a. Posthatch development of mucosal function in the broiler small intestine. *Poultry Science*. 77: 75-82.
- Uni, Z., Y. Noy, and D. Sklan. 1995. Posthatch changes in morphology and function of the small-intestines in heavy-strain and light-strain chicks. *Poultry Science*. 74: 1622-1629.
- Uni, Z., Y. Noy, and D. Sklan. 1999. Posthatch development of small intestinal function in the poultry. *Poultry Science*. 78: 215-222.
- Valancony, H., F. Humbert, J. Rukelibuga, M. Bougon, L. Balaine, and F. Lalande. 2001. Comparison of some substitutes for antibiotic additives in diets for turkey poults. effects on production and on resistance to salmonella colonization. *Sciences and Techniques Avicoles*. 35: 25-34.
- van der Wielen, P. W. J. J., D. A. Keuzenkamp, L. J. A. Lipman, F. van Knapen, and S. Biesterveld. 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology*. 44: 286-293.

- van der Wielen, P. W. J. J., S. Biesterveld, S. Notermans, H. Hofstra, B. A. P. Urlings, and F. van Knapen. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. *Applied and Environmental Microbiology*. 66: 2536-2540.
- van Dijk, A., E. J. A. Veldhuizen, A. J. A. M. van Asten, and H. P. Haagsman. 2005. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. *Veterinary Immunology and Immunopathology*. 106: 321-327.
- van Dijk, A., E. J. A. Veldhuizen, S. I. C. Kalkhove, J. L. M. Tjeerdsma-van Bokhoven, R. A. Romijn, and H. P. Haagsman. 2007. The beta-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. *Antimicrobial Agents and Chemotherapy*. 51: 912-922.
- Van Immerseel, F., J. De Buck, I. De Smet, J. Mast, F. Haesebrouck, and R. Ducatelle. 2002. Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a salmonella enteritidis strain. *Developmental & Comparative Immunology*. 26: 355-364.
- Van Miert, A. S. J. P. A. M. 1995. Pro-inflammatory cytokines in a ruminant model: Pathophysiological, pharmacological, and therapeutic aspects. *Veterinary Quarterly*. 17: 41-50.
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe et al. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 3: 0034.1-0034.11.
- Vaneechoutte, M., L. Dijkshoorn, A. Nemeč, P. Kampf, G. Wauters. 2011. *Acinetobacter*, *Chryseobacterium*, *Moraxella* and other nonfermentative gramnegative rods. In Versalovic J, Carroll K, Funke G, Jorgensen J, Landry M, Warnock D (ed), *Manual of Clinical Microbiology*, 10th Edition. ASM Press, Washington, DC. 714-738.
- Vaughn, L. E., P. S. Holt, R. W. Moore, and R. K. Gast. 2006. Enhanced gross visualization of chicken peyer's patch: Novel staining technique applied to fresh tissue specimens. *Avian Diseases*. 50: 298-302.
- Veldman, A., and P. J. Van der Aar. 1997. Effects of dietary inclusion of a natural clinoptilolite (mannelite (TM)) on piglet performance. *Agribiological Research-Zeitschrift Fur Agrarbiologie Agrikulturchemie Okologie*. 50: 289-294.
- Verrey, F., E. I. Closs, C. A. Wagner, M. Palacin, H. Endou, and Y. Kanai. 2004. CATs and HATs: The SLC7 family of amino acid transporters. *Pflugers Archiv-European Journal of Physiology*. 447: 532-542.
- Vissek, W. J. 1978a. Diet and cell-growth modulation by ammonia. *American Journal of Clinical Nutrition*. 31: S216-S220.

- Visek, W. J. 1978b. Mode of growth promotion by antibiotics. *Journal of Animal Science*. 46: 1447-1469.
- Vispo, C. and Karasov, W. H. 1997. The interaction of avian gut microbes and their host: An elusive symbiosis. In: *Gastrointestinal Microbiology. 1. Gastrointestinal Ecosystem and Fermentations*. (ed.) Mackie, R. J. and White, B. A. Chapman and Hall comp., New York
- Wagner, D. D., and O. P. Thomas. 1978. Influence of diets containing rye or pectin on intestinal flora of chicks. *Poultry Science*. 57: 971-975.
- Wagner, R. D. 2008. Effects of microbiota on GI health: Gnotobiotic research. *Advances in Experimental Medicine and Biology*. 635: 41-56.
- Walker, D., D. T. Thwaites, N. L. Simmons, H. J. Gilbert, and B. H. Hirst. 1998. Substrate upregulation of the human small intestinal peptide transporter, hPepT1. *Journal of Physiology-London*. 507: 697-706.
- Wang, L., J. Li, Q. Li, J. Zhang, and X. L. Duan. 2003. Morphological changes of cell proliferation and apoptosis in rat jejunal mucosa at different ages. *World Journal of Gastroenterology*. 9: 2060-2064.
- Warrington, J. A., A. Nair, M. Mahadevappa, and M. Tsyganskaya. 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiological Genomics*. 2: 143-147.
- Watson, A. J. M., and D. M. Pritchard. 2000. Lessons from genetically engineered animal models VII. apoptosis in intestinal epithelium: Lessons from transgenic and knockout mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 278: G1-G5.
- Wehkamp, J., J. Harder, K. Wehkamp, B. Wehkamp-von Meissner, M. Schlee, C. Enders et al. 2004. NF-kappa B- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by escherichia coli nissle 1917: A novel effect of a probiotic bacterium. *Infection and Immunity*. 72: 5750-5758.
- Weil, M., M. D. Jacobson, and M. C. Raff. 1998. Are caspases involved in the death of cells with a transcriptionally inactive nucleus? sperm and chicken erythrocytes. *Journal of Cell Science*. 111: 2707-2715.
- Werling, D., and T. J. Coffey. 2007. Pattern recognition receptors in companion and farm animals - the key to unlocking the door to animal disease? *Veterinary Journal*. 174: 240-251.
- Wiech, N. L., J. G. Hamilton, and O. N. Miller. 1967. Absorption and metabolism of dietary triglycerides in germfree and conventional rats. *Journal of Nutrition*. 93: 324-&.

- Wild, G. E., and D. Murray. 1992. Alterations in quantitative distribution of Na⁺/K⁺-ATPase activity along crypt villus axis in animal-model of malabsorption characterized by hyperproliferative crypt cytokinetics. *Digestive Diseases and Sciences*. 37: 417-425.
- Williams, P. E. V. 1995. Digestible amino-acids for non-ruminant animals - theory and recent challenges. *Animal Feed Science and Technology*. 53: 173-187.
- Willing, B. P., and A. G. Van Kessel. 2007. Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig. *Journal of Animal Science*. 85: 3256-3266.
- Willing, B. P., and A. G. Van Kessel. 2009. Intestinal microbiota differentially affect brush border enzyme activity and gene expression in the neonatal gnotobiotic pig. *Journal of Animal Physiology and Animal Nutrition*. 93: 586-595.
- Willing, B. P., and A. G. Van Kessel. 2010. Host pathways for recognition: Establishing gastrointestinal microbiota as relevant in animal health and nutrition. *Livestock Science*. 133: 82-91.
- Windhorst, H. -. 2006. Changes in poultry production and trade worldwide. *Worlds Poultry Science Journal*. 62: 585-602.
- Wise, M. G., and G. R. Siragusa. 2007. Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology*. 102: 1138-1149.
- Withanage, G. S. K., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers et al. 2005. Cytokine and chemokine responses associated with clearance of a primary salmonella enterica serovar typhimurium infection in the chicken and in protective immunity to rechallenge. *Infection and Immunity*. 73: 5173-5182.
- Wostmann, B. S. 1973. Intestinal bile-acids and cholesterol absorption in germfree rat. *Journal of Nutrition*. 103: 982-990.
- Wostmann, B. S. 1981. The germ-free animal in nutritional studies. *Annual Review of Nutrition*. 1: 257-279.
- Wostmann, B. S., Pleasant, Jr., and P. Bealmear. 1971. Dietary stimulation of immune mechanisms. *Federation Proceedings*. 30: 1779-&.
- Wright, E. M. 1993. The intestinal Na⁺/glucose cotransporter. *Annu. Rev. Physiol.* 55: 575-589.
- Wright, E. M., and D. D. F. Loo. 2000. Coupling between Na⁺, sugar, and water transport across the intestine. *Epithelial Transport and Barrier Function: Pathomechanisms in Gastrointestinal Disorders*. 915: 54-66.

- Wright, E. M., and E. Turk. 2004. The sodium/glucose cotransport family SLC5. *Pflügers Archiv-European Journal of Physiology*. 447: 510-518.
- Xiao, Y. J., Y. B. Cai, Y. R. Bommineni, S. C. Fernando, O. Prakash, S. E. Gilliland et al. 2006. Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. *Journal of Biological Chemistry*. 281: 2858-2867.
- Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Zhan, and M. Q. Wang. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry Science*. 82: 1030-1036.
- Yalcinkaya, I., T. Guengoer, M. Basalan, and E. Erdem. 2008. Mannan oligosaccharides (MOS) from *saccharomyces cerevisiae* in broilers: Effects on performance and blood biochemistry. *Turkish Journal of Veterinary & Animal Sciences*. 32: 43-48.
- Yamada, H., D. Chen, H. J. Monstein, and R. Hakanson. 1997. Effects of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. *Biochemical and Biophysical Research Communications*. 231: 835-838.
- Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda et al. 2002. Cutting edge: A novel toll/IL-1 receptor domain containing adapter that preferentially activates the IFN-beta promoter in the toll-like receptor signaling. *Journal of Immunology*. 169: 6668-6672.
- Yamauchi, K. E., and Y. Isshiki. 1991. Scanning electron-microscopic observations on the intestinal villi in growing white leghorn and broiler-chickens from 1 to 30 days of age. *British Poultry Science*. 32: 67-78.
- Yan, X. X., J. Najbauer, C. C. Woo, K. Dashtipour, C. E. Ribak, and M. Leon. 2001. Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. *The Journal of Comparative Neurology*. 433: 4-22.
- Yang, D., A. Biragyn, L. W. Kwak, and J. J. Oppenheim. 2002. Mammalian defensins in immunity: More than just microbicidal. *Trends in Immunology*. 23: 291-296.
- Yang, Y., M. Choct, and P. A. Iji. 2005. Effect of dietary mannanoligosaccharide level on performance and gross morphology of digestive tract segments of broiler chickens. *Proceedings of the 17th annual Australian poultry science symposium, Sydney, NSW, Australia*. Poultry Research Foundation, University of Sydney, Australia.
- Yang, Y., P. A. Iji, A. Kocher, E. Thomson, L. L. Mikkelsen, and M. Choct. 2008b. Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilisation, nutrient digestibility and intestinal microflora. *British Poultry Science*. 49: 186-194.
- Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2007b. Effects of mannanoligosaccharide on growth performance, the development of gut microflora, and gut

- function of broiler chickens raised on new litter. *Journal of Applied Poultry Research*. 16: 280-288.
- Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008a. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility and gut development of broilers given different cereal-based diets. *Journal of Animal Physiology and Animal Nutrition*. 92: 650-659.
- Yang, Y., P. A. Iji, and M. Choct. 2007a. Effects of different dietary levels of mannanoligosaccharide on growth performance and gut development of broiler chickens. *Asian-Australasian Journal of Animal Sciences*. 20: 1084-1091.
- Yang, Y., P. A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: A review of the role of six kinds of alternatives to in-feed antibiotics. *Worlds Poultry Science Journal*. 65: 97-114.
- Yason, C. V., B. A. Summers, and K. A. Schat. 1987. Pathogenesis of rotavirus infection in various age-groups of chickens and turkeys - pathology. *American Journal of Veterinary Research*. 48: 927-938.
- Yegani, M., and D. R. Korver. 2008. Factors affecting intestinal health in poultry. *Poultry Science*. 87: 2052-2063.
- Yitbarek, A., H. Echeverry, J. Brady, J. Hernandez-Doria, G. Camelo-Jaimes, S. Sharif et al. 2012. Innate immune response to yeast-derived carbohydrates in broiler chickens fed organic diets and challenged with clostridium perfringens. *Poultry Science*. 91: 1105-1112.
- Yolton, D. P., C. Stanley, and D. C. Savage. 1971. Influence of indigenous gastrointestinal microbial flora on duodenal alkaline phosphatase activity in mice. *Infection and Immunity*. 3: 768-773.
- Yoshimura, Y., H. Ohashii, K. Subedi, M. Nishibori, and N. Isobe. 2006. Effects of age, egg-laying activity, and salmonella-inoculation on the expressions of gallinacin mRNA in the vagina of the hen oviduct. *Journal of Reproduction and Development*. 52: 211-218.
- Yuan, Q., and W. A. Walker. 2004. Innate immunity of the gut: Mucosal defense in health and disease. *Journal of Pediatric Gastroenterology and Nutrition*. 38: 463-473.
- Zhang, G. L., and S. Ghosh. 2002. Negative regulation of toll-like receptor-mediated signaling by tollip. *Journal of Biological Chemistry*. 277: 7059-7065.
- Zhong, B., P. Tien, and H. Shu. 2006. Innate immune responses: Crosstalk of signaling and regulation of gene transcription. *Virology*. 352: 14-21.

Zhu, L., L. Wang, X. Luo, Y. Zhang, Q. Ding, X. Jiang et al. 2012. Tollip, an intracellular trafficking protein, is a novel modulator of the transforming growth factor-beta signaling pathway. *Journal of Biological Chemistry*. 287

Zhu, X. Y., T. Y. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Applied and Environmental Microbiology*. 68: 124-137.